

## The methanol stem extract of *Moringa oleifera* mitigated isoproterenol-induced myocardial infarction through cardiac troponin and NF- $\kappa$ B signaling pathways in rats

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**Abstract:** Cardiovascular diseases remain a health challenge globally, affecting approximately one third of the world's human population with a larger percentage occurring in developing countries. While the pathogenesis of this disease condition is yet to be fully elucidated, many studies have shown that isoproterenol provides a standard model that closely mimics human myocardial infarction by inducing myocardial ischemia, hypoxia, cell death, and reduced myocardial compliance. This study was designed to describe the protective effect of methanol stem extract of *Moringa oleifera* on isoproterenol-induced myocardial infarction in rats. Thirty-five adult male wistar rat were randomly sorted into five groups of seven animals each. Control received distilled water alone, Untreated group received single Isoproterenol (100 mg/kg) subcutaneously, two groups were pretreated orally with 50 and 100 mg/kg of *Moringa oleifera* respectively for seven days and then injected with a single Isoproterenol (100 mg/kg) subcutaneously on day eight. Another group received oral administration of *Moringa oleifera* alone at 100 mg/kg for seven days. Twenty-four hours after Isoproterenol was administered, blood pressure and electrocardiogram values were taken. Serum and cardiac homogenate analysis revealed that isoproterenol induced significant increases in creatine kinase myocardial band, nitric oxide, malondialdehyde, protein carbonyl, myeloperoxidase, advanced oxidative protein products alongside a decrease in superoxide dismutase, reduced glutathione, glutathione peroxidase and an increase in the expression of cardiac Troponin I and NF- $\kappa$ B with immunohistochemistry. Treatment with *Moringa oleifera* at both doses used brought on a reversal of these. Collectively, these submit that *Moringa oleifera* exerts its cardioprotective effect via antioxidant/NF- $\kappa$ B/Cardiac troponin signaling pathways.

**Key words:** Oxidative stress, inflammation, myocardial damage, isoproterenol

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### I. Introduction:

Worldwide, myocardial infarction induced mortalities remain one of the leading causes of death, it occurs as a result of an imbalance between blood demand and supply in the coronary vessels (Chatterjee et al., 2018). The heart is an organ that requires a lot of energy mostly derived from fatty acid metabolism and in cases of myocardial injury, the myocardium is unable to obtain the full benefits of fatty acid metabolism (Yuan et al., 2008). The formation of reaction oxygen species, products of lipid peroxidation, release of pro-inflammatory cytokines, and imperfections in the antioxidant defense are characteristics of myocardial infarction (Zhang et al., 2018). It is usually accompanied by pathological lesions and is a major cause of death and disability worldwide (Zhang, et al., 2019).

*Moringa oleifera*, commonly known as horse radish tree is a popular plant in tropical and sub-tropical regions of the world with every part appropriate for both commercial and nutritional purposes (Gopalakrishnan et al., 2016). The plant is rich in minerals and phytochemicals such as tannins, saponins and alkaloids, it also contain linoleic, oleic and palmitic acid (Sánchez-Machado et al., 2010). *Moringa oleifera* has been regarded as a cure-all, reported to possess various therapeutic properties including antidiabetic, anticancer, neuroprotective and anti-infective properties (Gopalakrishnan et al., 2016).

In this study, Isoproterenol was used to induce myocardial infarction, this is an ideal and conventional model of instigating myocardial infarction. Isoproterenol works by triggering widespread strain to the myocardium with subsequent lesions that mimics infarcts, it also restricts the supply of energy to the myocardial cells resulting in the compromise of the structural and functional integrity of the heart (Khan et al., 2019). Isoproterenol is also said to cause myocardial infarction through the generation of reactive oxygen species

including lipid peroxidation products (Fan, 2019). The pathology caused by isoproterenol closely resembles the lesions that accompanies myocardial infarction in humans hence the choice of its use in myocardial infarction studies (Kumar et al., 2019).

## **II. Materials and Methods**

Thirty (35) healthy, adult male Wistar rats were randomly separated into 5 groups of 7 animals each. Group A (Normal control) animals were given normal saline. Group B (toxicant) were given 100 mg/kg of isoproterenol injection subcutaneously on day 8. Groups C and D were given 50 mg/kg and 100 mg/kg of *Moringa oleifera* extract respectively for 7 days and then on day 8 were challenged with 100mg/kg of isoproterenol. Animals in Group E were administered 100mg/kg of *Moringa oleifera* only for 7 days. Twenty four hours after the last drug administration, their blood pressure and electrocardiogram (ECG) were taken. Blood sample was taken for serum analysis, thereafter they were sacrificed via cervical dislocation and heart harvested for biochemical assays, histology and immunohistochemistry

### *Serum preparation*

Approximately three milliliters of blood were collected by retro-orbital venous puncture using plain capillary tubes into plain bottles and allowed to clot. The clotted blood was then centrifuged at 4,000 revolutions per minute (rpm) for 10 minutes. Clear serum was separated with Pasteur pipette into another plain tube and then stored at 4°C until needed.

### *Cardiac homogenate preparation*

The hearts were excised, rinsed and homogenized using 50 mM Tris-HCl buffer (pH 7.4) containing 1.15% KCl. The homogenates were subjected to cold centrifugation at 4°C using a speed of 10,000 for 15 minutes. The post mitochondrial fractions (PMFs) obtained from cardiac homogenates were used for biochemical assays.

### *Biochemical analysis*

#### **Cardiac makers of oxidative stress**

Hydrogen peroxide generation was determined according to the method of Wolff (1994). The reaction mixture was subsequently incubated at room temperature for 30 minutes. The mixtures were read at absorbance at 560 nm and H<sub>2</sub>O<sub>2</sub> generated was extrapolated from H<sub>2</sub>O<sub>2</sub> standard curve. The Malondialdehyde (MDA) content as an index of lipid peroxidation was quantified in the PMFs of renal tissue according to the method of Varshney and Kale (1990). The absorbance was measured against a blank of distilled water at 532 nm. Lipid peroxidation was calculated with a molar extinction coefficient of  $1.56 \times 10^5$  M/cm. Protein carbonyl (PCO) contents in the renal tissues were measured using the method of Reznick and Packer (1994). The absorbance of the sample was measured at 370 nm. The carbonyl content was calculated based on the molar extinction coefficient of DNPH ( $2.2 \times 10^4$  cm<sup>1</sup>M<sup>1</sup>) and expressed as nmol/mg protein. The advanced oxidation protein product (AOPP) contents were determined as described by Kayali et al. (2006). Briefly, 0.4 ml of renal PMFs were treated with 0.8 ml phosphate buffer (0.1 M; pH 7.4). The absorbance of the reaction mixture was immediately recorded at 340 nm wavelength. The content of AOPP for each sample was calculated using the extinction coefficient of 261 cm<sup>-1</sup> mM<sup>-1</sup> and the results were expressed as μmol/mg protein.

#### **Cardiac antioxidants markers**

The Superoxide dismutase (SOD) assay was carried out by the method of Misra and Fridovich (1972), with slight modification (Oyagbemi et al., 2015). The increase in absorbance at 480 nm was monitored every 30 s for 150 s. The one unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome. Reduced glutathione (GSH) was estimated by the method of Jollow et al. (1974). Glutathione peroxidase (GPx) activity was also measured according to Beutler et al. (1963). Glutathione-S-transferase (GST) was estimated by the method of Habig et al. (1974) using 1-chloro-2, 4-dinitrobenzene as substrate. The protein and non-protein thiol contents were determined as described by Ellman (1959). Protein concentration was determined by the Biuret method of Gornal et al. (1949), using bovine serum albumin (BSA) as standard.

#### **Determination of serum markers of cardiac damage and inflammation**

The serum nitric oxide concentrations were measured spectrophotometrically at 548 nm according to the method of Olaleye et al. (2007). The serum myeloperoxidase (MPO) activity was determined according to the method of Xia and Zweier (1997). Creatine kinase myocardial band was carried out using Randox kits according to the manufacturer's instruction.

### **Histopathology**

Small pieces of kidney were fixed in 10% formalin, embedded in paraffin wax, and sections of 5-6 mm in thickness were made and thereafter stained with hematoxylin and eosin for histopathological examination according to the methods as previously described (Drury et al., 1976). Thereafter, the sections were examined with light microscopy.

### **Immunohistochemistry**

The immunohistochemistry was described as earlier reported by Oyagbemi et al., (2017). To determine the expression of cardiac troponin I, and nuclear factor kappa beta (NF-kB) in the heart, fixed tissues were embedded in paraffin and sectioned at a thickness of 5 nm. The sections were subsequently deparaffinized in xylene and rehydrated with graded alcohol. Antigen retrieval was carried out by immersing the slides in 10 mM citrate buffer at 95-100 ° C for 25 minutes with subsequent peroxidase quenching in 3% H<sub>2</sub>O<sub>2</sub>/methanol solution. The sections were blocked in goat serum followed by an overnight incubation at 4°C in the rabbit anti-CTnI and anti- NF-kB primary antibodies. Detection of bound antibody was carried out using biotinylated (goat anti-rabbit, 2.0 µg/mL) secondary antibody and subsequently, streptavidin peroxidase (Horse Radish Peroxidase-streptavidin) according to manufacturer's protocol (HistoMark<sup>®</sup>, KPL, Gaithersburg, MD, USA). Reaction product was enhanced with diaminobenzidine (DAB, Amresco<sup>®</sup>, USA) for 6-10 min and counterstained with high definition hematoxylin (Enzo<sup>®</sup>, NY-USA), with subsequent dehydration in ethanol. The sections were subsequently dehydrated in ethanol, cleared in xylene. The slides were covered with coverslips and sealed with resinous solution. The immunoreactive positive expressions of CTnI and NF-kB anti-rabbit intensive regions were viewed starting from low magnification on each slice then with 400× magnifications using a photo microscope (Olympus) and a digital camera (Toupcam<sup>®</sup>, Touptek Photonics, Zhejiang, China).

### **III. Results**

In this study, significant decreases in cardiac SOD, GPx, GST, reduced Glutathione, protein thiols and non-protein thiol levels were reported in the isoproterenol alone group when compared with the control and treated groups, whereas treatment with *Moringa oleifera* exhibited amelioration of these enzymatic and non-enzymatic antioxidant markers (Figures 1-6).

Again, significant elevations in hydrogen peroxide, malondialdehyde, protein carbonyl, and advanced oxidative protein product were reported in cardiac homogenate of the isoproterenol alone group whereas treatment with *Moringa oleifera* caused significant decreases when compared with the isoproterenol alone group (Table 1).

In this study, isoproterenol alone group showed significant increases in cardiac markers of inflammation and damage such as myeloperoxidase, creatine kinase myocardial band, and nitric oxide. Treatment with methanol stem bark extract of *Moringa oleifera* resulted in decrease levels of these markers (Table 2).

Isoproterenol caused moderate infarction of the endocardium and myocardium and ventricle, infarction of the myocardium while treatment with *Moringa oleifera* moderated these lesions (Figure 7). Isoproterenol alone exhibited highest expression of CTnI whereas the extract treated groups and control showed lesser expressions. Isoproterenol alone exhibited highest expression of NF-kB whereas the extract treated groups and control showed lesser expressions (Figures 8 and 9).

### **IV. Discussion and Conclusion**

Cardiovascular diseases remain a health challenge globally, affecting approximately one third of the world's human population with a larger percentage occurring in developing countries. While the pathogenesis of this disease condition is yet to be fully elucidated, many studies have shown that isoproterenol provides a standard model that closely mimics human myocardial infarction by inducing myocardial ischemia, hypoxia, cell death, and reduced myocardial compliance (Derbali et al., 2015).

Studies have elucidated the role of free radicals and reactive oxygen species in the progression of myocardial infarction. Oxidative stress facilitates myocardial damage in a variety of ways, it results in cellular and organelle damage leading to cell death (Shahzad et al., 2019). In this study, isoproterenol generated significant increases in levels of malondialdehyde; a product of lipid peroxidation but treatment with *Moringa oleifera* lowered the values. Lipids have the ability to modify the stability of cellular membranes and thus are known to play important roles in the pathogenesis of cardiovascular diseases. Lipid peroxides have also been previously reported in isoproterenol induced myocardial infarction (Khalil et al., 2015). Protein carbonyl, a non-reversible product of protein oxidation was also significantly increased in the isoproterenol alone group when compared with the control and extract treated groups, this finding indicates that reactive oxygen species led to the oxidation of proteins in the myocardium (Shahzad et al., 2019). The reduction in levels of protein carbonyl

in the treated group thus suggests that *Moringa oleifera* has the ability to prevent excessive oxidation of protein in the myocardium.

Antioxidant enzymes such as SOD, GPx, GST were depleted in the isoproterenol alone group when compared with the groups that were treated with *Moringa* and the control group. The depletions in the antioxidant enzymes is buttressed by the finding of elevations in reactive oxygen species such as malondialdehyde, protein carbonyl, advanced oxidative products and hydrogen peroxide. The elevations in these enzymes caused by *Moringa oleifera* points out the free radical scavenging activity of this plant extract. Isoproterenol has been previously reported to deplete antioxidant enzymes (Goyal et al., 2015).

Thiols, non-protein thiols and reduced glutathione were also markedly reduced in the isoproterenol alone group, these molecules are usually utilized because of their thiol (SH) content and their active removal of reactive oxygen species (Shahzad et al., 2019). The decrease in these molecules is most likely due to the oxidants generated by isoproterenol.

An increased concentration of creatine kinase-myocardial band (CK-MB) is an age-long diagnostic indicator of myocardial infarction. This enzyme is released into the bloodstream during myocardial injury (Derbali et al., 2015). The increase in CK-MB in the isoproterenol alone group and the ameliorated levels in the treated groups further lend credence to the protective effect of *Moringa oleifera* against myocardial infarction.

Myeloperoxidase (MPO) is a leukocyte-derived enzyme involved in the generation of a number of reactive oxidant species. Besides being a component of the innate immune system, it is now known that MPO-derived oxidants contribute to tissue damage during inflammation. The reactions catalyzed by MPO have been attributed to biological activities involved in cardiovascular diseases (Nicholls & Hazen, 2005). In this study, isoproterenol significantly increased MPO levels while *Moringa oleifera* moderated the levels, suggesting that beyond the antioxidant property shown by *Moringa oleifera*, it also possesses anti-inflammatory property.

Isoproterenol is known to induce pathological lesions such as infiltration of inflammatory cells, fragmentation of myofibrils, necrosis and edema in the cardiac tissue, which are all suggestive of myocardial damage (Shahzad et al., 2018).

Oxidative stress induces inflammation through the initiation of NF- $\kappa$ B signaling and other transcription factors, this in turn leads to the invasion of cytokines and chemokines (Zhang et al., 2019), this ferocious cycle of proinflammatory cytokines further worsens myocardial disorder (Verma et al., 2019). In this study NF- $\kappa$ B was upregulated by isoproterenol and the ISO alone group showed highest expression, administration of *Moringa oleifera* mitigated this. We thus postulate that *Moringa oleifera* acts an anti-inflammatory agent by inhibiting NF- $\kappa$ B signaling pathway thus protecting against myocardial infarction.

Owing to their high sensitivity and specificity for myocardial damage, evaluation of cardiac troponin levels has proven valuable and is firmly established in the management of patients with cardiac disease. Troponin measurements have been shown to have the potential to be sensitive indicators of myocardial injury due to both cardiac and non-cardiac disease processes (Wells & Sleeper, 2008). In this study, CTnI expression was highest in the isoproterenol alone group whereas *Moringa oleifera* moderated its expression.

Taking all these findings together, we thus conclude that the methanol stem extract of *Moringa oleifera* mitigated isoproterenol-induced myocardial infarction through cardiac troponin and NF- $\kappa$ B signaling pathways, and by its antioxidant property.

#### Conflict of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

### Acknowledgement

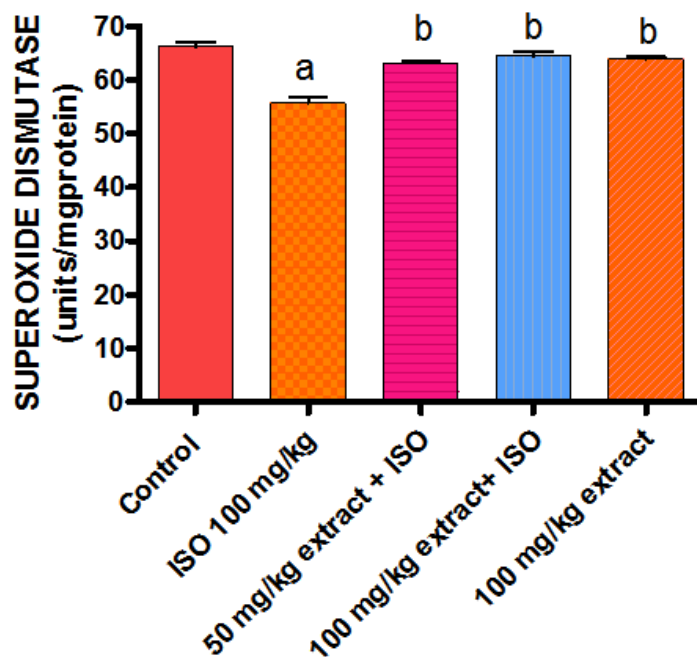
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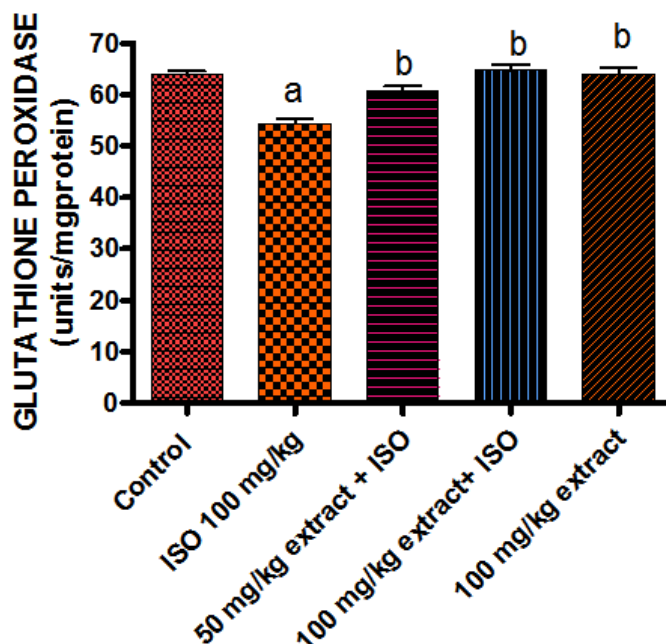
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**Figure 1:** Effect of methanol extract of stem bark of *Moringa oleifera* on cardiac enzymatic and non-enzymatic antioxidant defense system



Superscripts (a) indicates significant difference ( $p < .05$ ) when compared with control, whereas superscripts (b) indicates significant difference ( $p < .05$ ) when compared with ISO treated only.



**Figure 2**

Superscripts (a) indicates significant difference ( $p < .05$ ) when compared with control, whereas superscripts (b) indicates significant difference ( $p < .05$ ) when compared with ISO treated only.

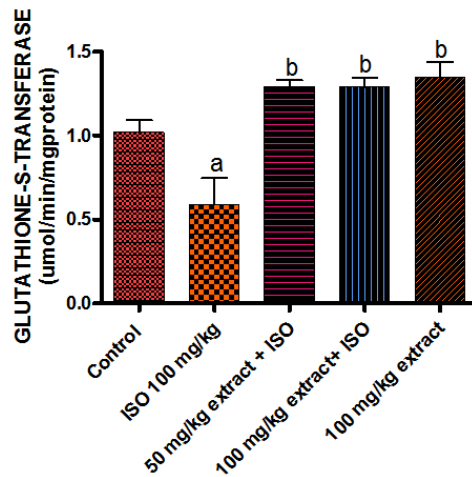


Figure 3

Superscripts (a) indicates significant difference ( $p < .05$ ) when compared with control, whereas superscripts (b) indicates significant difference ( $p < .05$ ) when compared with ISO treated only.

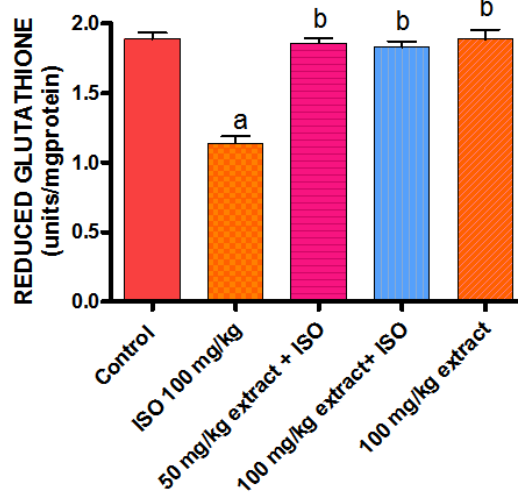


Figure 4

Superscripts (a) indicates significant difference ( $p < .05$ ) when compared with control, whereas superscripts (b) indicates significant difference ( $p < .05$ ) when compared with ISO treated only.

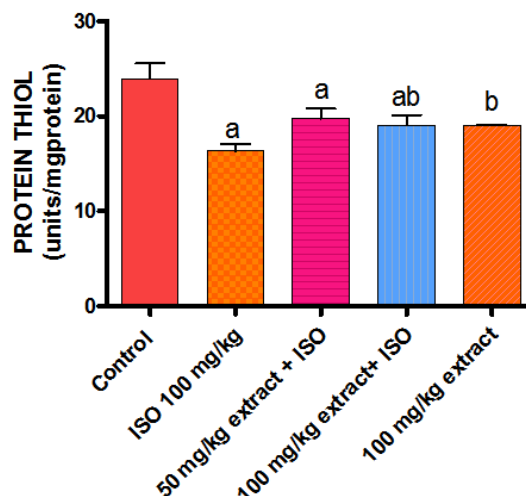


Figure 5

Superscripts (a) indicates significant difference ( $p < .05$ ) when compared with control, whereas superscripts (b) indicates significant difference ( $p < .05$ ) when compared with ISO treated only.

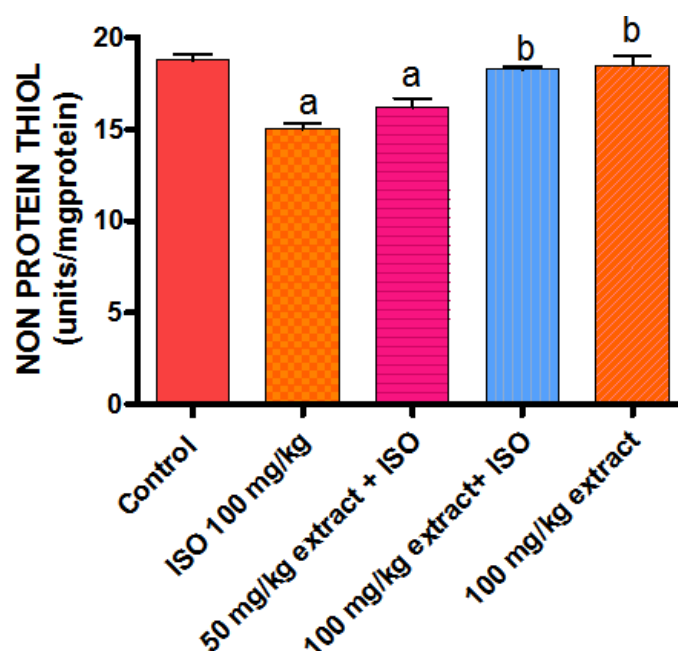


Figure 6

Superscripts (a) indicates significant difference ( $p < .05$ ) when compared with control, whereas superscripts (b) indicates significant difference ( $p < .05$ ) when compared with ISO treated only.

Table 1: Effect of methanol extract of stem bark of *Moringa oleifera* on cardiac markers of oxidative stress.

Parameters	Control	ISO 100 mg/kg	50mg/kg extract + ISO	100 mg/kg extract + ISO	100mg/kg extract
H <sub>2</sub> O <sub>2</sub> (umol/mgprotein)	32.64±0.6	36.65±1.38 <sup>a</sup>	32.20±0.65 <sup>b</sup>	30.81±0.94 <sup>b</sup>	32.02±0.77 <sup>b</sup>
MDA (umol/mgprotein)	2.10±0.43	4.19±0.22 <sup>a</sup>	2.10±0.13 <sup>b</sup>	2.08±0.16 <sup>b</sup>	2.33±0.15 <sup>b</sup>
PC(nmoles/mgprotein)	17.02±2.5	25.98±3.82 <sup>a</sup>	20.24±2.02 <sup>b</sup>	19.29±3.60 <sup>b</sup>	18.16±1.87 <sup>b</sup>
AOPP(nmoles/mgprotein)	10.80±2.00	14.00±2.08 <sup>a</sup>	11.95±1.7 <sup>b</sup>	12.86±0.15 <sup>b</sup>	10.49±1.02 <sup>b</sup>

Values are presented as mean ± standard deviation. Superscripts (a) indicates significant difference ( $p < .05$ ) when compared with control, whereas superscripts (b) indicates significant difference ( $p < .05$ ) when compared with ISO treated only.

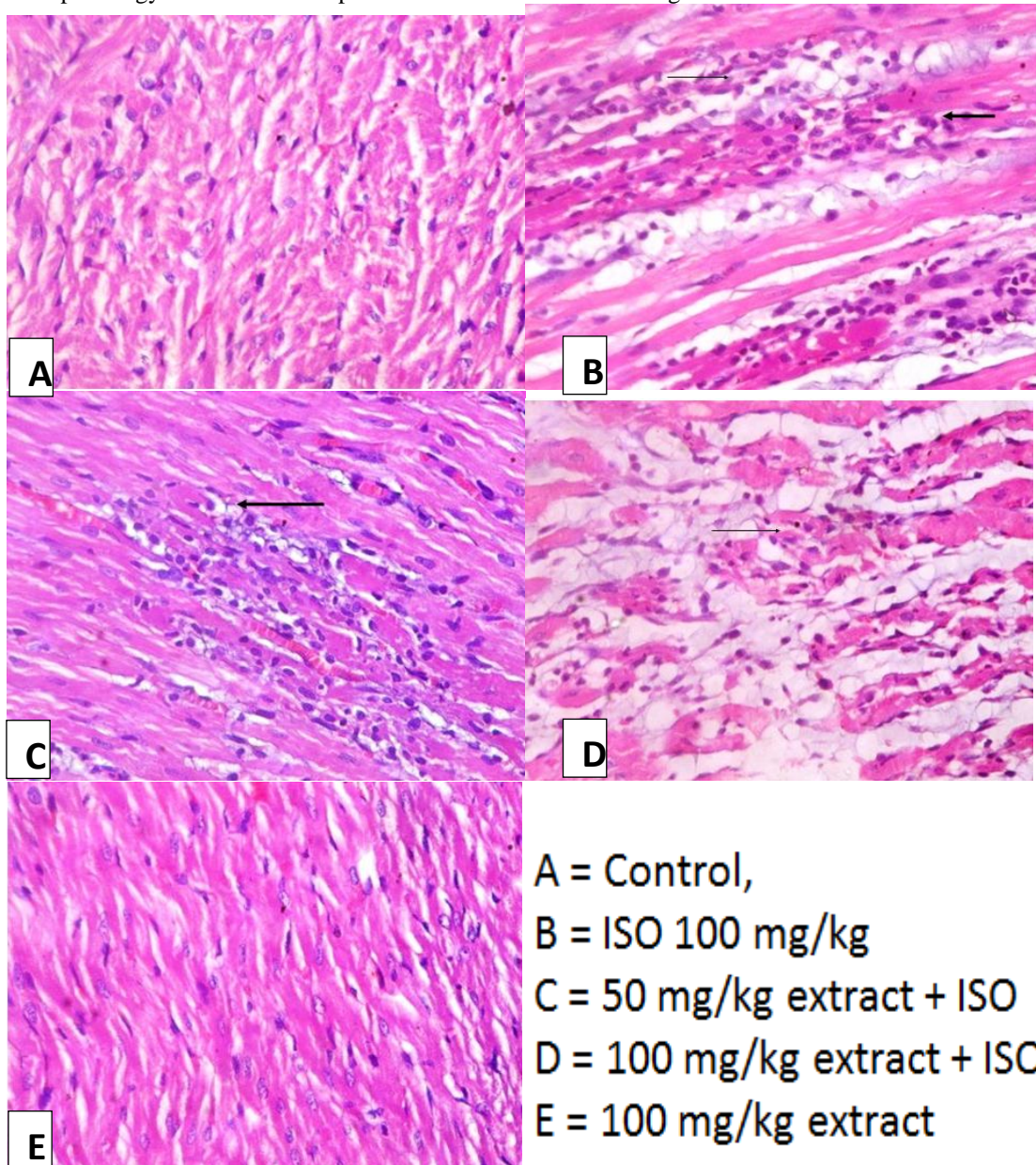
Table 2: Effect of methanol extract of stem bark of *Moringa oleifera* on cardiac markers of inflammation and damage

	Control	ISO 100 mg/kg	50 mg/kg extract + ISO	100 mg/kg extract + ISO	100mg/kg extract
MPO (U/mgprotein)	0.32±0.08	0.81±0.10 <sup>a</sup>	0.55±0.04 <sup>b</sup>	0.35±0.09 <sup>b</sup>	0.32±0.05 <sup>b</sup>
CK-MB (U/L)	2.75±0.95	8.26±1.65 <sup>a</sup>	4.13±1.65 <sup>b</sup>	3.85±0.95 <sup>b</sup>	2.20±0.95 <sup>b</sup>
NO (U/mgprotein)	0.46±0.02	0.67±0.03 <sup>a</sup>	0.49±0.05 <sup>b</sup>	0.45±0.08 <sup>b</sup>	0.44±0.05 <sup>b</sup>

Values are presented as mean ± standard deviation. Superscripts (a) indicates significant difference ( $p < .05$ ) when compared with control, whereas superscripts (b) indicates significant difference ( $p < .05$ ) when compared with ISO treated only. MPO= Myeloperoxidase, CK-MB = Creatine Kinase Myocardial Band, NO = Nitric Oxide

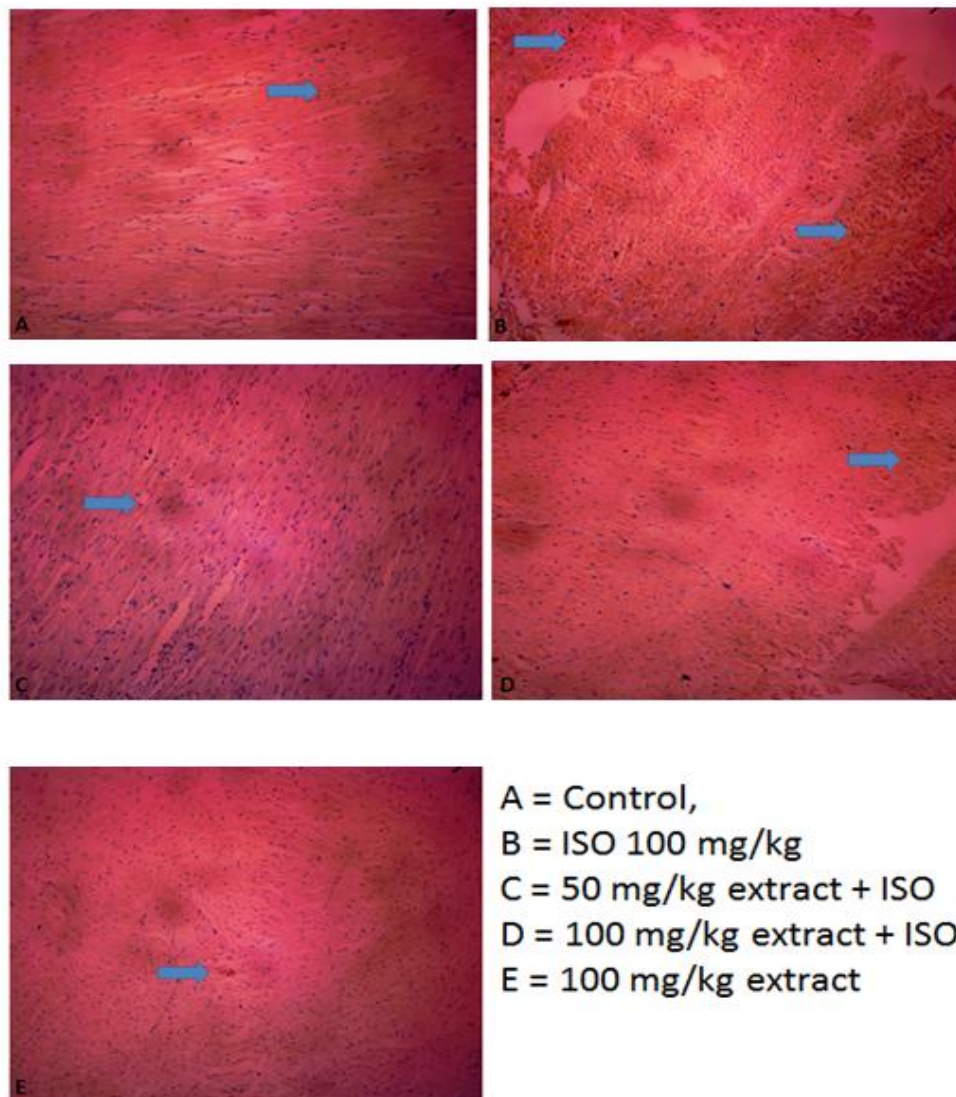


Histopathology of rat heart in isoproterenol-induced cardiac damage

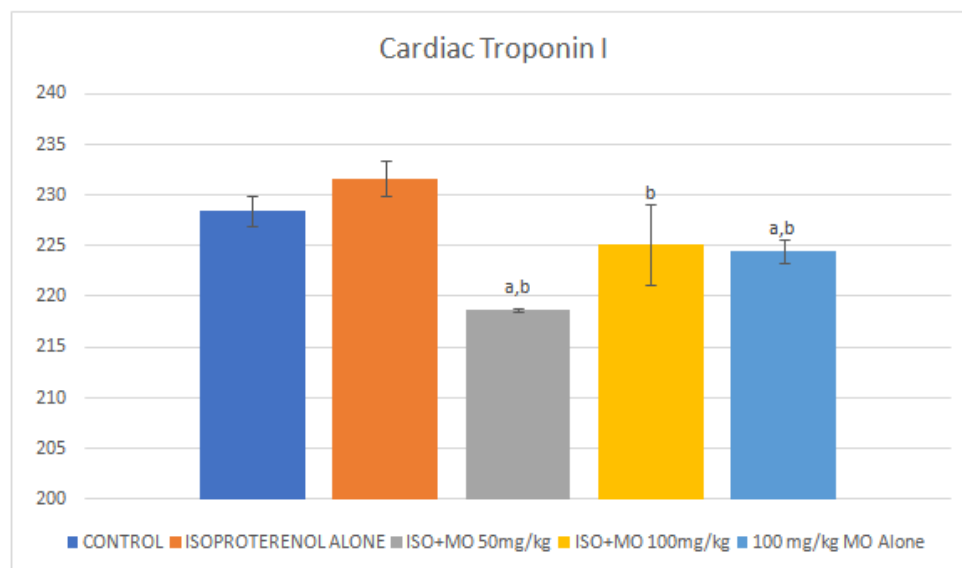


**Figure 7:** Photomicrographs of cardiac tissue at x400. **A** shows no significant lesion. **B** shows moderate infarction of the endocardium (thin arrow) and myocardium (black arrow) of ventricle. **C** shows mild infarction of the myocardium (black arrow). **D** shows moderate infiltration of inflammatory cells (thin arrow) to myocardium. **E** shows no significant lesion seen

**CARDIAC TROPONIN**

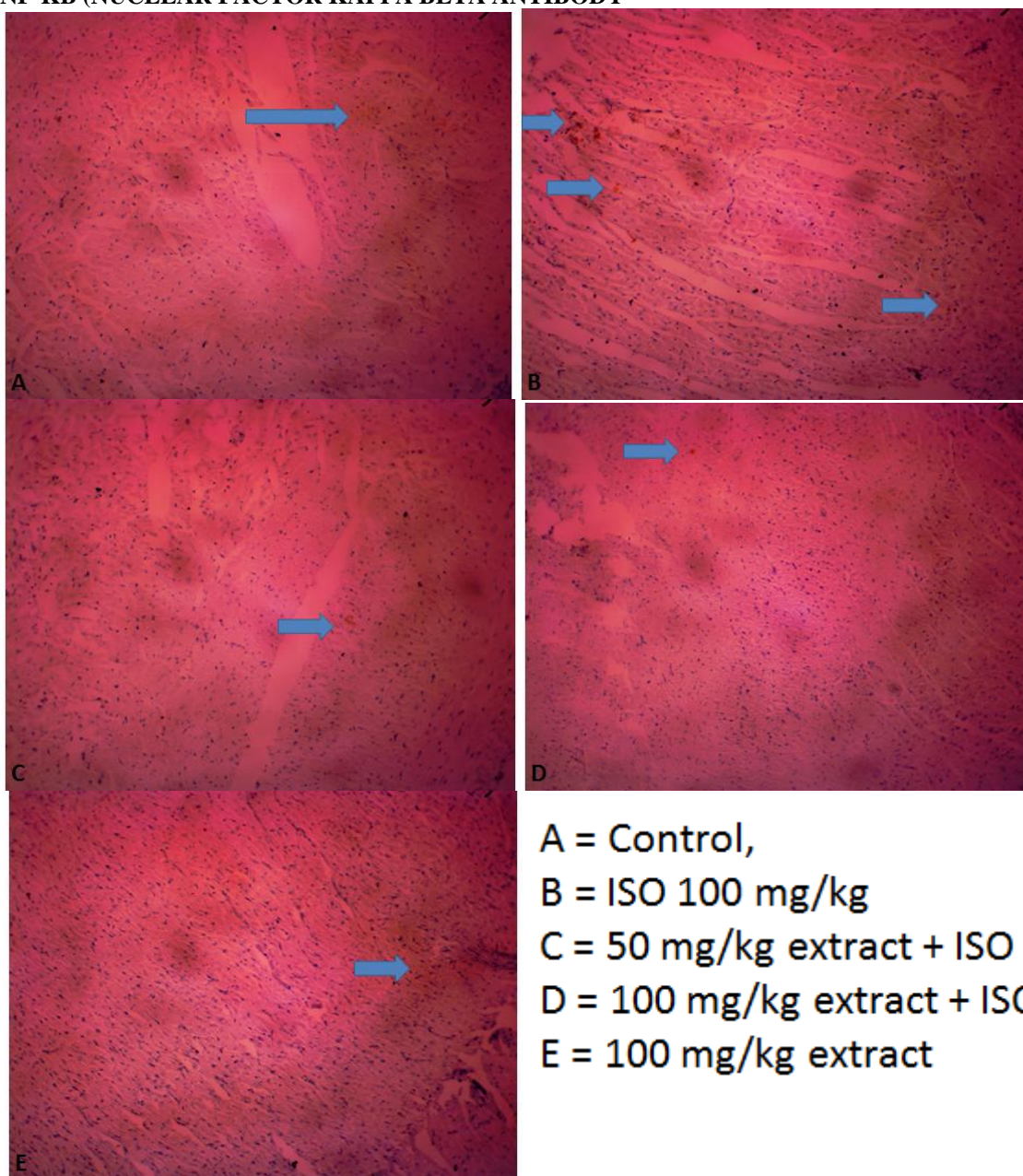


**Figure 8:** A shows least expression of CTn I in the heart section. B shows highest expression of CTn I when compared with A, C, D, and E.



**Figure 8a:** Cardiac troponin quantification as in Figure 8

**NF-KB (NUCLEAR FACTOR KAPPA BETA ANTIBODY**



**Figure 9:** A shows least expression of NF-KB in the heart section. B shows highest expression of NF-KB when compared with A, C, D, and E.

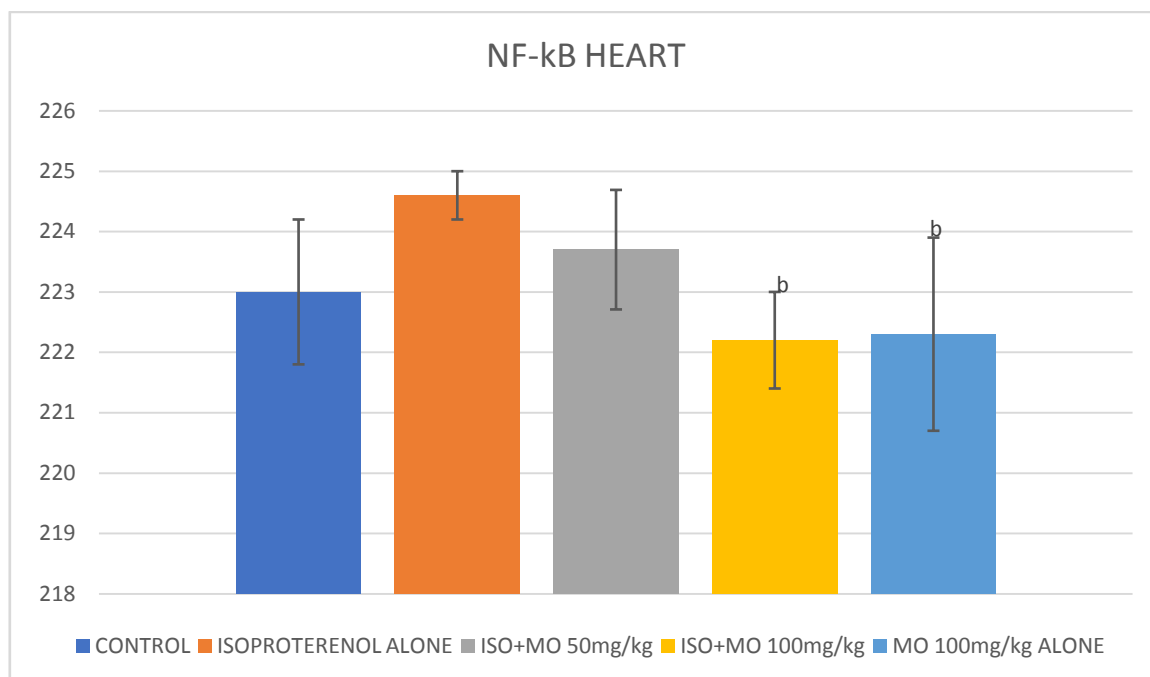


Figure 9a: NF-kB quantification as in figure 9.

Adeolu Adedapo, etal. "The methanol stem extract of *Moringa oleifera* mitigated isoproterenol-induced myocardial infarction through cardiac troponin and NF-kB signaling pathways in rats ." *IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)*, 15(2), (2020): pp. 65-76.