Histopathology of Liver and Cardiac Muscle of Alloxan-Induced Diabetic Rats (*Rattus norvegicus*) After Administration of Bosibosi (*Timonius flavescens* (Jacq.) Baker) Leaves Ethanol Extract

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Abstract. This research was aimed to study the effect of ethanol extract of bosibosi leaves (*Timonius flavescens*) (EEBL) on histopathology of liver and cardiac muscles of diabetic male rats. Animals (25) were randomly distributed into groups of NC (normal + NaCl), DC (diabetes + NaCl), DM (diabetes + metformin), DE300 (diabetes + EEBL 300) and DE500 (diabetes + EEBL 500 mg/kg bw/day); treated for 28 consecutive days and sacrificed for tissue collection. Tissues were processed (formalin, paraffin, H & E) and examined for histopathology. All diabetic rats showed hepatic tissue necrosis which is characterized by pycnotic nuclei, karyorhexis and karyolysis. The order of groups based on the percentage tissue damage is NC (27.61±2.44), DM (38.62±3.87), DE500 (45.15±3.76), DE300 (61.20±5.51) and DC (75.17±3.21). Cardiac muscle shows cell damage form mild degeneration to necrosis. The results indicate the potential of EEBL to repair tissues damaged by diabetes.

Keywords: Bosibosi (*Timonius flavescens*), rats, diabetes, liver and heart histopathology.

1 Introduction

Diabetes mellitus (DM), a metabolic disease, is characterized by hyperglycemia due to deficiencies in insulin secretion or action or both, which in turn affects the metabolism of carbohydrates, lipids, proteins and nucleic acids. DM is one of the most serious chronic diseases today and causes life-threatening complications, paralysis, and reduces life expectancy [1]. DM sufferers have increased drastically throughout the world with a projection of reaching 700 million patients in 2045 from 463 million patients in 2019 [2]. Intensive lifestyle intervention is a cornerstone in diabetes prevention and management [3]. According to latest findings by International Diabetes Federation, on average, one out of 11 adults aged between 20-79 currently have diabetes [4].

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Depending on the pathomechanism, DM can be classified into two main types, namely type-1 and type-2. Type-2 diabetes, the most common form of diabetes, is dominated by hyperinsulinemia, hyperglycemia, and dyslipidemia. Type-1 diabetes, occurs due to autoimmune-mediated destruction of the islets of Langerhans cells in the pancreas, is characterized by insulinopenia and hyperglycemia. In addition, the onset of type-1 diabetes is acute, unlike type-2 diabetes, which is characterized by a period of insulin resistance, hyperinsulinemia, and euglycemia before the onset of hyperglycemia [5, 6].

Increasing evidence shows that DM, both type 1 and type 2, can result in changes in the structure and function of internal organs, including the liver and heart [7][8]. Epidemiological studies suggest that diabetes itself promotes cardiac dysfunction directly or premature atherosclerosis, indirectly [7, 9]. Risk factors and incidence of heart failure are increased in DM regardless of hypertension, obesity, hyperlipidemia, and underlying coronary heart disease [10]. It is also known that metabolic changes in the heart muscle induced by both types of DM are associated with cardiomyopathy, which is characterized by a weakening of the heart's ability to pump blood, which can increase the risk of heart failure in diabetes sufferers [11]. The mechanism by which cardiomyopathy develops in diabetics is not yet fully understood. However, it has been revealed that metabolic abnormalities will produce reactive oxygen and nitrogen species which result in increased oxidative stress in both types of DM. Oxidative stress results in increased formation of free radicals and decreased antioxidant potential [12]. Autoxidation of glucose, cellular oxidation and increased blood glucose by proteins in blood vessel walls contribute to the increased formation of oxidative stress in DM [13]. These reactive species then trigger increased states of nitrooxidative stress, cardiomyocyte hypertrophy, profibrotic signaling, and myocardial remodeling along with apoptosis [14, 15, 16]. With the predicted high growth rate of diabetes in the future, early diagnosis, searching for alternative medicinal ingredients, and good treatment are very important.

Medicinal plants are currently in great demand and their acceptance has increased significantly [18]. Studies on secondary metabolites in plants in recent years have shown that plant secondary metabolites are a good source for pharmaceuticals [19]. Currently, people are returning to using medicinal plants because they have minimal side effects and the cost of synthetic drugs is expensive [20]. Laboratory data reveals that herbs containing strong antioxidant compounds can suppress oxidative stress disorders caused by DM [21]. Indonesia is a country with many medicinal plants and famous among them is bosibosi (*Timonius flavescens*). Bosibosi (Rubiaceae), is traditionally known to have the properties of increasing breast milk production after giving birth [22], anti-inflammatory [23], and inhibiting the activity of the lipoxygenase enzyme [24].

Bosibosi leaves are known to contain active secondary metabolite compounds in the form of flavonoids, terpenoids and saponins [25]. Analysis with GC-MS shows that there are more than 40 compounds in the ethanol extract of bosibosi leaves, ten of which are compounds that have antidiabetic properties, namely: (3β)-stigmast-5-en-3-ol, 3β.-(acetyloxy)-15α .-hydroxy-5.α.-cholesta-8(14),9(11)-dien-7-one, alpha-tocopherol, hexade-canoic acid, nonanoic acid, phytol, 2,3-dihydrobenzofuran, heptanoic acid, neophytadiene, and campesterol [26]. The ethanol extract of bosibosi leaves is known to reduce the blood sugar of alloxan-induced diabetic rats to a level equivalent to the blood sugar of diabetic rats given metformin [27]. It was also revealed that the extract could increase the relative weight of lymph nodes and the

number of lymphocytes compared to normal, non-diabetic rats [27]. Flavonoid compounds such as quercetin are known to improve endothelial dysfunction while narigenin has been shown to have high superoxide scavenging activity [28]. In addition, tannins can increase insulin secretion and reduce hyperglycemia in experimentally induced diabetic rats [29].

Complications of diabetes by investigating the long-term complications of untreated diabetes on histopathological changes in the liver and heart muscle in rats is very important. This research was aimed to study the effect of ethanol extract of bosibosi leaves (*Timonius flavescens* (Jacq.) Baker) on histopathological features of the liver and cardiac muscles of alloxan-induced diabetic male rats.

2 Methodology

2.1 Plant samples

Bosibosi (*Timonius flavescens* (Jacq.) Baker) leaf samples were taken from Siatas Barita Hills, Tarutung, North Tapanuli, North Sumatra, Indonesia. The plant was identified by the expert (reference number of No. 0087/UN33.4.8.3/LB/2021) as described previously [26]. The samples were washed and air-dried (room temperature), blended (100 mesh), then the simplicia were packaged in airtight plastic container, and stored (room temperature) until extracted.

2.2 Extraction of plant leaves

Simplicia was extracted with 95% ethanol using a soxhlet apparatus for 18 hours. The solvent was then evaporated (rotary evaporator; 40-45^{o}C). The remaining solvent was removed using a water bath at 50^{o}C. The final result, a concentrated ethanol extract from bosibosi leaves (EEBL), was filtered using coarse filter paper, placed in a sealed bottle and then stored at 4^{o}C until used in experiments.

2.3 Animals and experimental design

The test animals in this study were male Wistar rats (*Rattus norvegicus*) (25: 5 normal and 20 diabetic) aged 2 – 3 months with a body weight of 150 – 200 g; obtained from the Pharmacy Laboratory of Universitas Sumatera Utara. The research was carried out following a completely randomized design, animals were randomly distributed into 5 different groups (5 animals/group) (Table 1). During the 28 days of treatment, animals were given food (202C pellets; Gold Coin, Deli Serdang) and water (tap water) in excess.

Table 1. Experimental design

Group	Treatment	Number of animal
NC	Normal (nondiabetic) control + 0.9% NaCl	5
DC	Diabetic control + 0.9% NaCl	5
DM	Diabetes + metformin 200 mg/kg bw/day	5
DE300	Diabetes + EEBL 300 mg/kg bw/day	5
DE500	Diabetes + EEBL 500 mg/kg bw/day	5

2.4 Induction of diabetes

Diabetes was induced with a single injection of alloxan monohydrate (Sigma Aldrigh, St. Louis, MO, USA) dissolved in 0.9% NaCl. After one week adaptation period followed by fasting for 12 hours all rats, except the normal control group (NC), were given a single intraperitoneal injection of 175 mg/kg body weight alloxan [30]. The normal control group was given an ip injection of 0.5 ml 0.9% NaCl. Animals with fasting blood glucose levels ≥ 200 mg/dL after 72 hours of alloxan administration were categorized as confirmed diabetes and included in the experiment (Table 1). Treatment for all groups of animals began seven days after alloxan injection.

2.5 Extract administration

Bosibosi leaf ethanol extract (EEBL) was given to diabetic rats at a dose of 300 mg/kg bw/day (DE300) or 500 mg/kg bw/day (DE500) by delivering it directly to the stomach, once a day (in the morning, at 10.00-12.00) for 28 consecutive days. The extract was dissolved in 0.9% NaCl to a final volume of 0.5 ml per animal. The same volume of NaCl was given to both normal-non diabetic rats (NC group) and diabetic rats (DC group). Positive control (DM group), diabetic rats were given 200 mg/kg bw/day metformin dissolved in 0.9% NaCl solution.

2.6 Histology preparations, parameters and data analysis

After 28 days of treatment, the rats were sacrificed using mild anesthesia (sodium pentobarbital 90 mg/kg) and then the liver and heart organs were removed. The tissue was fixed with 10% formalin for 48 hours, dehydrated through graded alcohols, embedded in paraffin, sectioned at a thickness of 4 - 5 μm (rotary microtome) and stained with Ehrlich haematoxylin and eosin (H & E). All stages of tissue processing are carried out at the Veterinary Center, North Sumatra, Indonesia. Histopathological observations were carried out using a light microscope at 400x. The observed liver histology parameters were mainly focused on karyolysis, karyorrhexis, and pyknosis. Karyolysis (lysis of the cell nucleus), the cell nucleus looks very pale due to chromatin damage which is possible due to the movement of DNAase and RNAase, or the nucleus appears to have lysed or disappeared. Karyorrhexis is characterized by the destruction of the cell nucleus and the presence of chromatin fragments. Pyknosis, characterized by compaction due to shriveled chromatin, is round, dark and homogeneous. Normal hepatocytes are characterized by a normal morphological structure, the cell nucleus appears irregularly shaped, not dark, and tends to be transparent (open face nucleus) with hematoxylin staining that appears clear and not pale, the nucleolus and chromatin that make up RNA are clearly visible [31]. The histopathological parameters of heart muscle observed in this study were the condition of cardiomyocytes and cell infiltration (inflammation). Histopathological slides were observed with a light microscope at 400x, five slides per group (one slide per animal) and five fields per slide were analyzed to quantify the number of normal and structurally abnormal cells.

Quantitative data were analyzed using one-way ANOVA and Tukey's test as further tests.

3 Result and Discussion

3.1 Results

The histopathological structure of the liver of alloxan-induced diabetic animals after treatment is shown in Figure 1. Liver tissue consists of polygonal hepatocyte cells with round nuclei, arranged regularly between the portal areas. In general, the liver tissue of all groups of experimental animals showed the presence of hepatocytes that experienced nuclear damage, pyknosis, karyorrhexis and karyolysis. However, the number of each type of cell that is damaged varies between group.

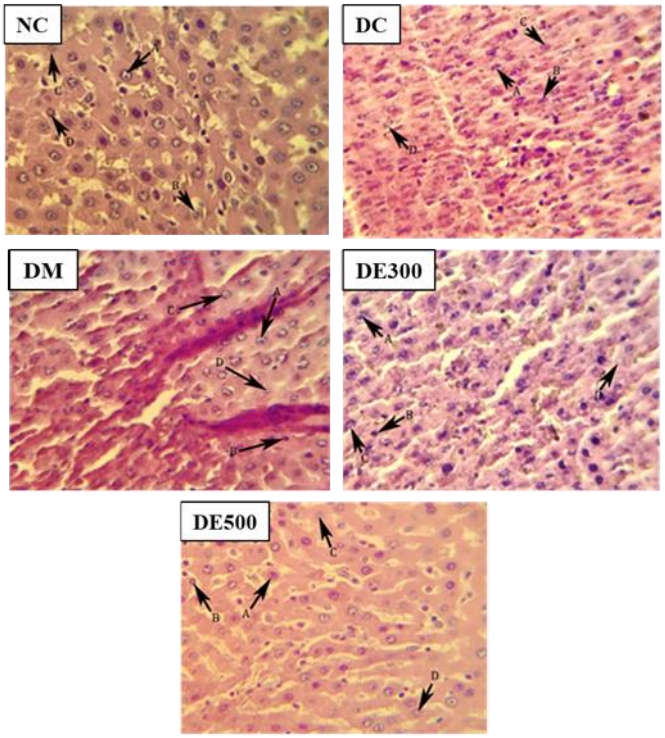

Figure 1. Histopathology of the liver of alloxan-induced diabetic rats (*Rattus norvegicus*) after 28 days administration of bosibosi leaves ethanol extract (NC = normal, nondiabetic control, DC = diabetic rats + 0.9% NaCl, DM = diabetic rats + metformin 200 mg/kg bw/day, DE300 = diabetic rats + 300 mg/kg bw/day extract, DE500 = diabetic rats + 500 mg/kg bw/day extract): The letters A, B, C or D at the base of the arrow indicate normal cells, pyknosis, karyorrhexis or karyolysis respectively. H & E staining, 400x.

The liver tissue of normal control group animals (NC group) was dominated by normal hepatocytes (more than 72.39%), only a small portion of hepatocyte cells experienced pyknosis (10.87%), karyorrhexis (9.45%) and karyolysis (7.29%) (Table 2). In contrast, in the group of alloxan-induced diabetic rats that were not given extract or metformin (DC group),

the liver tissue was dominated by damaged liver cells (pyknotic 31.31%, karyorrhexis 30.31%, and karyolysis 13.86); only 24.62% of hepatocytes were shows normal structure. Administration of metformin (in the DM group) turned out to be able to restore the hepatocyte composition close to normal, although it could not reach exactly the same composition as the normal and non-diabetic rats. Giving the extract to the diabetic rats (especially a dose of 500 mg/kb bw/day in the DE500 group) can increase the number of normal hepatocytes by reducing the number of pyknosis, karyorrhexis and karyolysis cells, although it cannot reach the composition achieved by the DM group (treated with metformin) especially in the composition of normal nondiabetic rats (NC group) (Table 2).

Table 2. Composition of normal and structurally abnormal hepatocytes in liver tissue of alloxan-induced diabetic rats (*Rattus norvegicus*) after 28 days administration of bosibosi leaves ethanol extract.

Structure of hepatocyte	Group*)				
	NC	DC	DM	DE300	DE500
Normal	72.39 ± 3.62^{a}	24.62 ± 5.85^{c}	60.38 ± 4.60^{ab}	38.80 ± 3.34^{c}	55.85 ± 8.06^{b}
Pyknosis[1]	10.87 ± 2.96^{c}	31.21 ± 2.46^{a}	15.06 ± 2.16^{c}	23.99 ± 8.43^{ab}	19.21 ± 0.96^{bc}
Karyorrhexis[2]	9.45 ± 2.41^{c}	30.31 ± 0.81^{a}	14.12 ± 3.73^{bc}	15.86 ± 0.35^{b}	15.51 ± 2.90^{b}
Karyolysis[3]	7.29 ± 2.08^{c}	13.86 ± 1.20^{b}	10.44 ± 0.71^{bc}	21.35 ± 1.26^{a}	10.43 ± 2.11^{bc}
Total 1 + 2 + 3	27.61 ± 2.44	75.17 ± 3.21	39.62 ± 3.87	61.20 ± 5.51	45.15 ± 3.76

*) NC = normal, nondiabetic control, DC = diabetic rats + 0.9% NaCl, DM = diabetic rats + metformin 200 mg/kg bw/day, DE300 = diabetic rats + 300 mg/kg bw/day extract, DE500 = diabetic rats + 500 mg/kg bw/day extract. Different letters on the same row indicate significantly different (Tukey HSD multiple range post hoc test, $p < 0.05$).

Microscopic examination of the musculature wall of the heart of the normal control rats (group NC) showed normal histological architecture with branching, anastomosing cylinders myocardium with centrally located oval basophilic nuclei (Figure 2). On the other hand, diabetic rats showed heart muscle experiencing cell damage such as mild degeneration to necrosis. Myocytes appear swollen and have vacuoles. Diabetic rats that were only given saline solution (DC group), and not given metformin or extracts, appeared to have relatively more necrosis and degenerative cells compared to the other groups. In diabetic rats given the extract (DE300 and DE500 groups) or metformin (DM group), cells undergoing degeneration and necrosis could still be found but in relatively small numbers compared to rats in the DC group.

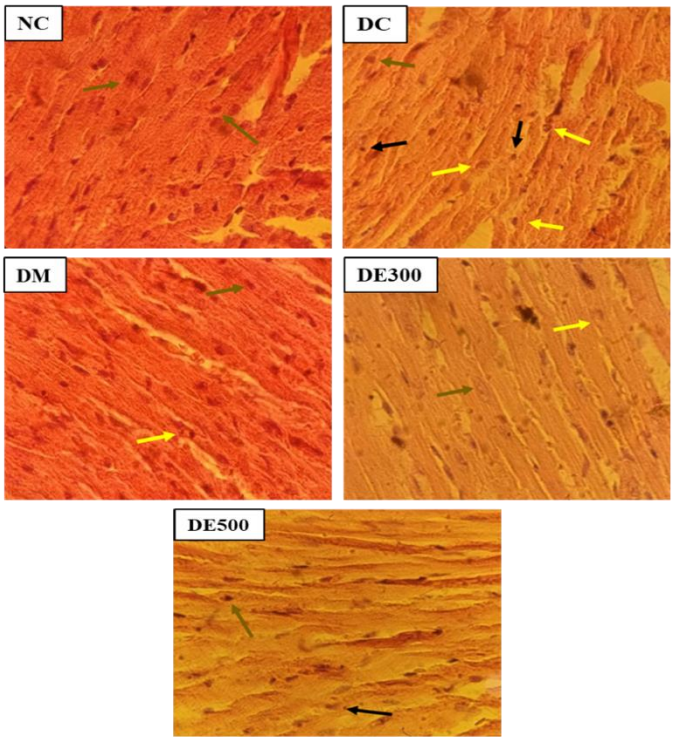

Figure 2. Histopathology of the cardiac muscles of alloxan-induced diabetic rats (Rattus norvegicus) after 28 days administration of bosibosi leaves ethanol extract (NC = normal, nondiabetic control, DC = diabetic rats + 0.9% NaCl, DM = diabetic rats + metformin 200 mg/kg bw/day, DE300 = diabetic rats + 300 mg/kg bw/day extract, DE500 = diabetic rats + 500 mg/kg bw/day extract). Green, yellow and black arrows indicate normal, degenerated and necrotic cells, respectively. H & E staining, 400x.

3.1 Discussion

Alloxan is one of the common diabetogenic agents that is often used to evaluate the antidiabetic or hypoglycemic potential of test compounds, both pure compounds and plant extracts, in studies using animal models of diabetes. It is well known that alloxan causes diabetes through a mechanism that essentially involves partial degradation of pancreatic islet beta (β) cells and subsequent reduction in the quality and quantity of insulin produced by these cells [32]. Alloxan and its reduction product, dialuric acid, form a redox cycle with the formation of superoxide radicals which then undergo dismutation to become hydrogen peroxide. After that, highly reactive hydroxyl radicals are formed via the Fenton reaction. The action of reactive oxygen species (ROS) together with a large increase in cytosolic calcium concentration causes rapid beta cell damage [30]. This compound causes oxidative stress in cells so that cells exposed to hyperglycemia will experience damage. Damage to pancreatic beta cells due to hyperglycemia causes insulin resistance and prevents the absorption of glucose by the body's cells. Insulin resistance will cause reduced use of glucose by peripheral tissues, decreased translocation of Glucose Transporter-4 (GLUT-4) and decreased glucose

oxidation. In this condition, glucose cannot enter cells or tissues so blood glucose levels will continue to increase [33].

The description above can explain well why the number of damaged liver tissue cells was very dominant and quite a large number of damaged heart muscle cells were found in alloxan-induced diabetic rats group (i.e. DC group) (Table 2, Figure 2). The types of liver cell damage observed in this study were pyknosis, karyorrhexis and karyolysis, while heart muscle cell damage generally took the form of degeneration and necrosis. The liver plays a pivotal role in glucose and lipid homeostasis, is severely affected by diabetes [34]. Al-Ani et al. [35] reported the presence of morphological and histological alterations in liver tissues of rats treated with alloxan, as indicated by the increase in liver weight, glycogen reduction, associated with lipid deposition, inflammatory cells infiltration and Kupffer cells hyperplasia. It has been previously reported that alloxan causes central venous congestion with significant dilatation of the sinusoidal spaces of liver tissue [36]. This results in swelling of the hepatocytes followed by hydropic degeneration, mild infiltration, necrosis and pyknosis of the hepatocyte nuclei and Kupffer cells [36]. Liver cell necrosis begins with pyknosis, namely damage characterized by the liver cell nuclei becoming smaller and darker in color. Karyorrhexis is characterized by the destruction of the nucleus into segments. Karyolysis is characterized by loss of color in the nucleus (fading), the cells appear empty when observed with a microscope [37]. Damaged cells will experience changes in structure and function until cell death [36]. Alloxan causes biochemical alterations in blood and pathophysiological variations in the liver of rats, varying from steatosis to steatohepatitis and liver fibrosis, and are similar to the modifications observed in human liver [38].

In heart muscle, alloxan is known to trigger histological changes in the form of disorganisation, distortion, degeneration and hyalinization of the myocardial fibers, increased interstitial space with chronic inflammatory infiltration, thickened vascular wall, dilated and congested blood vessels with eosinophilic material deposition [39]. The diabetic cardiomyopathy is characterized in part by disarray and collapse of myofibers, myocardial degeneration and cardiomyocyte hypertrophy [40].

Giving metformin to diabetic rats was able to reduce the number of cells damaged by the presence of alloxan, both in liver tissue and heart muscle tissue (DM group) (Table 2 and Figure 2). Metformin is one of the antidiabetic drugs that is widely used today. Metformin prevents prolonged insulin resistance, increases insulin signaling thereby triggering glucose absorption, increases GLUT-4 translocation, increases glycogen levels and glucose oxidation and repairs liver cells in diabetic rats. Physiologically, metformin has been shown to reduce hepatic glucose production, acting through both AMP-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms; by inhibition of mitochondrial respiration or mitochondrial glycerophosphate dehydrogenase, and mechanisms involving lysosomes [41].

Administration of the extract, especially a dose of 500 mg/kg bw/day (DE500 group) can repair liver tissue damage (Table 2). This dose of extract can increase the normal hepatocyte population by reducing the number of pyknotic cells, karyorrhexic cells and karyolysis. The repair efficacy of this extract is almost the same as metformin, although the improvement conditions obtained are never the same as those found in normal non-diabetic rats (NC group). The ethanol extract of bosibosi leaves contains various secondary metabolites such as flavonoids, terpenoids, saponins, and phenolic compounds [25][26][42]; which are known to

have biological activity that can reduce blood sugar levels [43]. Flavonoids are one of the natural secondary metabolite compounds contained in many plants and can be used as alternative compounds for diabetes treatment with low side effects. This compound is known to have a clinical role as anti-inflammatory, cardioprotective, antiviral, antibacterial, antidiabetic and anticancer [44]. Flavonoids act as hepatoprotectors and scavenging agents which are able to inhibit oxidation reactions and break the bonds of radical compounds so that free radicals are reduced [45]. Therefore, it makes sense why administering bosibosi leaf extract can repair tissue damaged by diabetes. Further research into the causes of damage to liver tissue, heart tissue and other tissues will help us uncover the pathogenesis of diabetes and its complications as well as search for and discover new drugs that are more effective and cheaper.

4 Conclusion

Diabetes induced by alloxan in rats resulted in damage to the liver tissue and heart muscle of experimental rats. Damage takes the form of pyknotic cell nuclei, karyorrhexis and karyolysis in hepatocytes or degeneration to necrosis which is characterized by swelling of myocytes, vacuolation and compaction of myocyte cell nuclei. Damage to hepatocytes and myocytes due to diabetes can be repaired by ethanol extract of bosibosi leaves.

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