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Protective effect of *Moringa oleifera* leaves extract on cardiac fibrosis of streptozotocin-induced diabetic rats

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ABSTRACT

Diabetes mellitus (DM) is a metabolic disease characterized by chronic hyperglycemia that induces excessive reactive oxygen species (ROS) production and causes oxidative stress. Diabetic cardiomyopathy is a diabetic complication characterized by structural and functional changes of the myocardium. Fibrosis is one of the pathological features of diabetic cardiomyopathy. *Moringa oleifera* leaves have been reported to possess antidiabetic and antioxidant activities which could prevent diabetic complications such as cardiomyopathy. A previous study reported that *M. oleifera* leaves extract have protective effects to the kidneys and liver of rats exposed to oxidative stress. This study aimed to investigate the protective effect of the *M. oleifera* leaves extract on cardiac fibrosis of rats induced by streptozotocin (STZ). This was an experimental study using a posttest-only control group design. Thirty-three male Wistar rats were randomly divided into three groups i.e. normal control group (Group 1) were administered normal saline, diabetic control group (Group 2) were administered normal saline, and diabetic treatment group (Group 3) were administered *M. oleifera* leaves extract. Diabetes induction of rats was conducted by intraperitoneally injection of STZ at dose of 45 mg/kg BW. The *M. oleifera* leaves extract at a dose of 1000 mg/kg BW was administered orally one time a day for 28 days. Statistical analysis was performed using the Kruskal-Wallis test followed by Mann Whitney. A significant difference in cardiac fibrosis occurrence between three groups was observed ($p < 0.05$). No cardiac fibrosis was observed in normal control group, In figure 3 we explained that fibrosis was observed in 8 rats of diabetic control group. Only 2 rats in the treatment group (G3) had cardiac fibrosis. In conclusion, *M. oleifera* leaves extract can inhibit cardiac fibrosis in STZ-induced diabetic rats.

ABSTRAK

Diabetes melitus (DM) merupakan penyakit metabolik yang ditandai dengan hiperglikemia kronis yang menyebabkan produksi spesies oksigen reaktif (ROS) berlebihan dan menyebabkan stres oksidatif. Kardiomiopati diabetik adalah komplikasi diabetes yang ditandai dengan perubahan struktural dan fungsional miokardium. Fibrosis adalah salah satu gambaran patologis kardiomiopati diabetik. Daun kelor (*M. oleifera*) dilaporkan memiliki aktivitas antidiabetik dan antioksidan yang dapat mencegah komplikasi diabetes seperti kardiomiopati. Penelitian sebelumnya melaporkan bahwa ekstrak daun kelor memiliki efek protektif terhadap ginjal dan hati tikus yang terpapar stres oksidatif. Penelitian ini bertujuan untuk mengetahui efek protektif ekstrak daun kelor terhadap fibrosis jantung tikus yang diinduksi streptozotocin (STZ). Penelitian ini merupakan penelitian eksperimental dengan menggunakan rancangan *posttest-only control group*. Tiga puluh tiga ekor tikus Wistar jantan dibagi secara acak menjadi tiga kelompok yaitu kelompok kontrol normal (K1) yang disuntik dan diberikan larutan garam normal, kelompok kontrol diabetes (K2) diberikan larutan garam normal, dan kelompok perlakuan diabetes (Kelompok 3) diberikan ekstrak daun kelor. Induksi diabetes pada tikus dilakukan dengan cara penyuntikan STZ secara intraperitoneal dengan dosis 45 mg/kg BB. Ekstrak daun kelor dosis 1000 mg/kg BB diberikan secara oral satu kali sehari selama 28 hari. Analisis statistik dilakukan dengan menggunakan uji Kruskal-Wallis yang diikuti oleh Mann Whitney. Terdapat perbedaan yang signifikan dalam kejadian fibrosis jantung antara tiga kelompok yang diamati ($p < 0,05$). Tidak ada fibrosis jantung yang diamati pada kelompok kontrol normal (K1), sedangkan fibrosis jantung diamati pada semua tikus (9 tikus) dari kelompok kontrol diabetes (K2). Hanya 2 tikus pada kelompok perlakuan (K3) yang mengalami fibrosis jantung. Kesimpulannya, ekstrak daun kelor dapat menghambat fibrosis jantung pada tikus diabetes yang diinduksi STZ.

Keywords:
diabetes;
oxidative stress;
cardiac fibrosis;
antioxidant;
Moringa oleifera

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both.[1] According to International Diabetes Foundation (IDF) in 2019, DM in Indonesia ranked seventh in the world with 10.7 million cases.[2] Uncontrolled DM can cause complications affecting the heart, kidneys, nerves and blood vessels.[3] Diabetic cardiomyopathy is a diabetic complication characterized by structural and functional changes of the myocardium, one of them is cardiac fibrosis.[4] The cardiac fibrosis can increase the risk of ventricular stiffness, which impairs cardiac contractility.[5]

According to a cohort study conducted over 5.5 years in 1.9 million patients with type 2 diabetes mellitus (T2DM), heart failure is the second most common manifestation of cardiovascular disease after peripheral arterial disease.[6] Another study reported that 32.3% of patients with T2DM experienced heart failure in less than 5 yr, and 67.7% of patients after 5 yr or more.[7] The cardiomyopathy also cause impairment of cardiac function which characterized by diastolic dysfunction. It was reported that the prevalence of diastolic dysfunction in patients with T2DM is between 40-60%.[8]

Hyperglycemia induces excessive production of reactive oxygen species (ROS) which further caused oxidative stress.[9] Oxidative stress has an important role in the pathogenesis of cardiac fibrosis through activation of the transforming growth factor (TGF)-β leads to the activation of the suppressor of mothers against decapentaplegic (SMAD) signaling pathway included SMAD-2/3.[10] In the nucleus, the SMAD-2/3 complexes regulate the transcription of target profibrotic genes encoding proteins involved in extracellular matrix production, including collagen.[11] Streptozotocin (STZ) is a diabetogenic agent that widely used in experimental studies.[12] Histopathological evaluation of the cardiac tissue of diabetic animal induced by STZ showed that diabetes causes structural changes in myocardium. These structural changes are caused by the excessive production of ROS in the myocardium due to hyperglycemic conditions.[13] A previous study also reported that diabetic rats induced by STZ show an increase in the collagen deposition.[14]

Moringa oleifera leaves have been reported to possess antidiabetic and antioxidant activities, which are helpful for the treatment of diabetes and its complications.[15] Quercetin in *M. oleifera* leaves extract was reported as one of the active constituents that is responsible for its high antioxidant activity.[16] *Moringa oleifera* exhibits cardioprotective effects in cardiac damage and vascular dysfunction due to its antioxidant activity.[17] In addition, the rich polyphenolic content of *M. oleifera* reduces the myocardial damage and decreases the oxidative stress.[18] Previous studies reported that administration of *M. oleifera* leaves extract at a dose of 1000 mg/kg BW exhibits protective effects to the kidneys and liver of rats exposed to oxidative stress.[19,20] The aim of this study was to investigate the protective effect of the *M. oleifera* leaves extract on cardiac fibrosis of rats induced by STZ.

MATERIAL AND METHODS

M. oleifera leaves extract preparations

The leaves of *M. oleifera* were obtained from a farmer in Wuluhan District, Jember Regency, and were authenticated at the Herbal Materia Medica Laboratory, Batu, East Java. The leaves were then washed and dried in oven at a temperature of 55-60°C. Once dried, the leaves were ground into a fine powder using a blender and

sieve. The dried and powdered leaves were extracted by maceration using 96% ethanol for 72 h. The mixture was stirred using an orbital shaker to facilitate the extraction process. After 72 h of maceration, the mixture was filtered using Whatman filter paper. The filtrate was then evaporated using water bath at a temperature of 70 ^{o}C. The dried extract obtained was then kept in a refrigerator until used.

Animals and design

This was an experimental laboratory study using a posttest-only control group design. Thirty-three male Wistar rats with weighing 200-300 g at 8-12 wk of age were randomly divided into three groups i.e. normal control rats' group (Group 1) were injected and administered normal saline, diabetic control rats' group (Group 2) were administered normal saline, and diabetic treatment rats' group (Group 3) were administered *M. oleifera* leaves extract. The rats were housed in individual cages, which were cleaned every 2-3 d in a wk. Rats were given standard feed and water *ad libitum*. The *M. oleifera* leaves extract at dose of 1000 mg/BW was orally administered daily started from day 4 and continued for the next 28 d. The rats' body weight was measured five times during the study using a digital scale. This study has been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, University of Jember, with ref:1599/H25.1.11/KE/2022.

Induction of diabetes by streptozotocin

Diabetes induction was performed by a single intraperitoneal injection of freshly STZ at a dose of 45 mg/kg BW by dissolved in 0.1 M of cold citrate buffer at pH 4.5 to overnight fasted male Wistar rats. After STZ induction, the rats were provided with 10% dextrose overnight to prevent hypoglycemic shock. Three days after STZ induction, the fasting blood glucose levels were measured using EasyTouch glucometer. Rats with fasting blood glucose level (BGL) of greater than 200 mg/dL were considered as diabetic rats and included in this study.

Histological examination

The histopathological examination of was conducted at the Biomedical Laboratory, Faculty of Dentistry, University of Jember. Rats were sacrificed by intraperitoneal injection of pentobarbital at dose of 150 mg/kg BW after 4 wk of treatment. The heart were cleaned with 0.9% NaCl solution to remove any remaining blood or dirt and were preserved in a jar containing a 10% buffer neutral formalin solution.[21] Masson's trichrome staining was used to identify collagen deposition in the cardiac tissue. Each preparation was examined under a microscope at magnification ranging from 100x to 400x and at five fields of view to evaluate the collagen deposition in cardiac tissue. Collagen deposition was stained blue, whereas the cardiac muscle was stained red.[22] The histopathological examination was carried out by two individuals using a single blind technique under the supervision of an anatomical pathologist.

Statistical analysis

Statistical analysis was performed using SPSS version 23 software. Statistical differences were assessed using the Kruskal-Wallis test, followed by Mann Whitney Test, due to not normal data distribution. Differences between groups were considered significant if a p value of <0.05.

RESULTS

Streptozotocin induction

The BGL of normal control group

(G1), diabetic control group (G2) and treatment group (G3) are presented in TABLE 1. The mean BGL of G1 is 108.91 ± 9.101 mg/dL (<200 mg/dL) which considered as normal rats. The mean BGL of G2 and G3 are 437.82 ± 38.126 mg/dL; 424.91 ± 41.469 mg/dL (>200 mg/dL) which considered as diabetic rats (TABLE 1).

Body weight of rats after STZ induction

The mean growth of rats body weight in all groups are presented in FIGURE 1. No significant difference in the body weight of rats in all groups in the week 0 ($p>0.05$). A significant difference in the body weight of rats between normal control group (G1) with diabetic control group (G2) and treatment group (G3) was observed ($p<0.05$). The growth of rats body weight was only observed in normal control group (G1), whereas in diabetic control group (G2) and treatment group (G3), the loss of rats body weight during study in the week 1 to 4 was observed.

Protective effect of *M. oleifera* leaves extract

Among 11 rats induced by STZ, only 9 rats in each group were survive and eligible for histopathological examination. Histopathological features of cardiac tissues of rats in each group are presented in FIGURE 2 and the results of histopathological examinations are presented in FIGURE 3. Significantly different in cardiac fibrosis between the three groups was observed ($p<0.05$) No fibrosis was observed in normal control group (G1), In figure 3 we explained that fibrosis was observed in 8 rats of diabetic control group and in 2 rats of treatment group (G3).

TABLE 1. Rats fasting BGL (mg/dL) in all of groups after STZ induction

Group	n	Mean ± SEM	Min	Max
Normal control group (G1)	11	108.91 ± 9.101	66	156
Diabetic control group (G2)	11	437.82 ± 38.126	248	600
Treatment group (G3)	11	424.91 ± 41.469	208	600

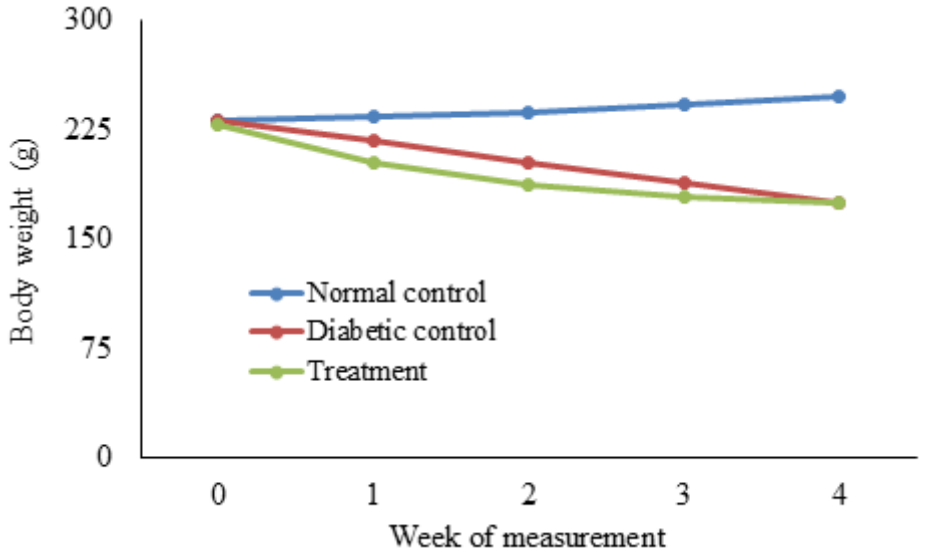
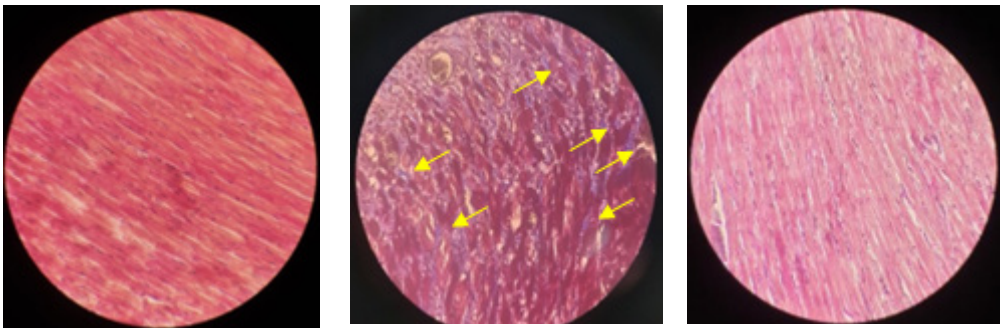


FIGURE 1. Mean body weight of rats



A. Normal control B. Diabetic control C. Treatment

FIGURE 2. Histopathological features of cardiac tissues of rats in each group after microscopic examination with 400x magnification. A. Normal control: no fibrosis was observed; B. Diabetic control: fibrosis was observed (yellow arrow); C. Treatment: no fibrosis was observed.

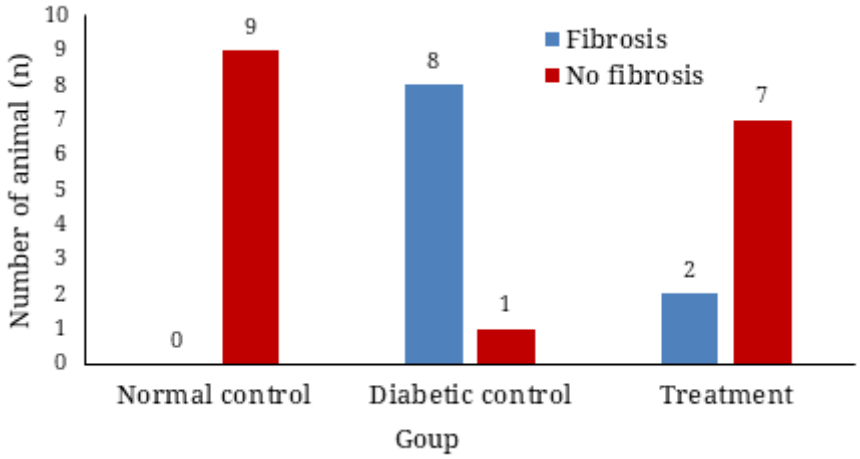


FIGURE 3. The results of histopathological examination of cardiac tissues of rats in each group.

DISCUSSION

In this study STZ is used to induce diabetes of rats. Streptozotocin-induced diabetes model is widely used in various studies related to diabetes in animal model. Streptozotocin is a naturally occurring chemical, a broad-spectrum antibiotic that is specifically toxic to the insulin-producing β-cells of the

pancreas lead to diabetes of the animal model.[23,24] Lew *et al*.[25] reported that the characteristics of STZ-induced diabetes rats include hyperglycemia, polyphagia, polyuria, polydipsia, and significant weight loss. The animal model is considered diabetes if the BGL greater than 200 mg/dL. This study showed that three days after STZ induction, the rats BGL of the diabetic control group (G2) and the treatment group (G3) range from 208 to 600 mg/dL (TABLE 1).

In addition, a significant weight loss was also observed in this study after STZ induction (FIGURE 1). The significant weight loss of the STZ-induced diabetes rats occurred due to an increased protein breakdown in muscle and tissue.[26] Diabetes leads to changes in protein metabolism.[27] The elevated protein catabolism for glucogenesis that cause weight loss occurs in diabetes.[28]

In figure 3 we explained that fibrosis was observed in 8 rats of diabetic control group and the part of rats in treatment group (G3) in this study (FIGURE 3). Cardiac fibrosis is an injury condition in the cardiac muscle that is characterized by excessive deposition of type 1 collagen.[29] Cardiac fibrosis in STZ-induced diabetic rats occurs due to the differentiation of cardiac fibroblast into myofibroblast.[30] Furthermore, the myofibroblasts secrete excessive amount of ECM such as collagen.[31] It was also reported that an increased expression of TGF-β, α-SMA, and connective tissue growth factor (CTGF) that contributed in the development of cardiac fibrosis are observed in the hearts of STZ-induced diabetic rats.[30,32,33] The CTGF is a fibrogenic factor that promotes fibroblast proliferation and interstitial collagen deposition.[34]

Hyperglycemia in STZ-induced diabetic rats increased ROS production which cause imbalance between ROS and antioxidant levels lead to oxidative stress.[9,13,24,34] The levels of MDA of diabetic rats increase lead to stimulate fibroblast proliferation and other fibrotic signaling pathways in cardiac tissue.[31,34] Oxidative stress triggers the activation of the TGF-β pathway, which, in turn, activates the SMAD signaling pathway, specifically SMAD-2/3.[10] Within the nucleus, SMAD-2/3 complexes govern the transcription of target profibrotic genes responsible for encoding proteins involved in extracellular matrix production, such as collagen.[11]

This study showed that the administration of *M. oleifera* leaves extract inhibits the incidence of cardiac fibrosis in diabetic rats. A better histological feature of cardiac tissue of diabetic rats treated by *M. oleifera* leaves extract was observed compared to that untreated diabetic rats. *Moringa oleifera* leaves extract can ameliorate the histological feature of cardiac tissue of diabetic rats as almost its normal condition. Previous study reported that *M. oleifera* leaves extract has protective effect on cardiac antioxidant status and lipid peroxidation in STZ-induced diabetic rats.[35]

Moringa oleifera leaves has been reported to contain active antioxidant compounds such as vitamin C, vitamin E, and flavonoids.[36-38] Quercetin, a potent antioxidant flavonoid found in high concentration in *M. oleifera*, has therapeutic activities.[39,18] The quercetin is responsible for free radical scavenging activity. Moreover, it was also reported that the quercetin in *M. oleifera* leaves extract reduce the cardiac necrosis biomarkers levels and normalize the myocardium structure in both *in vitro* and *in vivo* stidies.[18]

Previous studies reported that *M.*

oleifera leaves extract at a dose of 1000 mg/kg BW significantly showed positive effects to the rat organs exposed to oxidative stress.[19,20,40] It was also reported that *M. oleifera* leaves extract has a renoprotective effect on the glomerulus of diabetic rats. The *M. oleifera* leaves extract restored the glomerulus damage from a score of 4 to a score of 1 to 0 or return to its normal condition. Another study reported that *M. oleifera* leaves extract has a hepatoprotective effect against oxidative stress by restoring the structure of liver tissue close to normal.[20] *Moringa oleifera* leaves extract restored the structure of pancreatic islet cells of hypercholesterolemic rats model.[40]

CONCLUSION

In conclusion, *M. oleifera* leaves extract can inhibit cardiac fibrosis in STZ-induced diabetic rats.

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