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Effect of *Averrhoa bilimbi* Leaf Extract on Blood Glucose Level, Hepatosomatic Index (HSI), and Liver Histology of Diabetic Mice

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ABSTRACT

***Averrhoa bilimbi* leaf extract (BLE) contains high antioxidant levels. Antioxidants can suppress reactive oxygen species produced during hyperglycemic conditions in diabetes mellitus (DM), which cause damage to liver tissue. This study aimed to determine the BLE effect on fasting blood glucose (FBG), Hepatosomatic Index (HSI), and liver tissue damage. We used 24 mice in 6 different groups, divided by N (normal), K- (DM), K+ (DM+glibenclamide 0.013mg/20gBW), E1 (DM+ extract 6.3mg/20gBW), E2 (DM+ extract 8.4mg/20gBW), and E3 (DM + extract 10.5mg/20gBW). We used an alloxan dose of 120mg/kgBW to induce the DM and then treated with BLE for 14 days. We measured the fasting blood glucose using a glucometer. In addition, we evaluated the liver tissue damage with HSI and Hematoxylin-Eosin (HE) stained histological slides. The results showed that BLE significantly reduced the percentage of FBG and liver tissue damage, while HSI showed no significant difference. The most optimal extract dose was 8.4 mg/20gBW (E2 group), with an FBG decrease of 26.44%, a normal cell percentage of 88.56%, and an HSI score of 6.18%. Based on this finding, we concluded that bilimbi leaf extract could lower blood glucose and improve liver histology of diabetic mice but did not significantly affect HSI.**

1. Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia due to secretion disruption and/or resistance of insulin hormone from pancreatic β-cells (Glovaci *et al.* 2019). Diabetes mellitus is divided into DM type I and type II (Dharmayudha and Anthara 2013). In DM type I, damage to pancreatic-β cells cause insulin production to decrease, whereas, in DM type II, insulin resistance occurs, which is caused by reducing the sensitivity of tissue organ to insulin stimulation (Perkumpulan Endokrinologi Indonesia 2021). This leads to increased insulin need, but the demand cannot be met by pancreatic β-cells (Petersmann *et al.* 2019). Diabetes mellitus type II was ranked 5th in deaths from non-infectious diseases (IDF 2017) and showed an increase in the number of patients with DM type II aged 15 years and over, from 6.9 to 8.5% (Kemenkes 2018).

Diabetes mellitus can cause various organ damage, such as kidney, pancreas, liver, and retina. This is due to the high glucose level in the blood causing decrease and blockages of the bloodstream and disruption in various organ functions (Suastika *et al.* 2011). In addition, hyperglycemia also encourages the formation of Reactive oxygen species (ROS) (Salway 2012; Szenroedi *et al.* 2012). Reactive oxygen species (ROS) is one of the oxidants produced by the body during oxygen metabolism. One of the organs most likely to be attacked by ROS is the liver (Sánchez-Valle *et al.* 2012). Reactive oxygen species (ROS) can damage cell membranes and produce malondialdehyde (MDA), a lipid peroxidation product. Radical compounds of ROS can also cause the liver to become resistant to insulin (Tangvarasittichai 2015). This resistance can cause the hyperglycemic condition to become worse, which can cause and exacerbate DM type II. In addition, insulin resistance can also cause the accumulation of lipids in hepatocytes, resulting in Nonalcoholic Fatty Liver Disease (NAFLD) (Szendroedi *et al.* 2012). Diabetes mellitus and its treatment can have unwanted side

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effects or outcomes, which might increase patient complications (Pandanwangi *et al.* 2018). One of the organs that can be affected is the liver due to ROS and NAFLD formation (Szendroedi *et al.* 2012). The liver is one of the most important organs and plays a significant role in gluconeogenesis and glycogenesis (Al Ansori and Lipoeto 2020). Disruption of these two processes in DM patients causes an increase in ROS, which can lead to cell death, fibrosis, and necroinflammation (Sundaram *et al.* 2013; Wainwright 2015). Therefore, DM treatment should not only focus on reducing blood glucose levels but also be able to maintain the condition of various other organs so that it can increase the success of DM recovery.

Various natural ingredients have been proven to help relieve DM symptoms with the help of bioactive compounds in them (Patel *et al.* 2012). The bioactive compounds can be found as antioxidants, such as alkaloids, saponins, flavonoids, and polyphenols. Antioxidants are needed to scavenge free radicals (Prakash *et al.* 2014). Antioxidants mainly break free radical chain reactions and neutralize them, protecting them from the detrimental effects of excessive oxidation reactions. In DM patients, the production of free radicals due to glucose autooxidation exceeds the cell's ability to neutralize it. The increase of untreated free radicals can cause irreversible cell damage. Therefore, due to DM, exogenous antioxidants can suppress the rise of free radical production (Setiawan and Suhartono 2005). One of the natural ingredients potentially containing a high level of antioxidants is the leaf of *Averrhoa bilimbi*. The antioxidant activity of the phytochemical compound of *A. bilimbi* leaf ethanol extract was classified as very high (Hasim *et al.* 2019). This study aimed to know the effect of bilimbi leaf ethanol extract on blood glucose levels, hepatosomatic index (HSI), and liver histology of alloxan-induced diabetic mice. This study was expected to provide information on alternative DM treatment from natural ingredients to lower blood glucose levels and play a role as a hepatoprotective agent so it can reduce the negative effect of complications due to hyperglycemia condition.

2. Materials and Methods

2.1. Bilimbi Leaf Extraction

Averrhoa bilimbi leaf was collected from bilimbi plants in Benjeng District, Gresik Regency. Bilimbi leaves were then let dry without sunlight and put into an oven for four days at 40°C. The dry bilimbi leaves were then mashed and macerated using 96% ethanol. Maceration was repeated thrice, each for 24 hours, with a ratio of 1:3, 1:2, and 1:2 (w/v). The maceration result was using a rotary vacuum evaporator until thick bilimbi leaf extract (BLE) was obtained.

2.2. Diabetes Mellitus Induction and Blood Glucose Measurement

Induction of alloxan was conducted after mice were acclimated for seven days. Alloxan was induced intraperitoneally with a 120 mg/KgBW dissolved in 0.5 M sodium citrate buffer pH 4.5. After 6 hours of alloxan induction, mice were given a liquid of 10% sucrose for 72 hours. Mice then fasted for 10 hours to measure fasting blood glucose (FBG H0). FBG was measured from peripheral blood via tail using a glucometer on the treatment period's 7th day (H7) and 15th day (H15). FBG H7 and FBG H15 were then calculated in percentage against FBG H0 using the formula below to determine FBG decline.

$$\text{Percentage of FBG decline (\%)} = \frac{\text{(FBG H0 - FBG H7 or H15)}}{\text{FBG H0}} \times 100\%$$

2.3. Bilimbi Leaf Extract Treatment

Bilimbi leaf extract was given orally for 14 days after mice had induced DM. Mice were divided into six groups; normal control (N), i.e. normal mice without any treatment. Positive control (K+), i.e. diabetic mice given glibenclamide (0.013 mg/20gBW), negative control (K-), i.e. diabetic mice group without any other treatment, and three groups of diabetic mice given bilimbi leaf extract, with three varying doses; 6.3 mg/20gBW (E1 group), 8.4 mg/20gBW (E2 group), and 10.5 mg/20gBW (E3 group) respectively.

2.4. Liver Collection

On the 15th day of treatment, mice were weighed and sacrificed. The liver was collected, weighed, and fixed in 10% neutral buffer formalin (NBF 10%). The Hepatosomatic index (HSI) was then calculated using the following formula based on mice weight after treatment and liver weight.

$$\text{Hepatosomatic index (HSI) (\%)} = \frac{\text{Liver weight (g)}}{\text{Total body weight (g)}} \times 100\%$$

2.5. Histological Slide Preparation and Evaluation

The liver was fixed in Neutral Buffer Formalin and processed into a histological slide using the paraffin

method, sectioned at 4 µm thickness and stained using hematoxylin-eosin (HE). The histological slide was evaluated using a light microscope with 400x magnification, including normal, degenerated, and necrosis cells. The rate of liver damage was then determined using the following formula (Januar *et al.* 2014).

$$\text{Liver damage (\%)} = \frac{\text{Damaged cells}}{\text{Total cells}} \times 100\%$$

2.6. Data Analysis

Blood glucose level, hepatosomatic index (HSI), and liver cell damage were analyzed statistically using the SPSS program. First, data was tested using the Kolmogorov-Smirnov test to determine normality and distribution and then continued with the Anova test and post-hoc Duncan's test to determine the difference between groups.

3. Results

3.1. Effects of BLE on Blood Glucose Level

The blood glucose level recording obtained from 6 treatment groups on day 0 (pre-treatment), 7th (in the middle of treatment), and 15th (post-treatment) is presented in Table 1.

The data in Table 1 shows that on day 0 (3 days after alloxan induction), the FBG of DM-induced treatment groups were not significantly different with FBG values > 120 mg/dl, while normal control was significantly different from the other groups. FBG on day 15 of treatment showed that all groups except three significantly differed from K-. However, there is a clear change in FBG of E3 and K+ groups from day 0 to day 15. The decline of FBG percentage on day 7 and day 15 against day 0 is presented in Figure 1.

Table 1. Fasting blood glucose level (FBG) is the average of treatment groups

Treatment Group	Fasting blood glucose level (mg/dl)		
	Day 0 ± SD	Day 7 ± SD	Day 15 ± SD
E1	126.75±8.14^{b}	124.75±20.76^{c}	97.00±9.13^{ab}
E2	127.25±14.5^{b}	106.5±9.15^{b}	93.00±11.16^{a}
E3	149.75±38.1^{b}	128.75±14.4^{c}	115.25±11.61^{bc}
K+	133±16.31^{b}	98.5±20.68^{b}	91.00±9.96^{a}
K-	124.25±13.52^{b}	114.75±12.68^{b}	123.75±9.24^{c}
N	86.00±10.23^{a}	60.5±4.79^{a}	92.75±11.08^{a}

E1: bilimbi leaf extract 6.3 mg/20gBW, E2: bilimbi leaf extract 8.4 mg/20gBW, E3: bilimbi leaf extract10.5 mg/20gBW, K+: glibenclamide 0.013 mg/20gBW, K-: diabetic control group, N: normal control

*Different letters on one column show significant differences between treatment groups on the same day ($p<0.05$)

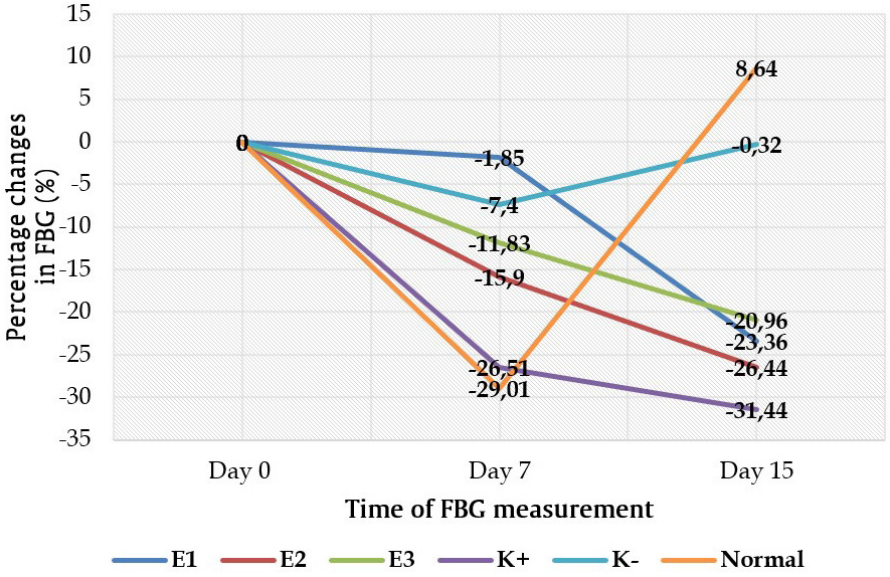

Figure 1. The decline rate of fasting blood glucose (FBG). E1: bilimbi leaf extract 6.3 mg/20gBB, E2: bilimbi leaf extract 8.4 mg/20gBB, E3: bilimbi leaf extract 10.5 mg/20gBB, K+: glibenclamide 0.013 mg/20gBW, K-: diabetic control group, N: normal control

Figure 1 shows that the biggest percentage decline in FBG was from the K+ group at 31.44%, followed by E2 at 26.44%, E3 at 23.36%, and E1 at 20.96%. In E1, E2, E3 and K+ group, FBG continued to decline, while in K- and Normal groups, the decline happened to the 7th day before FBG increased up to the 15th day. Thus, bilimbi leaf extract treatment had a significant effect in decreasing FBG.

3.2. Effects of BLE on Hepatosomatic Index (HSI)

Table 2 shows the mice's recorded body weight, liver weight, and HSI after treatment. No significant difference between groups was found for body and liver weight, however HSI was significantly different for E2 group.

3.3. Effects of BLE on Liver Histology

Table 3 presents the percentage of liver tissue damage in the different treatment groups. The results of histological slide evaluation observed using a 400x magnification light microscope are shown in Figure 2.

Histological observation of the liver showed that oral treatment of bilimbi leaf extract for 14 days significantly lowered tissue damage in hyperglycemic mice (Table 3, Figure 2). The diabetic control group (K-) had a lower percentage of normal cells and significantly differed from the other treatment groups (Table 3). Based on the data presented in Table 3, the highest normal cell average was group E2 at 88.56%, while the lowest was K- at 59.30%. Group E1, E2, E3, K+ and N was found to have >80% normal cells, while K- group had significantly different normal cell percentage of 59.30%. This shows that bilimbi extract treatment had the potential as a hepatoprotective agent in the diabetes model.

4. Discussion

Glibenclamide and BLE oral treatment significantly reduced FBG (Figure 1). Glibenclamide is an oral antidiabetic hypoglycemic drug derived from sulfonylurea that can actively lower blood glucose levels (Rabbani *et al.* 2010). Glibenclamide works by stimulating insulin secretion from the secretory granules of pancreatic β cells through the interaction of ATP-sensitive K channels on the pancreatic cell membrane. This causes the cell membrane to depolarize, and Ca2+ ions enter the pancreatic β cells, stimulating the granules to secrete insulin (Solikhah *et al.* 2020).

Table 2. Average hepatosomatic index (HSI) after administration of bilimbi leaf extract in diabetic mice

Treatment	Body weight (g)	Liver weight (g)	HSI (%)
E1	30.31±2.34^{a}	1.45±0.16^{a}	4.78±0.26^{ab}
E2	29.38±4.01^{a}	1.84±0.73^{a}	6.18±1.81^{b}
E3	30.98±3.89^{a}	1.48±0.33^{a}	4.79±1.09^{ab}
K+	26.72±3.41^{a}	1.27±0.26^{a}	4.78±0.91^{ab}
K-	31.95±2.83^{a}	1.41±0.18^{a}	4.41±0.21^{a}
Normal	31.15±1.80^{a}	1.75±0.13^{a}	5.64±0.60^{ab}

*Different letters on one column show the significant difference between the treatment group
E1: bilimbi leaf extract 6.3 mg/20gBW, E2: bilimbi leaf extract 8.4 mg/20gBW, E3: bilimbi leaf extract 10.5 mg/20gBW, K+: glibenclamide 0.013 mg/20gBW, K-: diabetic control group, N: normal control

Table 3. Percentage of mice hepatocytes with histopathological structural changes

Treatment group	Observation results in damage liver tissue (%)			
	Normal cell ± SD	Hydropic degeneration cell ± SD	Fat and Parenchymatous Degeneration Cells ± SD	Necrosis ± SD
E1	86.14±5.98^{a}	2.46±0.72^{b}	2.03±0.42^{ab}	9.37±5.61^{a}
E2	88.56±2.04^{a}	3.36±2.09^{b}	1.58±0.73^{a}	6.49±0.93^{a}
E3	87.75±7.50^{a}	2.21±1.08^{b}	1.04±0.47^{a}	8.99±6.8^{a}
K+	80.34±4.16^{a}	3.65±1.89^{b}	2.32±1.91^{ab}	13.8±3.21^{a}
K-	59.30±5.33^{b}	7.49±1.50^{a}	3.39±1.06^{b}	29.82±6.47^{b}
Normal	84.94±6.69^{a}	2.79±0.74^{b}	1.34±0.34^{a}	10.93±6.53^{a}

*Different letters on one column show the significant difference between the treatment group
E1: bilimbi leaf extract 6.3 mg/20gBW, E2: bilimbi leaf extract 8.4 mg/20gBW, E3: bilimbi leaf extract 10.5 mg/20gBW, K+: glibenclamide 0.013 mg/20gBW, K-: diabetic control group, N: normal control

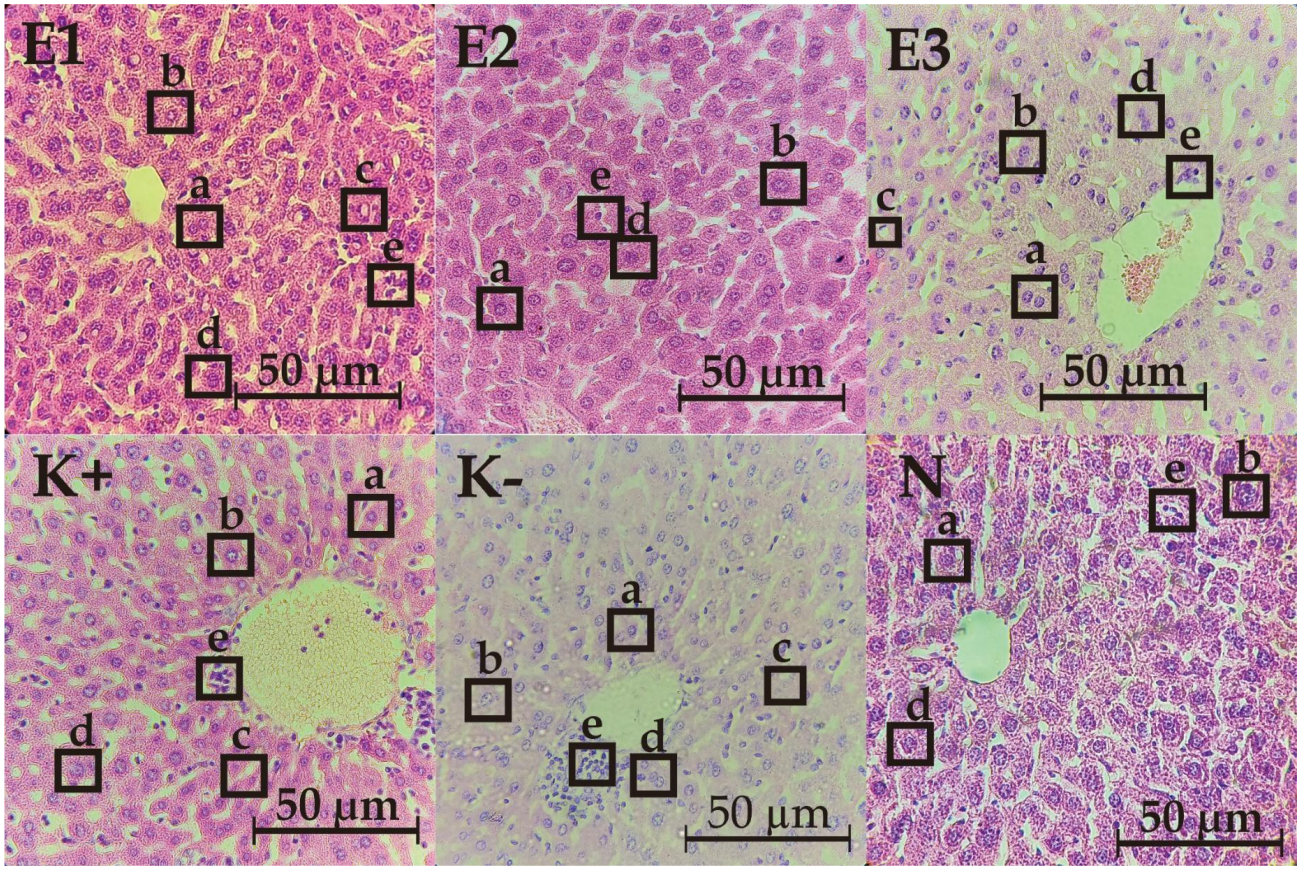

Figure 2. Histopathological description of liver mice post-treatment in 400x magnification. Description: Normal cells (a) hydropic degenerate cells, (b) fat degenerating cells, (c) parenchymatous degenerate cells, (d) necrosis Cell, (e) E1 = bilimbi leaf extract 6.3 mg/20 gBW, E2 = bilimbi leaf extract 8.4 mg/20 gBW, E3 = bilimbi leaf extract 10.5 mg/20 gBW, K+ = glibenclamide 0.013 mg/20 gBW, K- = diabetic control group, N =normal control

The antidiabetic activity of bilimbi leaf extract comes from its active compounds. The secondary metabolite compounds in bilimbi leaf have strong antioxidants and anti-inflammatory activities (Hasim *et al.* 2019). Antioxidant activity can suppress the activity of the α-glucosidase enzyme, which increases blood glucose level (Islam *et al.* 2020). The synergistic effect of several secondary metabolites also can decrease blood sugar levels (Solikhah *et al.* 2020; Nufus *et al.* 2021). The secondary metabolite content of starfruit leaf extract includes saponins, tannins, steroids, flavonoids, triterpenoids, and alkaloids (Hasim *et al.* 2019; Yanti and Vera 2019; Prabhu *et al.* 2021). Saponin compounds could stimulate increased insulin secretion and inhibit the action of the α-glucosidase enzyme (Rajalakhsmi *et al.* 2015). Tannin compounds can induce the phosphorylation of insulin receptors. Steroids can increase the effectiveness of insulin, which works at the cellular level and suppress glycogenolysis and gluconeogenesis in the liver (Chikhi *et al.* 2014). Flavonoids and alkaloids can maintain the process of pancreatic cell regeneration against free radicals and increase insulin release by stimulating Ca2+ absorption (Solikhah *et al.* 2020).

Data analysis of FBG change in the N group showed decrease on the 7th day and an increase on the 15th day (Figure 1). A drastic reduction in FBG might be caused by high-intensity physical activity before blood glucose level was recorded. The FBG increase on day 15 might be caused by fewer experimental animal activities before measuring FBG or stress factors that cause an increase in cortisol and aldosterone hormones, thereby increasing blood glucose levels (Lee 2007; Rifdiyani 2018).

Due to autoxidation reactions, hyperglycemic conditions induce ROS production, causing oxidative stress. Untreated oxidative stress will result in cell damage. One of the organs affected a lot by oxidation

reactions is the liver (Szendroedi *et al.* 2012). The liver plays an important role in gluconeogenesis and glycogenesis, on top of its role in detoxification (Al Ansori and Lipoeto 2020). Disruption of these two processes in DM patients causes an increase in ROS and NAFL which results in cell death, fibrosis, and necroinflammation (Sundaram *et al.* 2013; Wainwright 2015).

Oxidative stress disrupts membrane permeability (Itri *et al.* 2014). This can lead to hydropic degeneration in liver (Farzanegi *et al.* 2019). Parenchymal degeneration is not much different from hydropic degeneration. Parenchymal degeneration is featured by larger cell size, granular cytoplasmic structure, and cloudier colour. Parenchymal degeneration occurs due to water accumulation, triggering cell oxidation failure and inhibiting protein transport (Januar *et al.* 2014). Although parenchymal degeneration has a lower level of damage, both types of cell damage are reversible. However, if degeneration continues to occur, cell death or necrosis will occur (D'Arcy 2019).

Evaluation of the histological slides of the normal group (N) found a small number of cells that experienced degeneration and necrosis. These findings are still considered normal because cells in the body undergo apoptosis or programmed cell death periodically (Mitchell and Cotran 2007). The stress conditions in mice can also exacerbate this damage. Stress conditions can trigger production of cortisol hormone (Rifdiyani 2018). Increase in cortisol level can cause elevated oxidative stress. Thus, stress conditions can indirectly affect the process of liver tissue destruction through increased oxidative stress (Kurniawan *et al.* 2021).

The hepatoprotective activity of bilimbi leaf extract comes from its high antioxidant activity. Antioxidants can reduce oxidative stress by binding free radical electrons to prevent chain reactions that lead to hydrogen peroxide formation, resulting in improved damaged tissues (Tandi 2017; Wahidah *et al.* 2019). Compounds contained in bilimbi leaf extract also have specific anti-inflammatory roles, such as flavonoids, tannins, and triterpenoids. Saponins and alkaloids can inhibit the activation of NF-kβ, thereby reducing the production of TNF-α, which leads to the mechanism of cell destruction through the inflammatory pathway (Bonrowski 2004; Kim *et al.* 2011). In addition, flavonoids can stimulate Kupffer cells to regenerate liver cells (Astuti *et al.* 2016).

The E2 group was found to have the most optimal effect in reducing FBG and protecting liver tissue (Figure 1 and Table 2). The higher the dose, the more active substances contained. This should make the E3 group optimal because it has more active substances than other extract doses. The dose of E3 does not exceed the dose of bilimbi leaf extract, which causes toxicity. Bilimbi leaf extract at a < 2,000 mg/KgBW did not cause toxicity or change the macroscopic organs of the liver, kidneys, and heart (Vina *et al.* 2018). Bilimbi leaf extract doses < 5,400 mg/KgBW also did not cause toxicity and mortality and did not affect changes in body weight and locomotor activity (Wulandari 2017). However, the percentage decrease of FBG and percentage of normal cells in E3 were lower compared to E2, which the high activity of the mice might cause before the FBG measurement, and the stress conditions experienced by the mice during the treatment. These things can make the measurement results of both FBG and liver tissue examination in the E3 group lower than they should be and lower than in the E2 group.

In conclusion, *Averrhoa bilimbi* or bilimbi leaf extract treatment was significantly successful. The most optimal dose of bilimbi leaf extract in reducing fasting blood glucose levels and the role of hepatoprotective agent was bilimbi leaf extract at the dose of 8.4 mg/20gBW (E2 group) with a decrease in the percentage of final fasting blood glucose level by 26.44%, HSI value by 6.18%, and percentage of cells normal is 88.56%.

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