

**Effect of *Moringa oleifera* Leaf Extract on TGF- $\beta$ 1 and Galectin-3 Levels and Cardiac Fibrosis in Diabetic Rat**Suryono Suryono<sup>1\*</sup>, Muhammad I. Amien<sup>2</sup>, Achmad I. Tohari<sup>2</sup>, Antonius D. Saputra<sup>2</sup>, Muhammad R. F. Hidayat<sup>2</sup>, Hazbina F. Ramadhan<sup>3</sup><sup>1</sup>Departement of Cardiology and Vascular Medicine, Faculty of Medicine, University of Jember/dr. Soebandi General Hospital Jember 68121, East Java, Indonesia<sup>2</sup>Faculty of Medicine, University of Jember, Jember 68121, East Java, Indonesia<sup>3</sup>Bina Sehat Hospital, Jember 68131, East Java, Indonesia

## ARTICLE INFO

## ABSTRACT

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Cardiac fibrosis is the most prevalent form of diabetic cardiomyopathy. Increased levels of transforming growth factor-beta 1 (TGF- $\beta$ 1) and galectin-3 are present in cardiac fibrosis, and these proteins might be potential therapeutic targets. Researches have shown that *Moringa oleifera* (MO) display numerous biological activities which could be harnessed for the treatment of a variety of ailments, including cardiovascular diseases. This study is aimed at evaluating the effect of MO on TGF- $\beta$ 1 and galectin-3 levels in diabetic rat model of cardiac fibrosis. Fifteen (15) Wistar rats were randomly divided into three groups: normal control group, administered normal saline; case group, administered normal saline and streptozotocin; and MO treatment group, administered streptozotocin and MO extract (1000 mg/kg) once daily for 28 days. Cardiac fibrosis was evaluated by histopathological analysis of the rats' myocardium. The levels of TGF- $\beta$ 1 and galectin-3 were investigated by enzyme-linked immunosorbent assay (ELISA). Histopathological examination revealed fewer cardiac fibrosis features in the myocardium of MO treated group compared to the case group. In addition, MO treatment resulted in a significant reduction in collagen decomposition in the left ventricle myocardium. ELISA revealed a significant decrease in the TGF- $\beta$ 1 and galectin-3 levels in the MO treated group compared to the case group (641.4 $\pm$ 94.0 ng/L vs. 852.3 $\pm$ 56.2 ng/L, and 1.53 $\pm$ 0.07 ng/L vs. 1.79 $\pm$ 0.166 ng/L, respectively). In conclusion, the beneficial effects of MO are likely related to its ability to decrease oxidative stress in the heart tissue and reduce the formation of fibrosis by suppressing the expression of TGF- $\beta$ 1 and galectin-3.

**Keywords:** Cardiac fibrosis, TGF- $\beta$ 1, Galectin-3, *Moringa oleifera*.**Introduction**

Diabetes mellitus (DM) is a serious health concern globally, and its occurrence has been progressively rising in recent decades. In 2021, the International Diabetes Federation (IDF) reported that over 537 million persons or 1 in 10 adults had diabetes globally. DM is linked to a higher risk of cardiovascular diseases, such as coronary artery disease, stroke, and heart failure.<sup>1</sup> Cardiovascular problems associated with diabetes are a significant contributor to the morbidity and mortality of individuals living with this condition. According to the American Heart Association, people with diabetes are at a much higher (estimated to be two- to four-fold higher) risk of mortality from cardiovascular disease compared to those without the condition.<sup>2</sup> Diabetic cardiomyopathy is a complex condition characterized by the presence of cardiac fibrosis (CF) and left ventricular (LV) failure. LV diastolic dysfunction is an early functional change observed in the progression of diabetic cardiomyopathy.<sup>3</sup>

The pathophysiology of diabetic cardiomyopathy involves the development of CF, which leads to structural and functional changes in the left ventricle. Deposition of the interstitial extracellular matrix contributes to the development of CF and decreases LV compliance, resulting in heart failure with preserved ejection fraction (HFpEF).<sup>4</sup> DM contributes to CF through hyperglycemia, which causes oxidative stress and activates the transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)–mothers against decapentaplegic (SMAD)2/3 and galectin-3 signaling pathways.<sup>5,6</sup> Within the nucleus, TGF- $\beta$ 1 controls the transcription process of specific profibrotic genes via the SMAD2/3 pathway. These genes encode proteins that play a role in the synthesis of the extracellular matrix, such as collagen, which is influenced by galectin-3.<sup>7</sup> To prevent CF, it is essential to regulate oxidative stress using substances with antioxidant properties. *Moringa oleifera* (MO), has been the focus of numerous studies in recent times. MO is effective as an antioxidant, antidiabetic, and in the treatment of cardiovascular illness, including CF and inflammatory diseases.<sup>8</sup> MO possesses essential bioactive substances; the leaves are the most commonly used part due to their high contents of vitamins, polyphenols, phenolic acids, flavonoids, alkaloids, tannins, and saponins.<sup>9,10</sup> Arising from its high content of antioxidant flavonoids, MO protects against chronic diseases related to oxidative stress, such as cancer and cardiovascular disease.<sup>11</sup> There are many models for CF, including the single-cell resolution of fibrosis-related cell types, fibrosis-on-a-chip technologies, and the use of human cardiac cells, but none has been able to reveal the complex pathophysiology of the development of CF *in vivo*.<sup>12-14</sup> Therefore, animal models continue to form an essential resource for preclinical drug research and in elucidating the molecular pathways underlying CF. The induction of experimental diabetes in rats represents a model to examine the effects of various substances and elucidate the pathophysiology and mechanism of the condition.<sup>15</sup> However, data on the potency of MO as an antioxidant in alleviating CF caused by

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diabetic oxidative stress is lacking. Therefore, this study aims to evaluate the effect of MO on TGF- $\beta$ 1 and galectin-3 in CF diabetic rat model.

## Materials and Methods

### Ethical approval

This study was approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, University of Jember, Indonesia (ref:1599/H25.1.11/KE/2022).

### Collection and identification of plant material

*Moringa oleifera* leaves were collected from Jember Regency, Indonesia in December, 2021. The plant material was identified and authenticated at the Herbal Materia Medica Laboratory, East Java, Indonesia (key determination: 1b-2b-3b-4b-6b-7b-9b-10b-11b-12b-13b-14a-15b-197b-208b-209b-210b-211b-214a: Moringaceae 1: *M. oleifera*) with voucher No. 074/031/102.7-A/2022.

### Extraction of *Moringa oleifera* leaf

The MO leaves were first washed and dried in a conventional oven (Quincy lab, Chicago, IL, USA, model 20GCE-LT) at 60°C and then ground into a fine powder using a mechanical grinder (Miller Cuisinart, Stamford, CT, USA, model DBM-8). Subsequently, 600 g of the powdered MO leaves were extracted by maceration in 6 L of 96% ethanol (Merck, Darmstadt, Germany) at room temperature (25–28°C) for 72 h with continuous stirring using an orbital shaker to facilitate the extraction process. Thereafter, the mixture was filtered using Whatman filter paper and then evaporated on a water bath (Memmert®) at 70°C. The concentrated extract was then refrigerated until further use.

### Animals

Fifteen male Wistar rats aged 8 - 12 weeks weighing between 200 - 300 g were used for the study. The animals were housed in spacious, hygienic polypropylene cages during the course of the experiment and were fed with rodent pellets and drinking water *ad libitum*. The rats' body weight was measured five times during the study using a digital scale.

### Experimental design

A post-test-only control group design was used in the study. The rats were randomly divided into three groups, consisting of a normal control group (Group 1), to which normal saline was orally administered; a case group (Group 2), orally given normal saline and an intraperitoneal injection of Streptozotocin (STZ); and a treatment group (Group 3), which received an intraperitoneal injection of STZ and an oral dose of MO leaf extract. The treatment group was given the MO leaf extract at an oral dose of 1000 mg/kg BW once daily for 28 days.

### Induction of diabetes

Diabetes was induced by a single intraperitoneal dose of STZ. STZ was dissolved in 0.1 M citrate buffer (pH 4.5). The buffer was prepared by mixing 0.1 M citric acid with 0.1 M sodium citrate and the pH of the solution was adjusted to 4.5 with distilled water. A single intraperitoneal injection of STZ was administered to the rats at a dose of 45 mg/kg BW. After STZ induction, the rats were given 10% dextrose overnight to prevent hypoglycemic shock. After 72 h of STZ

administration, the fasting blood glucose of all the rats was checked using an EasyTouch glucometer.

### Histopathological studies

The LV myocardia of the rats were cleaned with a 0.9% NaCl solution to remove any remaining blood or debris, and then preserved in a jar containing a 10% neutral buffered formalin solution. Collagen deposition in the LV myocardium was identified using Masson's trichrome staining (SkyTek Laboratories). Each preparation was examined under a microscope at magnifications ranging from 100x to 400x and at five fields of view to evaluate the collagen deposition in the LV myocardium. The collagen deposition was stained blue, whereas the cardiac muscle was stained red.

### Assessment of serum levels of TGF- $\beta$ 1 and galectin-3

The levels of TGF- $\beta$ 1 and galectin-3 in the serum of each of the three experimental groups of rats were evaluated using the enzyme-linked immunosorbent assay (ELISA) kit (rat-TGF- $\beta$ 1 and rat-galectin-3 BT Laboratory™). All tests were performed in the Biochemical Laboratory, Faculty of Medicine, University of Jember, Indonesia. After preparing all the reagents needed for each marker, the relevant serum sample and 10  $\mu$ L of the antibody (anti-TGF- $\beta$ 1 and anti-galectin-3) were added to the plate for each marker, after which 50  $\mu$ L of streptavidin-HRP was mixed into each plate. Subsequently, 50  $\mu$ L of substrate A, 50  $\mu$ L of substrate B, and 50  $\mu$ L of the stop solution were added to each plate, and the absorbance was measured at 450 nm using a fluorometer.

### Statistical analysis

Statistical analyses were conducted using R 4.3.1 software. Numerical data were presented as the mean  $\pm$  standard deviation (SD), and analyzed using a one-way analysis of variance (ANOVA), followed by the post hoc pairwise T-test with Bonferroni corrections. For the descriptive data, the analysis was performed using Fisher's exact test. Statistical significance was set at  $p < 0.05$ .

## Results and Discussion

### Fasting blood glucose of STZ induced rats

The case and treatment groups received an intraperitoneal injection of STZ (45 mg/kg) to induce diabetes leading to myocardial fibrosis. After 24 h, STZ successfully induced diabetes in the case and treatment groups with blood glucose level above 500 mg/dL in both groups (Table 1).

### Effect of *Moringa oleifera* on histopathological findings of LV myocardium in rats

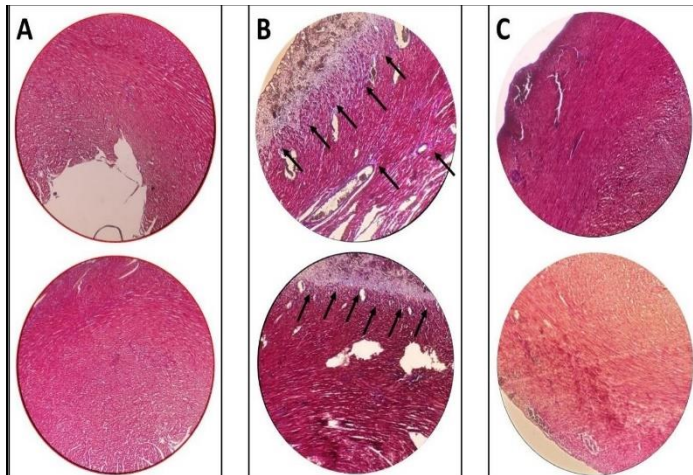
The histopathological results from the myocardia of the rats in each group are presented in Figure 1. From the histopathological features, myocardial fibrosis was observed in all rats in the case group, which were administered STZ. As expected, no fibrotic feature of the myocardium was observed in the normal control group. Whereas, in the treatment group, myocardial fibrosis was observed in only one rat out of the five rats in the group (Supplementary Figure 1).

**Table 1:** Fasting Blood Glucose Levels after 24 Hours of STZ Induction

Group	Number of Samples	Fasting Blood Glucose (mg/dL)
Case	5	539.8 $\pm$ 65
Treatment	5	506.8 $\pm$ 82
Normal Control	5	89.2 $\pm$ 9.70

Data represents mean  $\pm$  SD.

CF in STZ-induced diabetic rats could arise from excessive amounts of extracellular matrix secretions such as collagen from myofibroblasts due to oxidative stress.<sup>16,17</sup> The levels of malondialdehyde in diabetic rats increase, stimulating fibroblast proliferation and other fibrotic signalling pathways in cardiac tissue.<sup>18</sup> Some previous studies have also reported an increase in TGF- $\beta$ 1 expression, which contributed to the development of CF in the hearts of STZ-induced diabetic rats.<sup>18</sup> The histopathological findings indicate that the administration of MO leaf extract could inhibit the incidence of CF in diabetic rats. In the MO treatment group, CF histopathological feature was observed in only one rat and not in the remaining four rats. A normal histological finding of the myocardium was found in diabetic rats treated with MO leaf extract, which was similar to that in the normal group without STZ induction.



**Figure 1:** Histopathological features of the myocardium of the rats in each group (400x magnification). (A) Normal control group: no fibrosis was observed; (B) case group: fibrosis was observed (black arrows); and (C) treatment group: no fibrosis was observed. Black arrows: deposition of collagen in the LV myocardial tissue, representing myocardial fibrosis.

#### Effect of *Moringa oleifera* on TGF- $\beta$ 1 and galectin-3

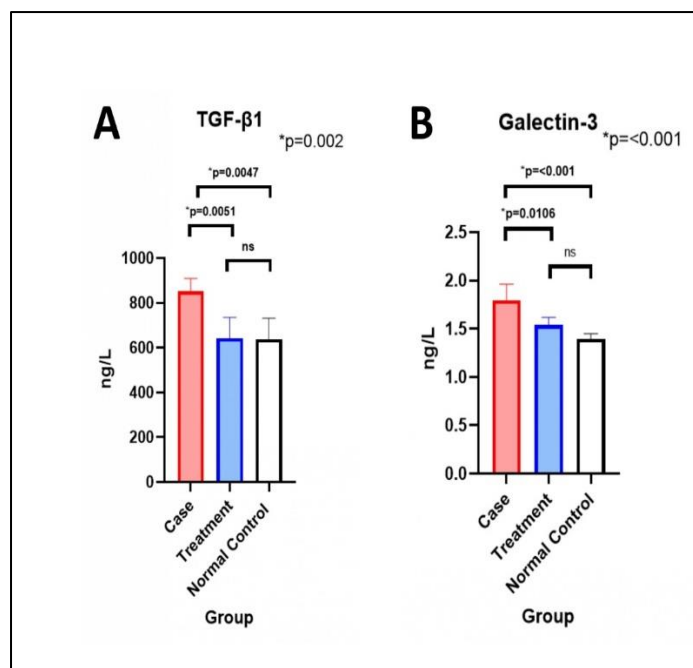
The serum levels of TGF- $\beta$ 1 in the rats are presented in Figure 2A. There was a significant difference ( $p = 0.004$ ) in the TGF- $\beta$ 1 levels in the case and normal control groups with the case group exhibiting higher mean TGF- $\beta$ 1 levels ( $852.3 \pm 56.2$  ng/L) than the normal control group ( $638.5 \pm 93.0$  ng/L). Similarly, TGF- $\beta$ 1 levels in the case group was significantly ( $p = 0.005$ ) higher than that in the treatment group which displayed a mean TGF- $\beta$ 1 levels of  $641.4 \pm 94.0$  ng/L. On the other hand, there was no significant difference in the mean TGF- $\beta$ 1 levels in the treatment group ( $641.4 \pm 94.0$  ng/L), and the normal control group ( $638.5 \pm 93.0$  ng/L).

Figure 2B presents the galectin-3 levels in the different experimental groups of rats. Just as it was observed for TGF- $\beta$ 1, the serum levels of galectin-3 in the case group was significantly ( $p < 0.05$ ) higher with mean value of  $1.79 \pm 0.166$  ng/L compared to the normal control and the treatment groups with mean galectin-3 levels of  $1.39 \pm 0.05$  ng/L and  $1.53 \pm 0.07$  ng/L, respectively. The results indicate that STZ administration stimulates an increase in the serum levels of TGF- $\beta$ 1 and galectin-3 in the rats, and MO administration significantly reversed the increased expression of TGF- $\beta$ 1 and galectin-3 to that of the normal control. TGF- $\beta$ 1 is a cytokine expressed by a variety of cells and may have an important function in cardiac illnesses, including myocardial infarction and CF.<sup>19-21</sup> In CF, TGF- $\beta$ 1 isoforms work with activins to stimulate signals within cells via SMAD2/3 due to the induction of oxidative stress. When TGF- $\beta$ 1 ligands bind to the TGF- $\beta$  receptor II, they phosphorylate the type I activin receptor-like kinase 5 (ALK-5). This enhances the binding of different ligands to TGF- $\beta$ 1 receptors on the cell surface, activating signaling effectors and SMAD2/3.



**Supplementary Figure 1:** Histopathological features of left ventricle myocardium of Rats in the different groups.

When TGF- $\beta$ 1, myostatin, or activin activate SMAD2, SMAD3, and NF- $\kappa$ B that may upregulate the expression of profibrotic genes,<sup>7,22</sup> transcription factors activate SMAD1, SMAD5, and SMAD8, which interact with bone morphogenetic proteins to modulate gene expression. The primary gene expression results involve various profibrotic gene expression, which includes collagens (COL1A1, COL3A1, COL5A2, COL6A1, COL6A3, and COL7A1), plasminogen activator inhibitor 1 (PAI-1), various proteoglycans, integrins, connective tissue growth factor (CTGF), and matrix metalloproteinases, which lead to CF.<sup>7</sup> Galectin-3 promotes fibrosis by upregulating genes such as transforming growth factor- $\beta$  (TGF- $\beta$ ) and stimulating the production of fibronectin and other extracellular matrix proteins.<sup>23</sup> Previous studies have linked galectin-3 to various processes involved in the development of CF and atherosclerosis, including inflammation and oxidative stress.<sup>24</sup> Galectin-3 is expressed to the greatest extent in tissue-resident macrophages.<sup>24</sup> It influences various aspects of macrophage activity, including phagocytosis and the efferocytosis of apoptotic neutrophils. In addition, it contributes to a profibrotic macrophage phenotype by binding to the transmembrane receptor CD98 and signals the activation of integrins via phosphatidylinositol-3-kinase (PI3K). Recent studies have observed an increase in galectin-3 and TGF- $\beta$ 1 expression in CF using *in vivo* and *in vitro* models; thus, these proteins may be potential therapeutic targets for CF.<sup>7,25-28</sup> Based on the aforementioned explanations, TGF- $\beta$ 1 and galectin-3 biomarkers may contribute to CF through oxidative stress. In this case, oxidative stress may increase due to hyperglycemia in subjects with DM. If the exposure to oxidative stress persists for a long time, it may trigger the TGF- $\beta$ 1 and galectin-3 signalling pathway, causing the decomposition of collagen, leading to CF. In addition, antioxidants are needed to reduce the amount of oxidative stress in the body. MO plays a role in inhibiting further oxidative stress that causes CF via its antioxidant properties at the beginning of the fibrosis pathway.



**Figure 2:** (A) TGF- $\beta$ 1 and (B) galectin-3 levels in rats in the different experimental groups. \* $p < 0.05$ ; ns = not significant.

Studies have shown that MO leaves contain active antioxidants such as vitamin C, vitamin E, and flavonoids. Quercetin, a powerful flavonoid and antioxidant, is present in high concentrations in MO<sup>29</sup> and is responsible for the elimination of free radicals. Furthermore, researchers have shown that quercetin in MO leaf extracts reduces cardiac necrosis biomarker levels and normalizes the myocardial structure in both *in vitro* and *in vivo* studies.<sup>30</sup> In this study, 1000 mg/kg MO leaf extract was used to assess its effect on TGF- $\beta$ 1, galectin-3, and LV myocardial fibrosis. In the treatment group, CF was observed in one of the rats. This phenomenon may be due to an imbalance between the oxidative stress and MO antioxidants or other complex factors. The responses of individuals to MO treatment may vary significantly, though this study has revealed that a 1000 mg/kg dose of MO may reduce the incidence of CF in rats in the treatment group. The dosage was based on previous research conducted by Giovani *et al.*, who found that MO leaf extracts at a 1000 mg/kg dose exhibit better protective effects than doses of 62.5, 125, 250, and 500 mg/kg.<sup>31</sup> Furthermore, another study reported that the administration of 1000 mg/kg MO leaf extract may decrease cardiac tissue fibrosis.<sup>32</sup> This study reported novel findings on the TGF- $\beta$ 1 and galectin-3 levels as well as the histopathology of LV myocardial fibrosis in diabetic rats treated with 1000 mg/kg MO leaf extract.

Several studies have confirmed that MO leaf extracts influence TGF- $\beta$ 1 and galectin-3 levels. In 2023, Thongrungrung *et al.* reported the use of MO leaf extracts in a diabetic nephropathy rat model and revealed that MO may downregulate TGF- $\beta$ 1 and reduce fibrosis in the kidney.<sup>33</sup> Aly *et al.* also reported the successful application of MO extracts in reducing TGF- $\beta$ 1 levels and liver fibrosis induced by acetaminophen.<sup>34</sup> Furthermore, other researchers have combined several antioxidant plants including *M. oleifera*, *G. lucidum*, and *S. platensis* to treat doxorubicin-induced cardiotoxicity, revealing a decrease in galectin-3, fibrosis, and hypertrophy in myocardial tissues.<sup>35</sup> The limitation of this study is that only a single dose (1000 mg/kg) of MO was administered; thus, it was not possible to compare the dose-dependent effects of MO. Therefore, further studies using a range of doses are recommended. Moreover, additional research is necessary to investigate the effectiveness of MO in suppressing CF by examining various molecular pathways and biomarkers.

## Conclusion

*Moringa oleifera* extract may inhibit CF in diabetic rat models via a decrease in the serum TGF- $\beta$ 1 and galectin-3 levels and a reduction in the collagen decomposition in the left ventricle. The beneficial effects of *Moringa oleifera* are likely related to its ability to decrease oxidative stress in the heart tissue and throughout the body and reduce the formation of fibrosis by suppressing the expression of TGF- $\beta$ 1 and galectin-3. This study provides evidence for the continued development of *Moringa oleifera* as a potential natural remedy for the treatment of heart disease.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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