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Antidiabetic activity of *Averrhoa bilimbi* fruit methanol extract through enhancement of GLUT4 protein expression in diabetes-induced mice

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ABSTRACT GLUT4, the glucose transporter responsive to insulin, is primarily found in muscle and adipose tissues. Diabetes can result from impaired insulin secretion and sensitivity. This study aims to evaluate the antidiabetic properties of Averrhoa bilimbi methanol extract by enhancing GLUT4 protein expression in mice with induced diabetes. Extraction was conducted via soxhletation using 96% methanol. Phytochemical analysis, employing qualitative tests and GC-MS, was performed. Antioxidant activity (IC_{50}) and toxicity (LD_{50}) were analyzed using DPPH and OECD methods. This research followed an experimental post-only control group design, with mice intraperitoneally injected with 150 mg/kg BW of alloxan monohydrate. A total of 24 mice were then divided into six groups: Normal, Negative Control, Positive Control (metformin), Low Dose (50 mg/kg BW), Medium Dose (250 mg/kg BW), and High Dose (300 mg/kg BW). Treatment lasted 21 days, with fasting blood glucose and body weight measurements taken every three days. On day 21, the liver and skeletal muscle were isolated, and blood was collected. Serum insulin and GLUT4 expression were assessed via ELISA and Western Blot, respectively. Phytochemical screening revealed flavonoids, saponins, terpenoids, tannins, and phenols and their derivatives. The IC_{50} value was 85 µg/mL, with an LD_{50} value of 1,000 mg/kg BW, indicating strong antioxidant activity and mild toxicity. The extract significantly reduced blood glucose levels but did not impact weight loss in diabetic mice. Average liver weight and index were highest in the Negative Control group, yet the lowest levels of hepatic and muscle glycogen were also observed in this group. Interestingly, insulin level and HOMA-IR decreased in diabetic mice, while the Medium Dose group exhibited the highest GLUT4 expression levels. In conclusion, medium doses of *A. bilimbi* methanol extract hold potential for diabetes treatment, with a probable mechanism of targeting GLUT4 protein expression.

KEYWORDS *Averrhoa bilimbi*; blood glucose; diabetes mellitus; GLUT4; insulin

1. Introduction

Glucose plays an important role as the primary energy source for the body and needs to be distributed by blood to energy-requiring tissues. GLUT4 is the major glucose transporter, insulin-responsive, and is expressed abundantly in muscle and adipose tissues. In response to insulin, GLUT4 is translocated to the plasma membrane surface, thereby regulating glucose transport into cells (Astuti et al. 2022). Impaired insulin secretion and sensitivity can lead to chronic hyperglycemia and, eventually, diabetes mellitus (DM) (Hajiaghaalipour et al. 2015).

Hyperglycemia in chronic conditions can cause diabetes mellitus (DM). Based on the data from the World Health Organization (2021), around 422 million people worldwide have diabetes, and 1.5 million deaths are directly related to DM each year. The number of cases and the prevalence of DM have continued to increase over the last few decades (International Diabetes Federation 2021). In 2021, International Diabetes Foundation reported that Indonesia ranks fifth in diabetes cases worldwide. DM can cause death due to diabetic complications, which include heart disease, stroke, nerve damage (diabetic neuropathy), kidney damage (diabetic nephropathy), and vision damage (diabetic retinopathy). DM with complications causes long-term failure and damage to various organ systems, which in turn can lead to death (Papatheodorou et al. 2018). The need for diabetes management causes bioprospection and exploration of plants that have the potential as antidiabetic to continue to be carried out, considering that the morbidity and mortality of DM continue to increase in developing countries, including Indonesia. Indonesia has a very large biodiversity and has the potential to become a drug producer (Sholikhah 2016). Therefore, the study on identification and investigation of Indonesian plants that have the potential as antidiabetic drugs is needed.

One of the endemic plants in Indonesia that has antidiabetic potential is *Averrhoa bilimbi*, which is locally known as "belimbing wuluh". *A. bilimbi* can grow well in Indonesia yet is still underutilized since it has acidic fruit. In contrast, plant parts such as bark, leaves, seeds, flowers, fruit, roots, and the whole plant have the potential as an alternative medicine to treat various diseases, including diabetes (Kumar et al. 2013). Research conducted by Kurup and Mini (2017) proved that the results of histopathological analysis showed the effect of *A. bilimbi* fruit extract in protecting hepatocytes from oxidative damage without affecting cellular function and structural integrity. The pancreatic recovery from oxidative stress and a significant reduction in blood glucose have shown after the treatment of diabetic rats with *A. bilimbi* fruit extract (Kurup and S 2017). Previous studies revealed that *A. bilimbi* fruit extract is rich in secondary metabolites such as flavonoids, phenols, saponins, alkaloids and coumarins (Abraham 2016; Suluvoy and Berlin Grace 2017). The antidiabetic activity of *A. bilimbi* can be caused by the presence of these bioactive compounds.

Induction of diabetes can be done by injection of alloxan monohydrate. Alloxan monohydrate is a diabetogenic agent that is toxic to pancreatic beta cells and has been used in various studies using various animal model species. Alloxan monohydrate disrupts the physiological function of the pancreatic beta cell with two different pathological effects. First, it selectively interferes with insulin secretion through its ability to specifically inhibit glucokinase, an enzyme responsible for the phosphorylation of glucose. Second, its specific chemical properties induce the formation of reactive oxygen species (ROS), such as superoxide radicals. Both mechanisms ultimately cause pancreatic beta-cell dysfunction and death (Lenzen 2008).

Studies related to the antidiabetic potential of *A. bilimbi* fruit is still limited to the effect of the fruit on blood glucose, enzymes involved in glucose metabolism in the liver, insulin secretion and pancreatic cells. Meanwhile, DM can occur due to impaired insulin signaling and the expression and translocation of glucose transporter proteins such as GLUT4. However, research that investigated the effect of *A. bilimbi* on GLUT4 protein expression has not yet been available. Therefore, this study aimed to determine the effect of methanol extract of *A. bilimbi* fruit on increasing GLUT4 protein expression in diabetes-induced mice.

2. Materials and Methods

2.1. Extraction and phytochemical analysis

A. bilimbi fruit in mature stages was harvested from Sukamaju Village, Pagerageung Tasikmalaya, West Java, Indonesia. Ripe fruit was identified by its yellowish-green color, soft texture, and some falls on the ground. The fruit was firstly washed in running water and stored for 24 h, then dried in the oven at 40–60 °C temperature until the moisture content was less than 10%. The fruits were then crushed with a blender to produce fine powder. Extraction was carried out according to the method described by Suluvoy and Berlin Grace (2017). Powder fruit of 25 g was used for the extraction with 250 methanol (96% v/v) in the soxhlet extractor. The remaining methanol was evaporated using a rotary evaporator at 55 °C. The semi-solid crude extract was obtained and stored at 2–4 °C for further use. Phytochemical analysis of the extract was carried out by qualitative tests including terpenoids, flavonoids, tannins, saponins, phenol, steroids test (Abraham 2016) and screening by GC-MS.

2.2. Experimental animal design and diabetic model induction

The design of this study was a post-test-only control design. Male Swiss Webster strain mice (*Mus musculus*), 2–3 months old, with an average weight of 37 ± 1 g, were obtained from the animal care center of the School of Life Sciences and Technology, Institut Teknologi Bandung. All animal experiments were in accordance with protocols approved by the local experimental ethics committee guidelines, Padjadjaran University Research Ethics Commission (80/UN6.KEP/EC/2022). The mice were kept in cages with a minimum cage area of 77.4 cm^2 and a height of 12.7 cm. The room temperature of the cage is 20–26 °C. Light and dark alternated every 12 h. The mice were fed ad libitum. The minimum number of mice used is determined using the Federer equation and follows the animal welfare principles of 3R5F.

After 1 week of acclimatization, mice were intraperitoneally injected with alloxan monohydrate freshly dissolved in 0.9% NaCl at a dose of 150 mg/kg of body weight (BW) to induce diabetes. Mice with blood glucose levels >200 mg/dL were classified as diabetic mice (Mouri and Badireddy 2020). Diabetic mice were randomly divided into five groups, with four mice in each group. These groups were the negative control diabetic group (NC), the positive control diabetic group, which was treated with metformin (PC) (150 mg/kg BW), and the treatment group, which was treated with methanol extract of *A. bilimbi* with low (50 mg/kg BW), medium (250 mg/kg BW) and high dose (300 mg/kg BW), namely LD, MD, and HD, respectively. Whereas 4 normal mice without diabetic induction were used as normal control (N). At the end of the experiment, mice were slaughtered using CO_2. Blood was collected by orbital puncture from the retro orbital sinus. Liver and skeletal tissues were isolated. Tissues and serum were stored at -80 °C for further study.

2.3. Antioxidant activity by DPPH radical scavenging

The antioxidant activity was determined by a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging test, which was carried out to determine the IC_{50} of the methanol extract of *A. bilimbi* fruit. The IC_{50} value indicates the concentration of the sample solution required to reduce DPPH free radicals by 50%. The antioxidant activity method was carried out and determined accord-

ing to the method proposed by Blois (1958), with modification. Five mg of DPPH solution was dissolved in 100 mL of methanol in a volumetric flask, then homogenized and placed in a dark glass bottle as a stock solution of 50 µg/mL. 5 mg of *A. bilimbi* fruit methanol extract was dissolved in methanol up to 10 mL in a volumetric flask, then homogenized, and 500 µg/mL stock solution was obtained. Then, the stock solution was diluted to obtain each concentration of 50, 100, 150 and 200 µg/mL with a volume of 10 mL. A total of 0.5 mL of each concentration was taken, and 3.5 mL of DPPH solution was added and homogenized.

2.4. Acute toxicity test

An acute toxicity test was carried out to determine the LD_{50} of the extract using the acute toxic class method (423) proposed by (OECD 2002). The final parameters for assessing acute toxicity were determined from the death of the test animals and followed the criteria for classifying extract according to (Indonesian Food and Drug Authority (BPOM) 2014).

The dose of the extract given the toxicity test was 5, 50, 300 or 2,000 mg/kg BW. Dosing is carried out in stages, starting with the initial dose, which is expected to result in mortality in some animals. There is no reference for the initial dose for the toxicity test of the extract, so the initial dose used was 300 mg/kg BW. Subsequent administration of the extract was carried out at higher or lower doses depending on the death that occurred.

2.5. Body weight and fasting blood glucose test

The mouse body weights and blood glucose levels were measured every 3 days during the treatment period. Blood was taken from the tail vein 10–12 h after fasting, and blood glucose levels were tested using the GlucoDr glucometer. The tip of the tail of the mice was cleaned first with 70% alcohol, then massaged slowly. Furthermore, the tip of the tail of the mouse was pricked with a small needle. The blood that comes out is then checked by touching it to the glucometer strip, within 11 s, the blood glucose level will be read on the glucometer screen and expressed in mg/dL.

2.6. Liver weight and liver index

The liver and muscles from the thigh (*regio femoralis*) were isolated. The liver was placed on ice and rinsed with a pre-cooled physiological solution, and filter paper was used to absorb excess water. Measurements of weight and liver index were carried out. The liver index of mice is the ratio of the fresh weight of an isolated liver to the body weight of mice on the last day (Huang et al. 2021), calculated by the following equation.

$$Liver\ index\ (\%) = \frac{liver\ weight}{bodyweight} \times 100\% \qquad (1)$$

2.7. Glycogen levels analysis

Analysis of glycogen levels was carried out using the anthrone-sulfate method proposed by Hassid and Abraham (Satyanarayana et al. 2015), with modification. The principle of measuring glycogen levels is that tissue samples containing glycogen are extracted with 30% KOH and precipitated with ethanol. The residue was treated with an anthrone-sulfate reagent, and the glucose in the hydrolysate was then determined as the reduced sugar.

Liver and muscle samples were transferred to a mortar to be pulverized. 5 mg of liver and muscle samples were taken from each mouse, and 1 mL of 30% KOH solution was incubated for 20 min in a water bath and then cooled. Then 1.5 mL of cold 95% ethanol was added, boiled again in a water bath, and cooled again. Then, it was centrifuged for 15 min at 2500 rpm, and the supernatant was discarded. The residue obtained was dissolved with 1 mL of distilled water, precipitated with 1 mL of 95% ethanol, centrifuged, and then separated again. The residue was dissolved in 5 mL of distilled water, and 5 mL of 0.2% anthrone-sulfate reagent was added. Standard solutions of 2 mL containing 0.025 mg, 0.05 mg, 0.1 mg, 0.15 mg, and 0.2 mg were prepared. A total of 5 mL of distilled water (blank) and a series of 35% standard solutions were added with 0.2% anthrone-sulfate reagent. The appearance of heat and a change in color to green indicates that the sample contains glycogen. The blank, standard solution and sample were heated in a bath for 10 min and then cooled to room temperature. The blank, standard solution and sample were transferred to the cuvette, and the absorbance was measured using a spectrophotometer at a wavelength of 620 nm.

The amount of glycogen is expressed as mg/g wet tissue (Satyanarayana et al. 2015). Glycogen levels (mg/g sample) were calculated using the following equation:

$$\frac{DU}{DS} \times 0.1 \times 10 \times 0.9 \qquad (2)$$

Whereas DU is the optical density of the sample, DS is the standard optical density, 0.1: mg of glucose in 2 mL of standard solution, 10: dilution factor, and 0.9: conversion factor of glucose value to glycogen value.

2.8. Serum insulin analysis

Measurement of serum insulin levels was performed using the Elabscience Mouse INS ELISA kit. Blood was taken before being given treatment (0 d) and after treatment (22 d). Blood samples were allowed to clot for 1 h at room temperature, then centrifuged for 20 min at 2,500 rpm. Then, the supernatant was collected to obtain serum.

The sandwich ELISA procedure begins with determining the wells for standards, blanks, and samples. A total of 100 mL of standards, blanks and samples were put into the appropriate wells. The plates were covered with the sealer provided in the kit and incubated for 90 min at 37 °C. The liquid from each well was discarded, and 100 mL of Biotinylated Detection antibody solution was added to each well. The wells were closed with new sealer and incubated for 1 h at 37 °C.

The solution from each well was discarded, and 350 mL of wash buffer was added to each well. Then, it was

soaked for 1 min, and the solution was removed from each well, then dried with absorbent paper. The washing step was repeated three times. A total of 100 mL of HRP Conjugate working solution was added to each well. The wells were covered with a new sealer and incubated for 30 min at 37 °C. Then, the solution from each well was removed, and the washing process was repeated 5 times.

A total of 90 mL of substrate reagent was added to each well. The wells were covered with a new sealer and incubated for approximately 15 min at 37 °C. Wells containing insulin, Biotinylated Detection antibody and Avidin-HRP conjugate will appear blue. Then, 50 mL of stop solution was added to each well, and the color changed to yellow. Then, read using a microplate reader. Optical density (OD) is calculated at a wavelength of 450 m. The concentration of insulin in the sample can be determined by comparing the OD of the sample with the standard curve. Insulin levels were expressed in units of ng/mL.

Serum insulin levels obtained were then used to determine insulin resistance. The degree of insulin resistance before and after treatment was determined by HOMA-IR. The HOMA-IR value is calculated using the following formula (Liu et al. 2019).

$$HOMA - IR = \frac{FINS\left(\frac{mIU}{L}\right) \times FBG\left(\frac{mmol}{L}\right)}{22.5} \quad (3)$$

2.9. Western Blot analysis

Detection and quantification of GLUT4 protein was carried out using the Western Blot method, according to Hnasko and Hnasko (2015), with modification. After 21 days of treatment, a total of 25 mg of muscle samples were taken and mashed. The sample was added with 200 µL Radioimmunoprecipitation assay (RIPA) lysis buffer (ratio of sample to buffer = 1:20). Then, it was homogenized with a homogenizer and centrifuged at 15,000 rpm for 5 min. The supernatant obtained was then added with 200 µL of Bicinchoninic Acid (BCA) consisting of Reagents A and B with a ratio of 1:50, namely 196 µL of Reagent A and 4 µL of Reagent B. Then incubated at 37 °C for 30 min. Sample and blank absorbance measurements were carried out with a spectrophotometer at a wavelength of 562 nm.

The sample volume was weighed, and lysis buffer was added in a ratio of 1:1 to separate the protein by breaking the disulfide bonds. The sample was heated at 95 °C for 5 min so that the protein denatured, then cooled with an ice pack until it turned blue. Afterwards, the gel and running buffer were put into the electrophoresis apparatus. Ten microliter sample and 2 µL loading ladder were put into each well. The electrophoresis apparatus was turned on at 80 V and gradually increased to 150 V for 2 h. A sandwich layer is made consisting of a sponge, filter paper, gel, membrane, filter paper and sponge. Electro transfer was performed to allow protein movement from the gel to the membrane with an electric current of 200 mA for 30 min. Then, the membrane was stained with Ponceau S. Stain, and the membrane was washed for 3×15 min with 0.1% PBST. The next stage was blocking with 0.75% skim milk and washing the membrane again for 3×15 min with 0.1% PBST. The membrane was incubated with primary antibody overnight. Subsequently, the membrane was washed for 3×15 min with 0.1% PBST. The membrane was incubated again with the secondary antibody for 2 h, and the membrane was washed again for 3×15 min with 0.1% PBST. Then, the substrate was added and incubated for 5 min, and the protein band thickness was detected.

2.10. Statistical analysis

Data were tested and visualized using the GraphPad 9.2. Data were first assessed for normality and homogeneity tests. Data were normally distributed and homogeneous, so further tests were performed. Blood glucose level data, body weight, insulin levels, and HOMA-IR values were analyzed using the Two-Way ANOVA method. Meanwhile, liver index data, liver and muscle glycogen levels, and expression of GLUT4 protein were analyzed using the One-Way ANOVA method. The analysis was continued with the Post Hoc Tukey test. The measurement results were then analyzed using the T-test to analyze the significance of the data before and after treatment. A p value of <0.05 was considered statistically significant.

3. Results and Discussion

3.1. Extraction and phytochemical analysis

A qualitative phytochemical analysis was carried out to identify the secondary metabolites present in the methanol extract of *A. bilimbi* fruit. The phytochemical qualitative analysis is shown in Table 1. Various compounds were detected from the qualitative analysis of the methanol extract of *A. bilimbi*, such as flavonoids, saponins, terpenoids, tannins, and phenols, while steroid was not detected.

The chromatogram of the GC-MS analysis result is shown in Figure 1. The result of the GC-MS phytochemical analysis is shown in Table 2. The GC-MS analysis revealed the presence of carboxylic acid derivative ester compounds in the methanol extract of *A. bilimbi* fruit, such as oxalic acid derivatives, caprylic acid derivatives, pelargonic acid derivatives and myristic acid derivatives.

3.2. Antioxidant activity by DPPH radical scavenging

An antioxidant activity test was carried out using DPPH to determine the DPPH free radical scavenging activity

TABLE 1 Phytochemical constituents of *A. bilimbi* fruit methanol extract.

No.	Bioactive compound	Result
1.	Flavonoids	+
2.	Terpenoids	+
3.	Phenol	+
4.	Tannins	+
5.	Saponins	+

(+) indicates the presence of a phytochemical compound.

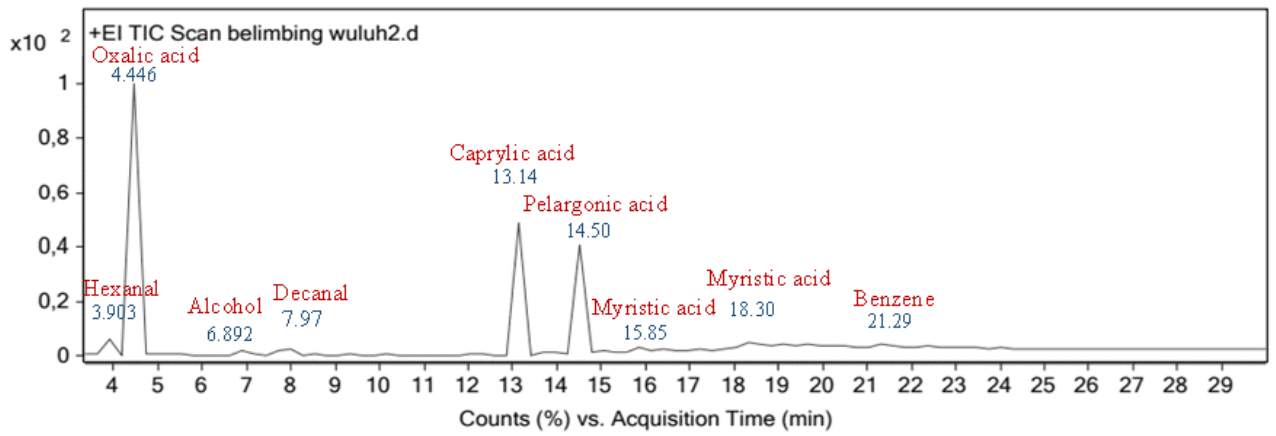


FIGURE 1 GC–MS chromatogram of *A. bilimbi* fruit methanol extract.

TABLE 2 Compounds identified by GC–MS in the *A.bilimbi* fruit methanol extract.

No.	Compound	Molecular formula	Molecular Mass	Retention time (min)	Area (%)
1.	Hexanal	$C_6H_{12}O$	100.16	3.903	564
2.	Ethanedioic acid, dimethyl ester	$C_4H_6O_4$	118.09	4.446	10000
3.	2-Nonen-1-ol, (E)-	$C_9H_{18}O$	142.24	6.892	438
4.	2-Decenal, (E)-	$C_{10}H_{18}O$	154.25	7.978	487
5.	Octanoic acid, methyl ester	$C_8H_{916}O_2$	158.24	13.14	4661
6.	7-Nonenoic acid, methyl ester	$C_9H_{16}O_2$	170.25	14.50	4269
7.	Z-8-Methyl-9-tetradecenoic acid	$C_{15}H_{28}O_2$	240.38	15.85	386
8.	cis-9-Tetradecenoic acid, isobutyl ester	$C_{18}H_{34}O_2$	282.50	18.30	393
9.	1,4-Bis(trimethylsilyl)benzene	$C_{12}H_{22}Si_2$	222.47	21.29	391

(IC_{50}) of the methanol extract of *A. bilimbi* fruit. Determination of IC_{50} value was carried out based on the absorbance measurements of the extract at different concentrations. The regression analysis curve obtained shows free radical scavenging (% inhibition) as the y-coefficient, while the log extract concentration is the x-coefficient. The scavenging activity curve of *A. bilimbi* fruit methanol extract is shown in Figure 2. The IC_{50} value of the methanol extract of *A. bilimbi* fruit is 85 µg/mL. Based on the antioxidant activity classification by Blois (1958), the IC_{50} value of A. bilimbi fruit methanol extract is in the range of 50–100 µg/mL. Therefore, it is classified as a compound with strong antioxidants. Quercetin was used as a positive control and was classified as a very strong antioxidant with an IC_{50} value of 6.141 µg/mL.

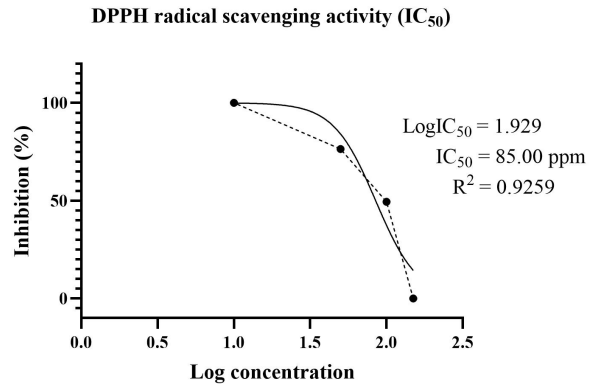


FIGURE 2 DPPH radical scavenging activity curve of *A. bilimbi* fruit methanol extract.

3.3. Acute toxicity

Treatment with *A. bilimbi* fruit methanol extract at a dose of 300 mg/kg BW as a start dose did not cause death in mice, so the treatment was repeated. After repetition, no toxic symptoms and death of mice were observed. Subsequent administration of the extract was carried out using a higher dose, 2,000 mg/kg BW. Observations were made for the initial 4 h after administration of the extract and every 24 h for 14 d. A total of 2 mice showed toxic symptoms such as weakness and tremors, which eventually died.

Based on the Globally Harmonized System (GHS) on 423 OECD (2002), methanol extract of *A. bilimbi* is classified into category 4 (dose >300–2000 mg/kg) with an LD_{50}-cut off value of 1000 mg/kg BW. Based on the classification by Indonesian Food and Drug Authority (BPOM) (2014), the methanol extract of *A. bilimbi* was categorized as slightly toxic. The acute toxicity procedure adapted from 423 OECD (2002) is shown in Figure 3.

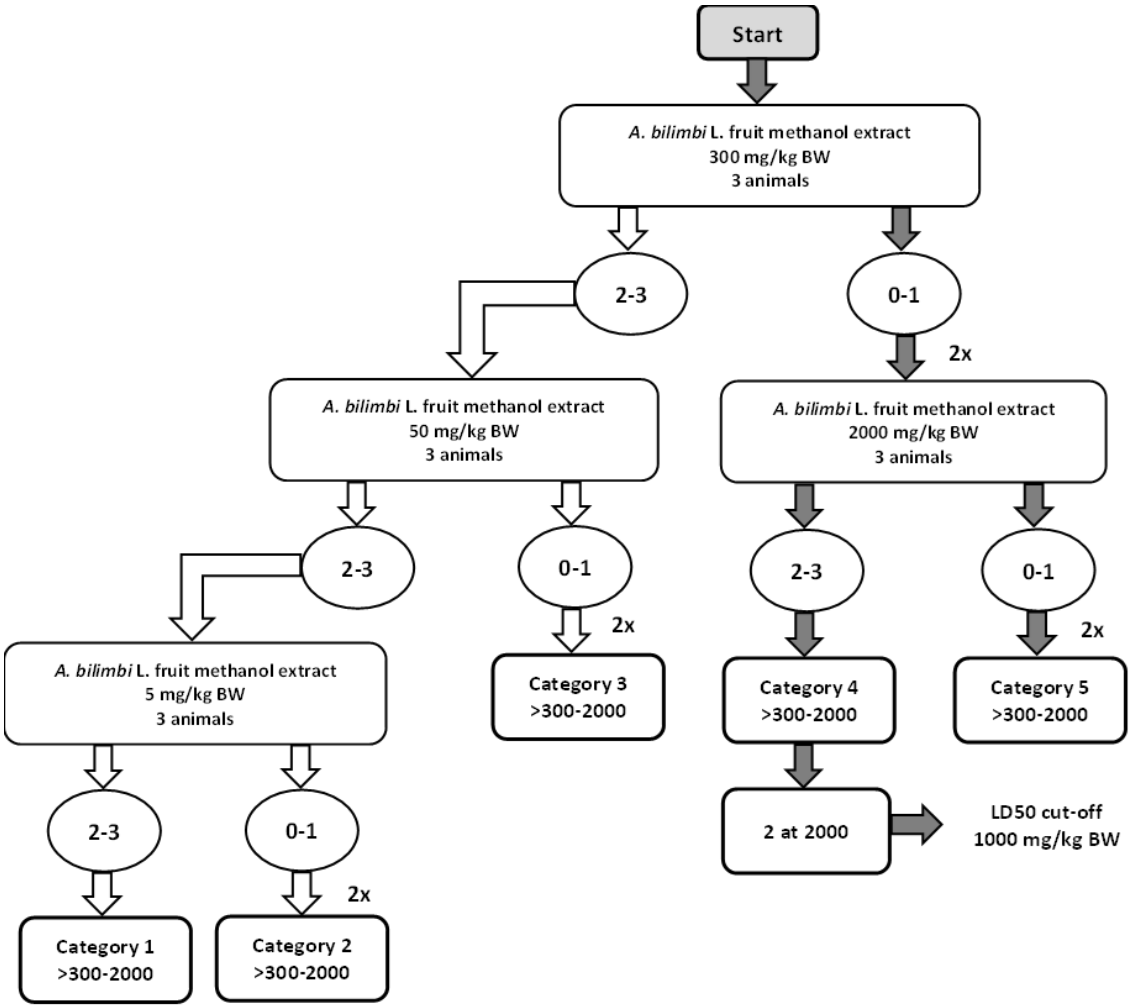


FIGURE 3 An acute toxicity test procedure shows the process for the determination of the LD_{50} cut-off value of *A. bilimbi* fruit methanol extract ((OECD 2002), with modification).

3.4. Body weight and fasting blood glucose

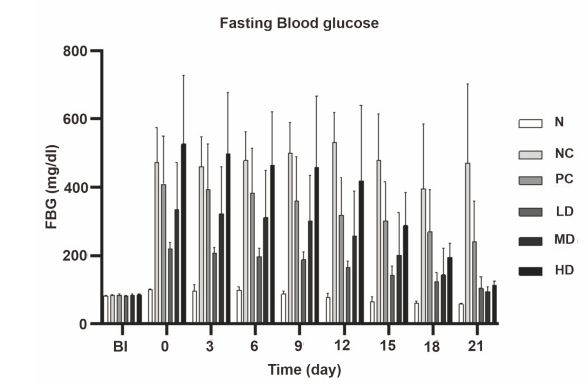
Mice were induced peritoneally with alloxan at a dose of 150 mg/kg BW before administration of *A. bilimbi* methanol extract. Mice were fasted for 12–18 h, then blood glucose levels were measured. There was a decrease in blood glucose levels in diabetic mice. The fasting blood glucose mean of mice before and after administration of *A. bilimbi* fruit methanol extract is shown in Figure 4a. The fasting blood glucose levels before and after treatment in each group were significantly different ($p < 0.05$), except for the NC group ($p > 0.05$). The results of the study showed that there was a significant effect of methanol extract of *A. bilimbi* at low, medium, and high doses on reducing blood glucose levels up to normal limits (<125 mg/dL). The greatest decrease in blood glucose levels was in the HD group, followed by the MD group, then the PC group, which was given metformin and the LD group ($p < 0.05$).

Weight loss was also found in diabetic-induced mice. The average body weight of mice before and after alloxan induction is shown in Figure 4b. There was a decrease in the body weight of diabetic mice, including the NC group, PC group, LD, MD, and HD group ($p < 0.05$). Meanwhile, the body weight of the N group did not show significant weight loss ($p > 0.05$).

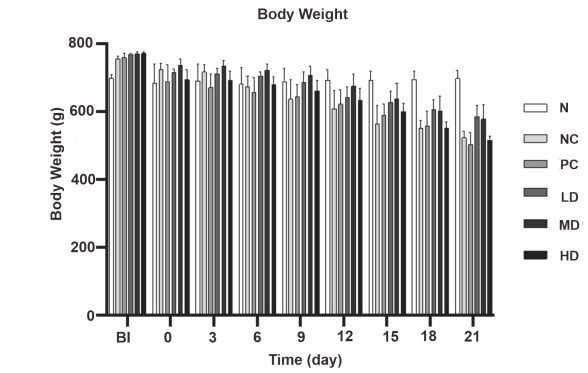
As shown in Figure 4b, the average body weight of mice before and after treatment in each diabetic group showed significant differences ($p < 0.05$). However, there was no significant difference in the Normal group ($p > 0.05$). The greatest weight loss was shown by the NC group, followed by the PC group and the HD group. The results showed that the administration of methanol extract of *A. bilimbi* did not have an inhibitory effect on weight loss in diabetic mice.

3.5. Liver weight and liver index

The average liver weight and liver index after administration of *A. bilimbi* fruit methanol extract are shown in Figure 5a and 5b. The liver index was found to be higher in the diabetic group than the N group, and among the diabetic group, the NC group had the highest liver index. A high liver weight-to-body ratio was observed in the NC group, even though there was a decrease in the body weight ($p > 0.05$).



(a)



(b)

FIGURE 4 (a) Fasting blood glucose levels; (b) Body weight. N: normal; NC: negative control; PC: positive control; LD: low dose; MD: medium dose; HD: high dose. BI: before diabetes induction.

3.6. Glycogen levels analysis

Figure 5c and 5d show that the hepatic and muscle glycogen in the diabetic group were significantly lower than that in the normal group ($p < 0.05$). Among the diabetic group, the highest hepatic and muscle glycogen levels were found in the MD group, followed by the HD group, the PC group, the LD group, and the NC group ($p < 0.05$).

The hepatic and muscle glycogen of the MD group were significantly higher than that in the NC group ($p < 0.05$). It showed the potential of methanol extract of *A. bilimbi* to increase glycogen synthesis and decrease glycogenolysis and gluconeogenesis. A significant difference was also shown by the MD group with the PC group ($p < 0.05$). The result also indicated a more optimal ability of the extract to increase the hepatic and muscle glycogen levels compared to metformin.

3.7. Serum insulin analysis

Serum insulin levels before and after administration of *A. bilimbi* fruit methanol extract are shown in Figure 6a. Serum insulin levels in diabetic mice before treatment were high. High serum insulin levels (hyperinsulinemia) together with high blood glucose levels (hyperglycemia) might indicate insulin resistance. Alloxan causes partial degradation and dysfunction of the beta cells in the pancreas and leads to inhibition of insulin production. In Figure 6a, the fasting blood glucose of the NC group after induction of alloxan shows a significant increase compared to the N group. Alloxan increases fasting blood glucose in mice. The fasting blood glucose in the N group remains normal because no alloxan is inducted.

Interestingly, the NC group showed higher insulin serum levels than the N group. The possible mechanisms underlying these conditions are the inhibition of glucose uptake in diabetic mice, causing impaired glucose metabolism that leads to lipolysis and an increase in free fatty acids (Jiang et al. 2020). Alternatively, high blood glucose can also cause DAG and ROS synthesis interfere with insulin signaling (Mukherjee et al. 2013) and result in insulin resistance.

There were significant differences in insulin serum levels before and after the treatment in the NC group and the LD, MD, and HD groups. It indicated the potential of methanol extract of *A. bilimbi* fruit to reduce hyperinsulinemia, which is one of the characteristics of diabetes. However, no significant difference was found between the PC group and the LD, MD, and HD groups. The methanol extract of *A. bilimbi* fruit with different doses might have the same effect on insulin levels in diabetes. Furthermore, there was a significant difference between the insulin levels before and after treatment in the PC group, LD, MD, and HD groups.

Insulin resistance before and after treatment was evaluated to calculate the HOMA-IR value. The HOMA-IR value is determined based on the value of insulin levels and blood glucose levels. As shown in Figure 6b, the highest HOMA-IR was found in the NC group, followed by the HD group and the PC group.

3.8. Western Blot analysis

Western blot analysis is shown in Figure 7. The analysis revealed that the thickest band was found in the MD group, followed by the LD group and the PC group. As well as the highest levels of GLUT4 protein expression relative to GAPDH were found in the MD group, followed by the LD group and the PC group ($p < 0.05$). A medium dose (250 mg/kg BW) of methanol extract of *A. bilimbi* shows the highest GLUT4 expression effect in diabetic mice. Meanwhile, the lower dose (50 mg/kg BW) and higher dose (300 mg/kg BW) are less optimal to increase GLUT4 protein expression.

3.9. Discussion

GLUT is a transmembrane protein that is responsible for transporting glucose across the cell membrane. Upon refeeding, an increase in blood glucose levels stimulates insulin secretion from pancreatic beta cells. The insulin receptor is a protein tyrosine kinase tetramer with two α-subunits and two β-subunits. When insulin binds to its receptor α-subunits on the cell membrane, receptor β-subunits auto phosphorylate each other. Then, the phosphorylated β-subunits recruit insulin receptor substrates (IRS) and phosphorylate them. The phosphorylated IRSs bind to and activate phosphatidylinositol 3-kinase (PI3K),

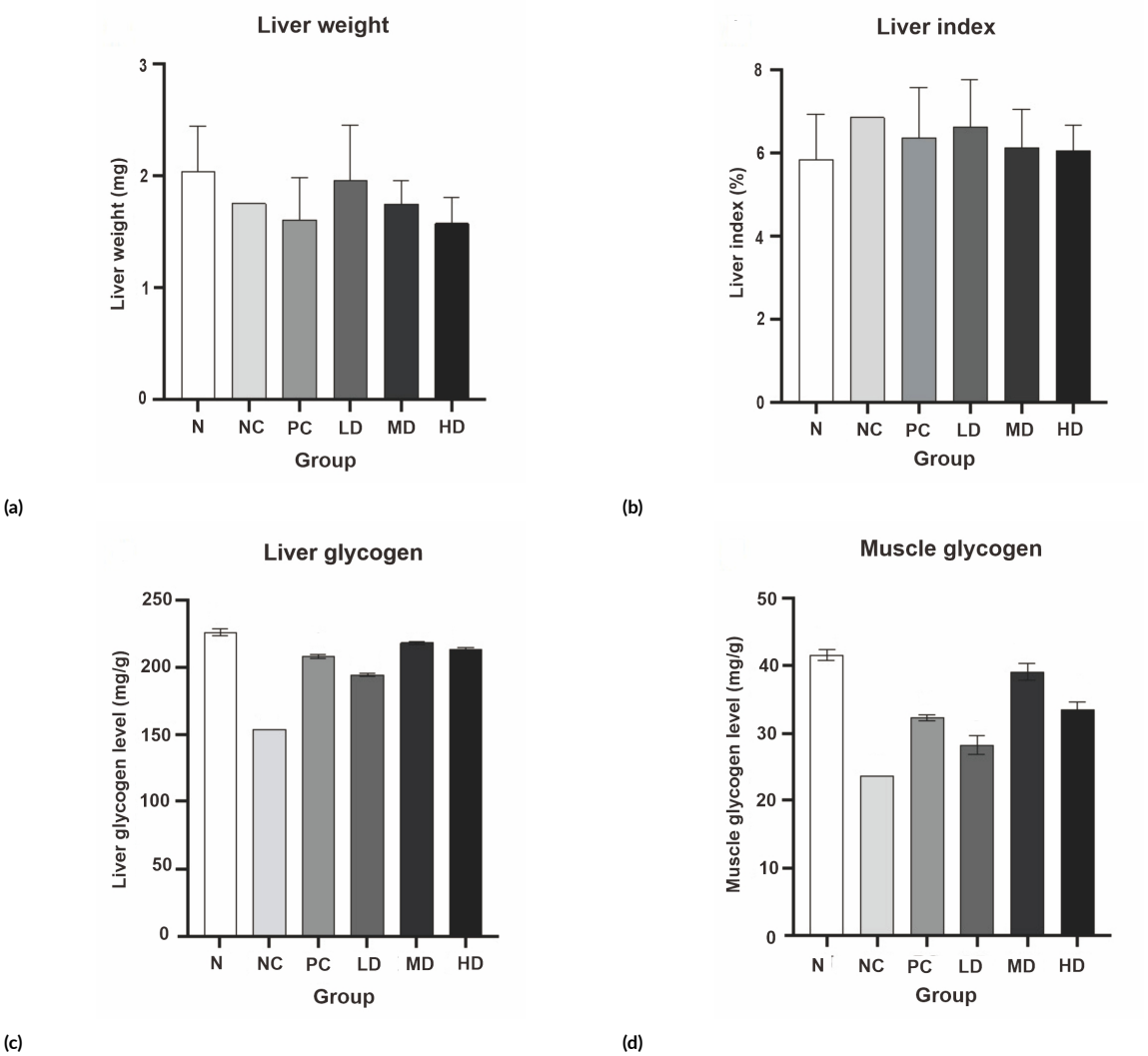


FIGURE 5 (a) Liver weight; (b) Liver index; (c) Liver glycogen levels; (d) Muscle glycogen levels. N: normal; NC: negative control; PC: positive control; LD: low dose; MD: medium dose; HD: high dose. a $p < 0.05$ indicates a significant difference with the normal group, b $p < 0.05$ indicates a significant difference with the negative control group, and c $p < 0.05$ indicates a significant difference with the positive control group.

which is recruited to the plasma membrane and converts phosphatidylinositol 4,5- bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). Then PIP3 activates AKT (protein kinase B, PKB), which triggers the translocation GLUT4 containing vesicles from intracellular compartments to the cell membrane. GLUT4 translocation facilitates glucose transport into cells and maintains glucose homeostasis (Mukherjee et al. 2013; Hajiaghaalipour et al. 2015; Md Sayem et al. 2018; Wang et al. 2020).

The phytochemical qualitative analysis revealed that the methanol extract of *A. bilimbi* fruit contains bioactive compounds, such as flavonoids, saponins, terpenoids, tannins, and phenols. Meanwhile, steroid compounds were not detected. The qualitative analysis result is in accordance with research conducted by Abraham (2016) and Suluvoy and Berlin Grace (2017). The GC-MS analysis revealed the presence of carboxylic acid derivative ester compounds in the methanol extract of *A. bilimbi* fruit, such as oxalic acid derivatives, caprylic acid derivatives, pelargonic acid derivatives and myristic acid derivatives. These compounds are classified into the medium-chain fatty acid (MCFA) group. Other compounds found in small quantities include aldehyde group compounds, such as hexanal and decanal, alcohol and benzene. Previous studies have shown that terpenoids can inhibit α-amylase and α-glucosidase (Ortega et al. 2022). These two enzymes play a major role in the hydrolysis of starch into glucose and can increase blood glucose levels. Based on previous research, flavonoids, phenols and terpenoids can reduce sodium–glucose cotransporter (SGLT1), causing inhibition of glucose absorption in the intestine (Ortega et al. 2022). Inhibition of starch hydrolysis and glucose absorption can inhibit the increase in blood glucose levels.

Previous studies showed that flavonoid compounds can increase glycogenesis and reduce gluconeogenesis in the liver and muscles (Hajiaghaalipour et al. 2015). Furthermore, medium-chain fatty acids (MCFA) compounds

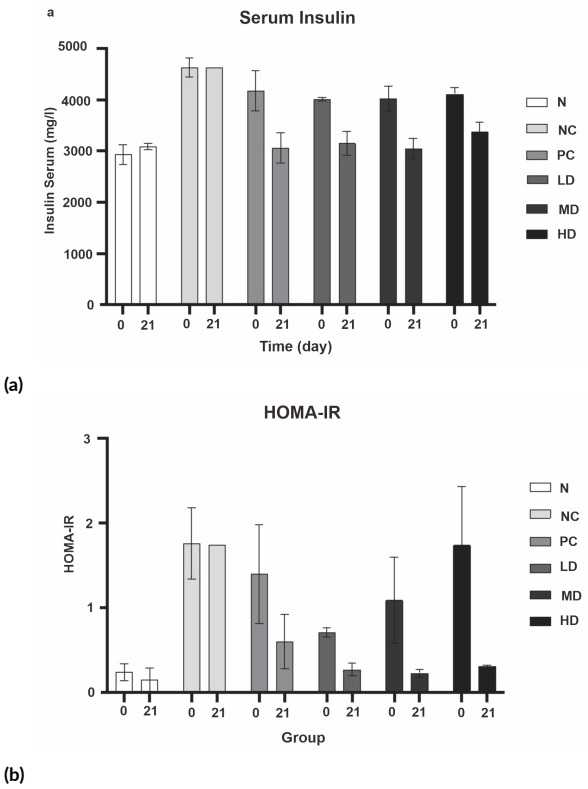


FIGURE 6 (a) Serum insulin levels; (b) HOMA-IR. a $p < 0.05$ indicates a significant difference with the normal group, b $p < 0.05$ indicates a significant difference with the negative control group, c $p<0.05$ indicates a significant difference with the positive control group.

such as caprylic acid (C8) and pelargonic acid (C9) can increase cell sensitivity to insulin through their ability to inhibit inflammation due to ROS (Thomas et al. 2019). Flavonoids and phenols can increase GLUT4 protein expression and insulin signaling by preventing decreased levels of phosphorylated tyrosine and insulin receptors and inhibiting the PI3K/AKT pathway (Hajiaghaalipour et al. 2015; Md Sayem et al. 2018). Based on previous studies, among the phytochemical compounds found, flavonoid compounds, phenols and MCFA derivatives in the methanol extract of *A. bilimbi* are thought to be able to regulate increased expression of GLUT4 protein (Hajiaghaalipour et al. 2015; Md Sayem et al. 2018; Thomas et al. 2019).

Based on the antioxidant activity classification table by Blois (1958), the IC_{50} value of the methanol extract of *A. bilimbi* fruit is in the range of 50–100 µg/mL. Therefore, it was classified as a compound with strong antioxidant. Oxidative stress was suggested as one of the mechanisms underlying alloxan-induced diabetes. It was initiated by the reduction of alloxan through interaction with intracellular thiols such as the tripeptide glutathione (GSH) to produce dialuric acid and oxidized glutathione (GSSG). Dialuric acid was oxidized back to alloxan which produces alloxan radicals and ROS such as superoxide radicals ($O2^{\cdot-}$), which undergo dismutation by superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2). Hydroxyl radicals ($OH^{\cdot-}$) can be formed in Fenton reactions with suitable metal catalysts and can ultimately cause damage and dysfunction of pancreatic β cells (Lenzen 2008). Antioxidants protect the cells from the harmful effects of free radical damage and oxidative stress. The presence of bioactive compounds such as flavonoids, phenols, terpenoids and tannins could probably cause the strong antioxidant activity of the methanol extract of *A. bilimbi* fruit. The antioxidant mechanisms of these compounds include direct scavenging or detoxification of reactive oxygen species (ROS), inhibiting the formation of ROS through inhibition of xanthine oxidase and NADPH oxidase, both of which are enzymes involved in the formation of ROS, as well as inducing the activity of antioxidant enzymes (Wojtunik et al. 2014). In addition, medium-chain fatty acid (MCFA) compounds such as caprylic acid (C8) and pelargonic acid (C9) can inhibit the formation of ROS (Thomas et al. 2019). The ability to inhibit ROS by this compound is due to increased expression of uncoupling protein 3, which acts as a carrier protein in the mitochondrial inner membrane and is involved in oxidative phosphorylation (Garvey 2003). Further research is needed for this finding.

Based on the Globally Harmonized System (GHS) on 423 OECD (2002), methanol extract of *A. bilimbi* fruit is classified into category 4 with an LD_{50}-cut off value of 1,000 mg/kg BW. Based on classification by Indonesian Food and Drug Authority (BPOM) (2014), the methanol extract of *A. bilimbi* fruit is categorized as mildly toxic, which is safe to use in this study.

Alloxan induction causes an increase in high blood glucose levels. As explained by Lenzen (2008), alloxan is a chemical compound that is diabetogenic. Alloxan selectively causes pancreatic β-cell dysfunction and death through inhibition of glucokinase and release of free radicals, which induce oxidative stress of pancreatic β-cells. Glucose concentrations that continue to rise above the physiological range produce the manifestations of diabetes. Induction of alloxan not only causes an increase in blood glucose levels but also causes disturbances in the body's metabolism. Weight loss in diabetic mice can occur due to impaired glucose metabolism. Failure to take up glucose by the tissues causes the body to lack energy sources from glucose, resulting in a lipid overhaul that can reduce body weight.

Furthermore, failure of glucose metabolism also causes a decrease in protein synthesis due to decreased uptake of amino acids by tissues. This imbalance in protein synthesis and protein catabolism also causes changes in body tissue metabolism, thus causing weight loss. Diabetes caused the death of three mice from the negative control group at the end of the treatment. The results of the observations showed the presence of symptoms of polyuria and polydipsia accompanied by high blood glucose levels. At the end of the treatment, diabetes caused the death of 3 mice in the Negative Control group. The death of mice was thought to be caused by complications of severe hyperglycemia. The surviving mice observed until the last day of treatment (day 21) were in accordance with previ-

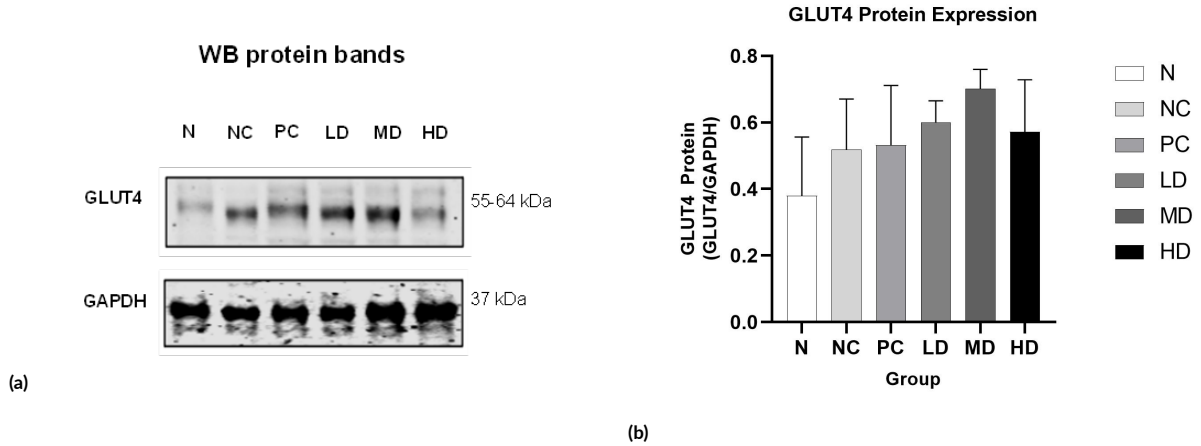


FIGURE 7 (a) GLUT4 protein bands; (b) GLUT4 protein expression density. N: normal; NC: negative control; PC: positive control; LD: high dose; MD: medium dose; HD: high dose.

ous findings that rats that died within five days after injection of alloxan monohydrate occurred due to acute toxicity of alloxan monohydrate. In contrast, rats that died more than 10 d after alloxan injection, it occurs due to complications of severe hyperglycemia (Nakahara et al. 2014). This study showed the potential of methanol extract of *A. bilimbi* fruit as an antidiabetic by reducing blood glucose levels but did not have an inhibitory effect on weight loss in diabetic mice.

The liver has a special role in glucose metabolism and is essential for systemic glucose homeostasis. The balance between the utilization and output in the liver and the regulation of skeletal muscle and adipose tissue are the most important factors in the blood glucose balance. The liver is also an important organ in lipid metabolism (Jiang et al. 2020). Glucose and lipid metabolism in the liver is a complex and inseparable process. Impaired insulin secretion, such as in diabetes, can inhibit glucose uptake by the liver and muscles. The effect of alloxan-induced diabetes on liver weight was not certain and still needs further investigation. However, many studies observed several pathological changes in the livers of alloxan-induced diabetic mice. Accumulation of fat in the liver could be an indicator of liver enlargement, and liver enlargement can be measured by liver index (Huang et al. 2021). Diabetic conditions create alterations in triglyceride secretion by liver cells. A large amount of glucose is transported to the liver, which eventually generates an additional stock of this carbohydrate in hepatocytes that is rapidly diverted to the synthesis of fatty acids (Lucchesi et al. 2015). This situation will increase lipolysis, the decomposition of fatty acids into free fatty acids (FFA), and some of the FFA will be taken to the liver to form triglycerides through the process of lipogenesis (Huang et al. 2021). Hyperglycemia induces the activation of transcription factors of hepatic metabolism, such as sterol regulatory element-binding proteins (SREBPs) and the carbohydrate response element-binding protein (ChREBP), which would promote hepatic lipogenesis (Lucchesi et al. 2015). It can be attributed to the increased accumulation of fat in the liver. However, the results showed that there was no significant effect of the administration of methanol extract of *A. bilimbi* fruit on decreasing the liver index in diabetic mice ($p > 0.05$).

Glycogen is the main storage form of carbohydrates in the body. Glycogen is found mainly in the liver and muscle, making up 3-6% and about 0.5% of the fresh weight of the liver and muscle, respectively (Jiang et al. 2020). Glycogen in the liver acts as the primary storage source that maintains blood glucose homeostasis, and glycogen in the skeletal muscles provides energy to the muscles during high-intensity activities. Net glycogen deposition in the liver and muscle depends on the coordinated inhibition of glycogenolytic molecules and the stimulation of molecular glycogen synthesis. Glucose is the main inhibitor of glycogen breakdown (glycogenolysis), and insulin is the main activator of glycogen synthesis (glycogenesis) (Jiang et al. 2020). Diabetic mice experience impaired glucose metabolism in the liver and muscles, which is characterized by decreased glycogen synthesis and glucose utilization through increased glycogenolysis and gluconeogenesis. Insulin not only plays a role in glucose uptake but specifically stimulates glycogen synthesis. Decreased glycogen synthesis is also associated with impaired insulin signaling. This study showed that there was a significant effect of *A. bilimbi* fruit methanol extract on liver and muscle glycogen levels ($p < 0.05$), even more optimal in increasing liver and muscle glycogen levels compared to metformin.

Serum insulin level before treatment was quite high. Hyperinsulinemia together with hyperglycemia indicates the possibility of insulin resistance. Insulin resistance is a pathophysiological condition characterized by impaired biological response to insulin stimulation of target tissues such as adipose, muscle and liver (Cerf 2013). Pancreatic β cells secrete a lot of insulin (hyperinsulinemia), while target tissues cannot respond properly to insulin, resulting in high blood glucose levels. This study shows the po-

tential of methanol extract of *A. bilimbi* fruit in reducing hyperinsulinemia.

The finding is supported by HOMA-IR values. The highest HOMA-IR value was found in the mice in the NC group. In humans, the HOMA-IR <1 indicates insulin sensitivity, while the HOMA-IR >1.4 indicates initial insulin resistance and risk of diabetes mellitus (Lee et al. 2016). The HOMA-IR value in normal mice was <0.7, while the HOMA-IR in diabetic mice was >1. A high HOMA-IR value indicates high insulin resistance or failure of insulin sensitivity (Huang et al. 2021). Hyperglycemia in diabetic conditions can induce DAG and ROS synthesis (Mukherjee et al. 2013), Thus stimulating the activation of PKC, NF-kB and IKK proteins, which can interfere with insulin binding to insulin receptors in the plasma membrane and ultimately cause insulin resistance. Administration of methanol extract of *A. bilimbi* fruit showed a decrease in the HOMA-IR value, which was thought to indicate a restoration of insulin resistance.

Western blot analysis showed that *A. bilimbi* fruit methanol extract at a dose of 250 mg/kg BW increased GLUT4 protein expression in diabetic mice. Meanwhile, the lower dose, 50 mg/kg BW, is less optimal in increasing GLUT4 protein expression. On the other hand, a dose of 300 mg/kg BW also did not show the ability to increase GLUT4 protein expression. The reason for the lesser effect of HD treatment on increasing GLUT4 protein expression is still uncertain. However, based on the acute toxicity test, the methanol extract of *A. bilimbi* was considered mild toxic with an LD_{50} value of 1,000 mg/kg BW. High doses of extract presumably have toxic effects, which reduce its ability to increase the expression of GLUT4 protein. Phytochemical analysis revealed the presence of several medium-chain fatty acid derivatives, which in high doses might affect increasing the free fatty acids. According to Mukherjee et al. (2013), free fatty acids have a disruption effect on the translocation of GLUT4 protein from intracellular storage vesicles to the cell membrane by inhibition of AKT/PKB activation.

Meanwhile, the GLUT4 protein expression in the Normal group was lower compared to all the diabetic groups, even the Negative Control. It might be due to the lack of stimulation of glucose and insulin in normal mice. According to Wang et al. (2020), in an unstimulated state, most GLUT4 protein is in the intracellular vesicle storage (GSV). The level of GLUT4 protein on the cell membrane is regulated by the rate of the movement from intracellular GSV to the cell membrane. GLUT4 cycles between the cytoplasmic storage site and cell membrane, and it is stimulated by elevated blood glucose and insulin signaling. Therefore, the GLUT4 protein expression in the Normal group was low. Clearly, further studies of the effect of the extract on GLUT4 expression and translocation are warranted in the future.

The result also demonstrated lower protein expression of GLUT4 in the Positive Control compared to the Negative Control. Minamii et al. (2018) study showed that metformin treats diabetes by inhibiting gluconeogenesis, thereby inhibiting liver glucose production and causing a decrease in blood glucose levels. The result is in line with Herman et al. (2022) which revealed metformin treatment in the skeletal muscle of subjects with newly diagnosed diabetes mellitus showed no effect on the expression of *GLUT4* mRNA. The administration of methanol extract of *A. bilimbi* fruit showed a higher density of GLUT4 protein expression than that of metformin, similar to previous study that was using a leaf extract of Artemisia annua (Kartikadewi et al. 2019). These results indicate that the methanol extract of *A. bilimbi* fruit is thought to be more optimal in increasing GLUT4 protein expression. However, the mechanism of action of metformin through the GLUT4 protein still needs further study.

Measurements of blood glucose levels, glycogen levels and insulin support GLUT4 protein expression finding. A decrease in blood glucose levels to normal limits (<125 mg/dL) was found in the group of mice given *A. bilimbi* fruit methanol extract at a dose of 250 mg/kg BW. Correspondingly, the highest levels of liver and muscle glycogen and the lowest levels of serum insulin and HOMA-IR were found in the group of mice given A. bilimbi fruit methanol extract at a dose of 250 mg/kg BW.

GLUT4 plays an important role in regulating glucose homeostasis in the body. GLUT4 protein is a glucose transporter that is insulin dependent, so the role of GLUT4 is closely related to insulin signaling and metabolism in various insulin-sensitive tissues, such as the liver and muscle (Md Sayem et al. 2018). When postprandial glucose

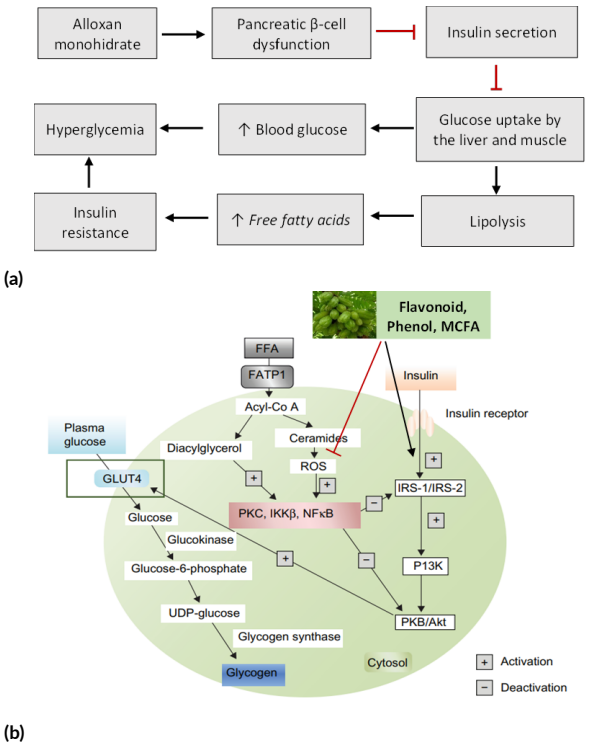


FIGURE 8 (a) Metabolic changes in diabetes; (b) Pathway of antidiabetic activity of methanol extract of *A. bilimbi* fruit through regulation of glucose transport by GLUT4 (Mukherjee et al. 2013), with modification).

levels increase, insulin binds to insulin receptors. It activates signaling cascades, which ultimately trigger translocation of the GLUT4 storage compartment to the cell membrane, thereby facilitating glucose transport into cells and glycogen storage in the liver and in muscle and regulating normal blood glucose levels. Metabolic changes in diabetes and the pathway of antidiabetic activity of methanol extract of *A. bilimbi* fruit through regulation of glucose transport by GLUT4 are summarized in Figure 8.

4. Conclusions

In conclusion, the medium dose of *A. bilimbi* methanol extract seems to have a potential effect on the treatment of diabetes with the probable mechanism of targeting GLUT4 protein expression.

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Authors' contributions

GYJ and AR designed the study. GYJ and HO carried out the laboratory work. GYJ and AR analyzed the data. GYJ wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interest.

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