Article

The methanol leaf extract of *Vernonia amygdalina* ameliorates cardiomyopathy in alloxan-induced diabetic rats

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Abstract

Vernonia amygdalina is a tropical plant with a lot of interesting biological and medicinal uses. The plant is relatively not toxic, hence safe for consumption and possesses a great potential as pharmaceutical leads for the treatment of diseases. It is for this that the methanol leaf extract was evaluated for its cardioprotective effects in rats. Rats were randomly allotted to five groups of ten animals each. Group A animals were not diabetic and normal saline and served as normal control, group B animals were diabetic rats that received alloxan alone, group C animals were diabetic rats but received glibenclamide at 4mg/kg. Groups D and E were also diabetic animals that received the methanol leaf extract of *Vernonia amygdalina* (MLVA) at 200mg/kg and 400mg/kg respectively. All treatments were done daily via the oral route and lasted for 28 days. Administration of alloxan caused a significant increase in the blood pressure parameters of diabetic control rats when compared with the normal control. Treatment with glibenclamide brought about a reduction in the blood pressure of treated diabetic rats when compared with the normal control. MLVA treated diabetic groups showed a significant lowering of all blood pressure parameters. Oxidative stress markers such as Myeloperoxidase (MPO), nitric oxide content (NO), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) all experienced significant increase in their levels in group B animals but corresponding decrease for extract-treated and glibenclamide-treated groups. The activities of antioxidant enzymes (SOD, GST and GPx) measured in this study showed significant reduction in the diabetic control group when compared to the normal control but following treatment with MLVA there were significant ($\alpha<0.05$) increase in their levels when compared with the diabetic control. The extract also appeared to result in a higher though not significant increase in SOD and GPx activities when compared with the glibenclamide treated group. Histopathologically, rats in control group show no visible lesions but diabetic rats in group B showed myocardial infarction. Treatment with glibenclamide showed no visible lesions but diabetic rats treated with MLVA at 200mg/kg showed focal area of lymphoid aggregate while those treated with MLVA at 400mg/kg showed a mild disseminated haemorrhagic lesion. Diabetic rats also showed higher expression of CRP and IL-1B in their cardiac tissues but down regulation of this protein in the cardiac tissues of extract- and glibenclamide-treated rats.

Keywords *Vernonia amygdalina*; cardiomyopathy; diabetes; anti-oxidants; immunohistochemistry; histopathology.

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1 Introduction

Cardiovascular pathologies are the most common cause of mortality in the world, and diabetes mellitus is one of the major risk factor for cardiovascular disease development. By 2025, diabetes is expected to affect 300 million people with a prevalence of 5.4%. The lifetime risk of cardiovascular disease in patients with diabetes is 67–78% (Fox et al., 2008). Diabetes affects the heart in 3 ways: (1) coronary artery disease (CAD) due to accelerated atherosclerosis; (2) cardiac autonomic neuropathy (CAN); and (3) diabetic cardiomyopathy.

Diabetic cardiomyopathy (DCM), one of diabetic complications, remains the major cause of mortality in people with diabetes (Tziakas et al., 2005). Lipid disorder, coagulation predomination, oxidative stress and inflammatory injury are important factors responsible for the development of diabetic cardiomyopathy because these factors promote the progression of premature atherosclerosis, coronary insufficiency and myocardial infarction (Reasner, 2008). The condition is also associated with important clinical consequences, such as increased susceptibility to hypertension-mediated damage, an increased mortality rate after acute myocardial infarction, and progression to symptomatic heart failure.

DCM is characterized by diastolic dysfunction, accumulation of extracellular matrix, activation of the inflammatory mechanisms, apoptosis and alterations in calcium homeostasis in the cardiomyocytes (Cai et al., 2006) The exact pathogenesis of DCM is complex and multifactorial, however, early factors leading to diabetic cardiac cell damage are documented to result from various stimuli such as hyperglycemia, glucose oxidation to reactive oxygen species (ROS), reactive nitrogen species, up-regulation of extracellular matrix proteins, myocardial fibrosis, activation of protein kinase C, cytokines and renin angiotensin system (Asbun & Villarreal, 2006). Hyperglycemia-induced overproduction of free radicals and reactive oxygen species (ROS) and oxidative stress can also cause cellular DNA damage and acceleration of cardiomyocyte necrosis and/or apoptosis (Ye et al., 2004; Zhou et al., 2008).

Hypertension affects approximately two-thirds of patients with diabetes (Kabakov et al., 2006; Ferrannini and Cushman, 2012) and is a significant contributing factor to cardiovascular complications (Sowers et al., 2001; American Diabetes Association, 2012). Lowering blood pressure (BP) has been shown to reduce cardiovascular events in patients with diabetes and to exert a renoprotective effect (Adler et al., 2000; Mancia et al., 2007; American Diabetes Association, 2012).

Hypertension aggravates the cardiovascular complications associated with diabetes and vice-versa. When occurring together, the two disorders result in devastating structural and functional cardiac impairments than are caused by either disease alone, or often leads to premature congestive heart failure, sudden cardiac death, and acute myocardial infarction (Sowers and Epstein, 1995; Alderman et al., 1999). It has been proposed that the structural myocardial damage in coexisting diabetes and hypertension may be attributed primarily to hypertension whereas the myocellular dysfunction primarily to diabetes (Grossman and Rosenthal, 1995). The combination of diabetes and hypertension impairs the myocardial contractile function synergistically, and

leads to sufficient organ damage to induce a severe, potentially fatal diabetic hypertensive cardiomyopathy (Fein et al., 1990; Rogers et al., 1991). Thus, any agent(s) with lipid-lowering, anti-coagulatory, anti-oxidative and/or anti-inflammatory and blood pressure lowering activities may potentially prevent or delay the occurrence of cardiovascular events in diabetes.

Vernonia amygdalina, family Asteraceae or Compositae is plants that is consumed locally as food and serve important ethnomedicinal uses. *V. amygdalina* is a tropical plant with a lot of interesting biological and medicinal uses. The plants are relatively not toxic, safe for consumption and possess a great potential as pharmaceutical leads for the treatment of diseases and beyond (Clement et al., 2014). Many parts of the plants are useful, they are used locally for the treatment of fever, Stomach disorder, jaundice, worm infestation, constipation, malaria, hiccups, kidney problems, amoebic dysentery, schistosomiasis, cough, wounds, diabetes, laxative, venereal diseases and other bacterial and protozoal infection (Clement et al., 2014)

Several research has shown that *Vernonia amygdalina* possesses the following activities; antidiabetic, (Ebong et al., 2008; Eteng et al., 2008) antioxidant (Oboh et al., 2008; Pownhall et al., 2010), anthelmintic (Huffman, 2001), hypolipidaemic (Adaramoye et al., 2008), anti-platelet and blood pressure lowering effects (Igboechi and Anuforo, 1984; Tona et al., 2004). However little has been reported about the protective effects of *Vernonia amygdalina* in diabetic cardiovascular events. This study was therefore carried out to evaluate the effects of *Vernonia amygdalina* on diabetes-induced cardiovascular complications.

2 Materials and Methods

2.1 Plant collection and preparation of extract

Fresh leaves of *Vernonia amygdalina* were collected from the community around the University of Ibadan, Ibadan, Nigeria. The leaves were dried under shade for about 14 days after which they were grounded to powder using an electric blender. 600g of the powdered material was soaked in 2.5 litres of methanol and shaken vigorously. The sample was then left for 72 hours with intermittent shaking. After 72 hours the mixture was filtered using a Buckner funnel and Whatman No. 1 filtered paper. The extract was further concentrated using a rotary evaporator. The weight of the resulting extract was 32.4grams.

2.2 Experimental animals

Seventy male albino rats (150–220 g) of the Wistar strain were obtained and kept in the experimental animal house of the Faculty of Veterinary Medicine, University of Ibadan throughout the period of this experiment. They were housed in rats' cages and were fed with standard rat diet. They were given access to clean water at all times and allowed to acclimatize for a period of two weeks. After acclimatization fasting blood glucose (pre-induction) was measured before induction of diabetes. All experimental procedures were in conformity with the University of Ibadan Ethics Committee on Research in Animals as well as internationally-accepted principles for Laboratory animal upkeep and use.

2.3 Induction of diabetes

Diabetes was induced in rats by a single intraperitoneal (I.P) injection of freshly prepared solution of Alloxan monohydrate (100mg/kg). Forty eight hours after induction fasting blood glucose level was assessed using ACCUCHEK active blood glucometer and a total of thirty six animals with blood glucose of 200mg/dl and above were selected for the study.

Rats were randomly allotted to five groups of ten animals each. Group A animals were not diabetic and received vehicle+ normal saline and served as normal control, group B animals were diabetic rats that received vehicle alone, groups C were diabetic rats that received glibenclamide at 4mg/kg, group D and E received the methanol leaf extract of *Vernonia amygdalina* at 200mg/kg and 400mg/kg respectively. All treatments were done daily via the oral route and lasted 28 days.

2.4 Blood pressure measurement

Indirect blood pressure parameters (systolic, diastolic, and mean blood pressure) were measured on day 27 without anaesthesia, by tail plethysmography using an electrosphygnomanometer (CODA, Kent Scientific, USA). The average of at least nine readings, taken in the quiescent state, following acclimatization, was taken per animal.

2.5 Blood collection and serum preparation

Blood was collected for serum biochemical assays on days 14 and 28 post-treatment. From each rat, fresh whole blood (3 ml) was collected through the retro-orbital venous plexus into sterile tube it was thereafter centrifuged at 4 000 rpm for 10 minutes. Serum was collected into sample bottles and stored at –20 °C till the time of analysis. The animals were not anesthetized

2.6 Tissue preparation

After collection of blood from each of the animals, all rats were sacrificed by cervical dislocation on the 28th day of the treatment and the heart was harvested. A portion was then cut into 10% formalin for histological evaluation and the remaining was placed on ice, rinsed and homogenized in aqueous potassium buffer (0.1 M, pH 7.4) and the homogenate was centrifuged at 10,000 rpm (4^oC) for 10 min to obtain the post mitochondrial fraction (PMF). The PMF obtained was then subsequently stored at –20 °C until the time of use.

2.7 Biochemical assays

2.7.1 Measurement of serum myeloperoxidase content

70ul of sample was pipetted into the cuvette, O- dianisidine, phosphate buffer was added and then H_20_2 inside a beaker Aspirate 2,000 ul of O- dianisidine and H_2O_2 mixture and add to the sample Take reading at 0 second, 30 seconds and 60 seconds. MPO generated was calculated as change in activity per mg protein multiplied by a factor of 10.

2.7.2 Measurement of serum nitric oxide (NO) content

Serum nitric oxide (NO) was measured as described by Olaleye et al. (2007) by indirectly measuring the nitrite concentration. After incubation at room temperature for 20 minutes, the absorbance at 540 nm was measured by spectrophotometer. The concentration of nitrite in the sample was determined from a sodium nitrite ($NaNO_2$) standard curve and was expressed as μmol nitrite/mg protein.

2.7.3 Determination of cardiac superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) was determined by the method of Misra and Fridovich (1972) with modification from our laboratory (Oyagbemi et al., 2015), giving credence to the validation of the method. Briefly, 100 mg of epinephrine was dissolved in 100 ml distilled water and acidified with 0.5 ml concentrated hydrochloric acid. This preparation prevents oxidation of epinephrine and is stable for 4 weeks. 0.01 ml of cardiac PMF was added to 2.5 ml 0.05 M carbonate buffer (pH 10.2) followed by the addition of 0.3 ml of 0.3 mM adrenaline. The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds. One unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome during 1 minute.

2.7.4 Determination of cardiac reduced glutathione (GSH)

The cardiac reduced glutathione (GSH) was estimated by the method of Jollow et al. (1974). Briefly, 0.5ml of 4% sulfosalicylic acid (precipitating agent) was added to 0.5 ml of PMF and centrifuged at 4 000 rpm for 5minutes. To 0.5 ml of the resulting supernatant 4.5 ml of Ellman's reagent (0.04 g of DTNB in 100 ml of 0.1 M phosphate buffer, pH 7.4) was added. The absorbance was read at 412 nm against distilled water as blank.

2.7.5 Determination of cardiac glutathione peroxidase (GPx) activity

The cardiac glutathione peroxidase (GPx) activity was also measured according to Buetler et al. (1963). The reaction mixtures contained 0.5 ml of potassium phosphate buffer (pH 7.4), 0.1 ml of sodium azide, 0.2 ml of

GSH solution, 0.1 ml of H_2O_2, 0.5 ml of cardiac PMF and 0.6 ml of distilled water. The mixture was incubated in the water bath at 37 °C for 5 minutes and 0.5 ml of trichloroacetic acid (TCA) was added and centrifuged at 4 000 rpm for 5 mins. One ml of the supernatant was taken and 2 ml of K_2PHO_4 and 1 ml of Ellman's reagent were added. The absorbance was read at 412 nm using distilled water as blank.

2.7.6 Determination of cardiac thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substance was quantified as malondialdehyde (MDA) in the cardiac PMF. The MDA was determined according to the method of Varshney and Kale (1990) To 1.6 ml of Tris-KCl, 0.5 ml of 30% TCA, 0.4 ml of samples and 0.5 ml of 0.75% thiobarbituric acid (TBA) prepared in 0.2 M HCl were added. The reaction mixture was incubated in the water bath at 80 °C for 45 mins, cooled on ice and centrifuged at 4 000 rpm for 15 minutes. The absorbance was measured against a blank of distilled water at 532 nm. Lipid peroxidation in units/mg protein was calculated with a molar extinction coefficient of 1.56×10^5 M/Cm.

2.7.7 Measurement of cardiac hydrogen peroxide (H_2O_2) generation

Hydrogen peroxide generation was determined according to Woff (1994). To 2.5 ml of 0.1 M potassium phosphate buffer (pH 7.4), 0.250 ml of ammonium ferrous sulphate (AFS), 0.1 ml of sorbitol, 0.1 ml of xylenol orange (XO), 0.025 ml of H_2SO_4 and 0.050 ml of cardiac PMF was added. The mixture was mixed thoroughly by vortexing till it foamed and a light pink color of the reaction mixture was observed. The reaction mixture was subsequently incubated at room temperature for 30 min. The absorbance was assessed at 560 nm, using distilled water as blank. The hydrogen peroxide (H_2O_2) generated was extrapolated from the hydrogen peroxide standard curve.

2.7.8 Protein determination

Protein concentrations were determined as described by Gornal et al. (1949). Briefly, 1 ml of diluted serum was added to 3 ml of the biuret reagent. The reaction mixture was incubated at room temperature for 30 minutes. The mixture was thereafter read with a spectrophotometer at 540 nm using distilled water as blank. The final value for total protein was extrapolated from the total protein standard curve.

2.8 Histopathology

Small pieces of the heart were collected in 10 % formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5–6 µm in thickness were made and stained with haematoxylin and eosin for histopathological examination (Drury et al., 1976).

2.9 Immunohistochemistry

The tissues were processed for immunohistochemistry based on the methods described by Todorich et al. (2010). Paraffin-embedded heart tissues were placed on charged slides and then dewaxed by immersion in xylene twice for 5 minutes each. Rehydration in ethanol of 100%, 90%, and 80% concentrations for 5 minutes each was then carried out. The slides were placed in distilled water tank for 5 minutes before carrying out peroxidase quenching with 3% H_2O_2/ Methanol (endogenous peroxidase) for 10 minutes. The slides were then rinsed with water and placed in wash buffer tank for 5 minutes and rinsed with distilled water. Antigen retrieval was done by boiling in citrate buffer pH 6.0 and allowed to cool. Slides were then rinsed and placed in distilled water tank for 2 minutes. The sections were blotted dry, and goat serum (KPL, Inc., Gaithersburg, Maryland, USA) added followed by incubation in a humidifying chamber for 15 minutes. After the incubation, the slides were shaken to remove excess goat serum, and incubated with IL-1B (1:200; Cell signaling Technology) antibody and CRP antibody (1: 200; Bioss Inc. Woburn, Massachusetts, USA), respectively, at 48^o C overnight in a humidifying chamber. The slides subsequently rinsed with wash buffer and placed in wash buffer tank for 5 minutes. Detection of bound antibody was carried out using biotinylated secondary antibody and subsequently, streptavidin Horse Radish peroxidase (HistoMark®, KPL, Gaithersburg MD, USA) for 30

minutes. Reaction product was enhanced with diaminobenzidine (DAB, Amresco®, USA) and counterstained with high definition haematoxylin (Enzo®, NYeUSA), with subsequent dehydration in ethanol. The slides were covered with coverslips and sealed with resinous solution. The immunoreactive positive expression of IL-1B and CRP intensive regions were viewed starting from low magnification on each slice then with 400× magnifications using a photo microscope (Olympus) and a digital camera.

2.10 Statistical analysis

Results were expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA), using GraphPad Prism version 6. The level of statistical significance was considered as $p<0.05$. Students t-test at 95% level of significance was used to assess significant difference between controls and treated group.

3 Results

3.1 Effects of *Vernonia amygdalina* on blood pressure of alloxan induced diabetic rats.

Administration of alloxan caused a significant increase in the blood pressure parameters of diabetic control rats when compared with the normal control. Treatment with glibenclamide brought about a reduction in the blood pressure of treated diabetic rats when compared with the normal control. However MLVA treated diabetic groups showed a significant lowering of all blood pressure parameters (Table 1).

Table 1 Effects of *Vernonia amygdalina* on blood pressure of alloxan induced diabetic rats.

GROUPS	SYSTOLIC BP	DIASTOLIC BP	MEAN ARTERIAL PRESSURE
A	129.33±6.12	97.17±4.17	107.89±4.70
B	174.83 ± 7.81^{abcde}	150.00 ± 14.37^{abcde}	157.56 ± 11.48^{abcde}
C	106.00 ± 2.68^{abde}	80.00 ± 8.02^{abde}	88.67 ± 5.53^{bc}
D	133.60 ± 11.28^{bc}	104.60 ± 10.01^{bc}	114.27 ± 9.14^{bc}
E	131.67 ± 9.89^{bc}	109.83 ± 7.83^{bc}	117.11 ± 8.51^{bc}

Values are mean ± SD, n =9, $^{a}\alpha < 0.05$ compared with control, $^{b}\alpha < 0.05$ compared with diabetic control, $^{c}\alpha < 0.05$ compared with glibenclamide, $^{d}\alpha < 0.05$ compared with MLVA, 200mg/kg, $^{e}\alpha < 0.05$ compared with MLVA, 400mg/kg.

3.2 Effect of *Vernonia amygdalina* on serum myeloperoxidase and nitric oxide content of alloxan induced diabetic rats

Myeloperoxidase a pro oxidant molecule released from activated neutrophils in inflammation was significantly increased in diabetic control group when compared with the normal control. Treatment with MLVA resulted in a dose dependent reduction in the myeloperoxidase generated in the diabetic rats. This reduction was also significant when compared with the glibenclamide treated group (Fig. 1).

Nitric oxide content which was measured indirectly by measuring the nitrite content of the serum showed significant increase in the MLVA treated diabetic group when compared with the diabetic control which showed a reduction in the nitrite levels when compared with the normal control (Fig. 2)

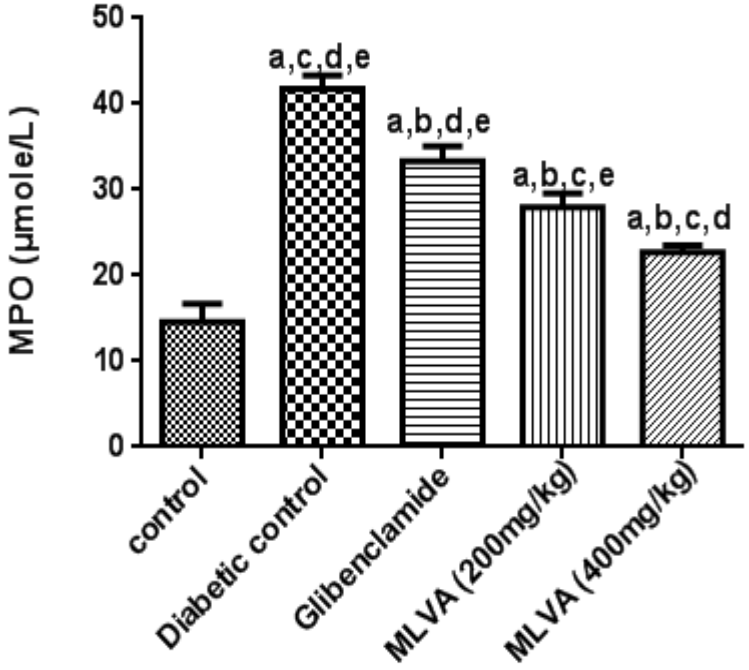

Fig. 1 Effect of *Vernonia amygdalina* on myeloperoxidase (MPO) in alloxan induced diabetic rats. Values are mean ± SD, n =10, **a** α≤ 0.05 compared with control, **b** α≤ 0.05 compared with diabetic control, **c** α≤ 0.05 compared with glibenclamide, **d** α≤ 0.05 compared with MLVA, 200mg/kg, **e** α≤ 0.05 compared with MLVA, 400mg/kg.

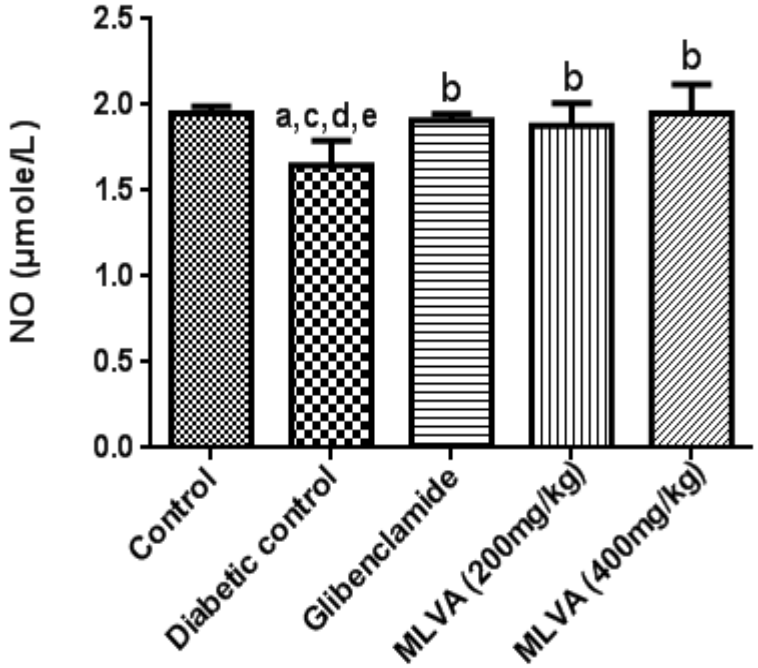

Fig. 2 Effect of *Vernonia amygdalina* on: Nitric oxide (NO) in alloxan induced diabetic rats. Values are mean ± SD, n =10, [a] α≤ 0.05 compared with control, [b] α≤ 0.05 compared with diabetic control, [c] α≤ 0.05 compared with glibenclamide, [d] α≤ 0.05 compared with MLVA, 200mg/kg, [e] α≤ 0.05 compared with MLVA, 400mg/kg.

3.3 Markers of oxidative stress

Hydrogen peroxide, a marker of oxidative stress was significantly reduced in glibenclamide and MLVA treated diabetic rats. In diabetic control H_2O_2 was increased but not significantly when compared with the normal control (Fig. 3).

Malondialdehyde (MDA), an end product of lipid peroxidation was significantly increased in the diabetic control when compared with the normal. On the other hand, treatment with MLVA produced a reduction in the MDA content of the cardiac tissue of treated diabetic rats. While the glibenclamide treated groups did not show any significant change in the MDA content when compared with the diabetic control and the normal control (Fig. 4).

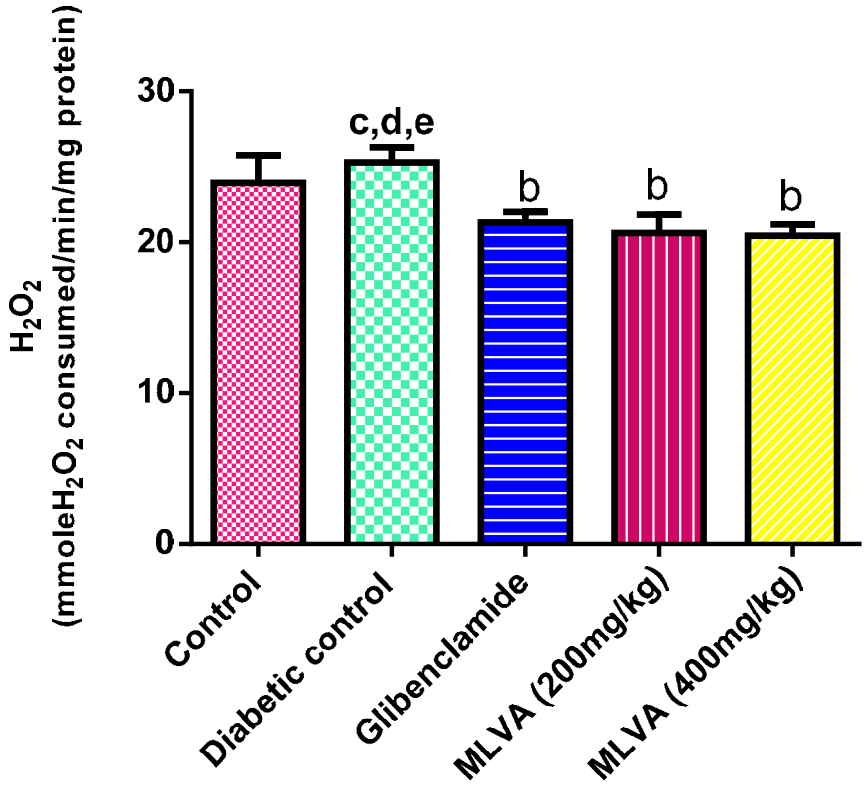

Fig. 3 Effect of MLVA on hydrogen peroxide level in cardiac post mitochondrial fractions (PMFs) of alloxan induced diabetic rats. Values are mean ± SD, n =10, [a] $\alpha \leq 0.05$ compared with control, [b] $\alpha \leq 0.05$ compared with diabetic control, [c] $\alpha \leq 0.05$ compared with glibenclamide, [d] $\alpha \leq 0.05$ compared with MLVA, 200mg/kg, [e] $\alpha \leq 0.05$ compared with MLVA, 400mg/kg.

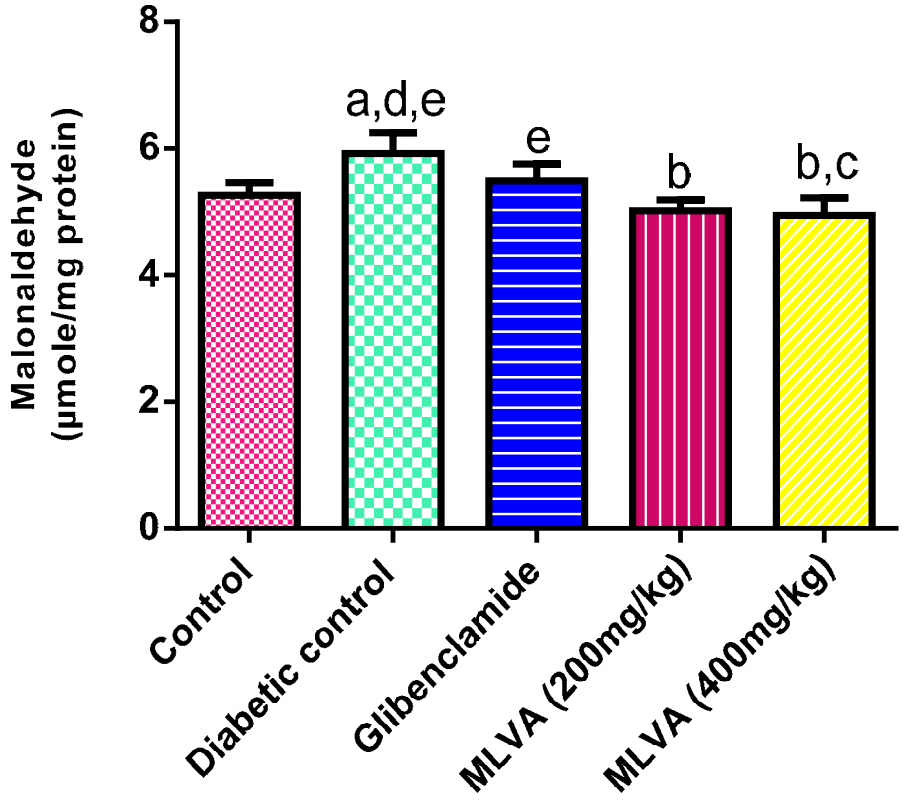

Fig. 4 Effect of MLVA on the malonaldehyde content of cardiac post mitochondrial fractions (PMFs) of alloxan induced diabetic rats. Values are mean ± SD, n =10, **a** α≤ 0.05 compared with control, **b** α≤ 0.05 compared with diabetic control, **c** α≤ 0.05 compared with glibenclamide, **d** α≤ 0.05 compared with MLVA, 200mg/kg, **e** α≤ 0.05 compared with MLVA, 400mg/kg.

3.4 Effect of *Vernonia amygdalina* on antioxidant systems of cardiac tissue of alloxan induced diabetic rats

Endogenous antioxidant defense mechanisms include both enzymatic and non-enzymatic pathways. Their functions in human cells are to counterbalance toxic reactive oxygen species (ROS). In this study, non enzymatic antioxidants (GSH, Protein thiol and Non protein thiol) showed significant (α<0.05) reduction in the diabetic control rats when compared to the normal control. But treatment with MLVA restored the levels of these antioxidants to values that were similar to those of the normal control. The glibenclamide treated group exhibited a higher though not significant increase in the level of protein thiol when compared with MLVA treated groups (Figs 5, 6 and 7).

The activities of antioxidant enzymes (SOD, GST and GPx) measured in this study showed significant reduction in the diabetic control group when compared to the normal control (Fig. 8, 9 and 10). Following treatment with MLVA there were significant (α<0.05) increase in the antioxidant enzymes of treated rats when compared with the diabetic control. The extract also appeared to result in a higher though not significant increase in SOD and GPx activities when compared with the glibenclamide treated group.

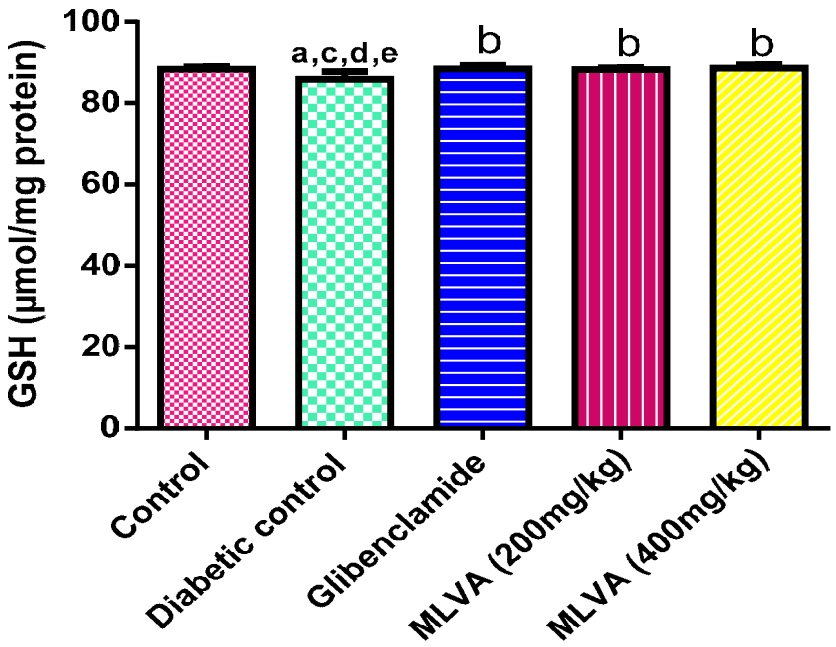

Fig. 5 Effect of MLVA on reduced glutathione (GSH) concentration in cardiac post-mitochondrial fractions (PMFs) of alloxan induced diabetic rats. Values are mean ± SD, n =10, **a** α≤ 0.05 compared with control, **b** α≤ 0.05 compared with diabetic control, **c** α≤ 0.05 compared with glibenclamide, **d** α≤ 0.05 compared with MLVA, 200mg/kg, **e** α≤ 0.05 compared with MLVA, 400mg/kg.

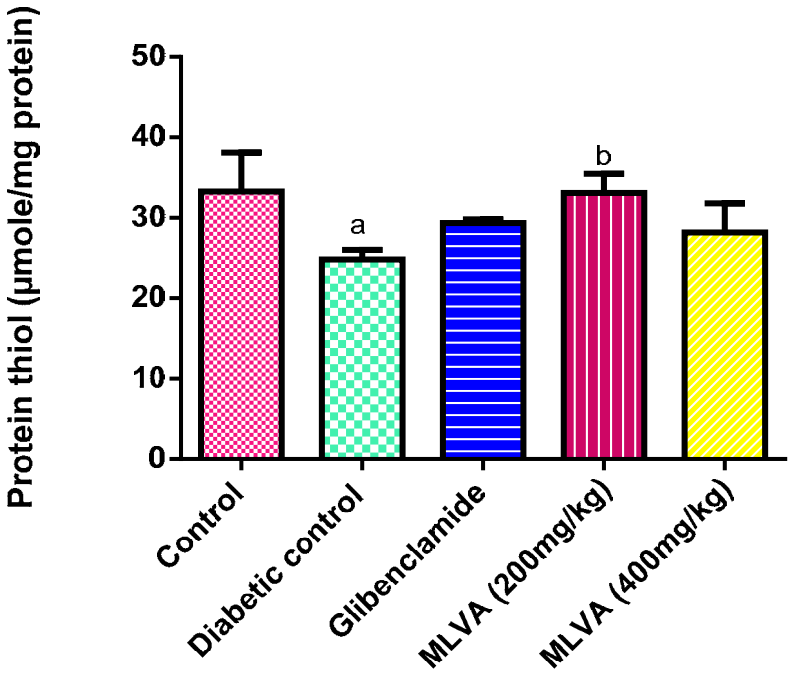

Fig. 6 Effect of MLVA on protein thiol concentration in cardiac post-mitochondrial fractions (PMFs) of alloxan induced diabetic rats. Values are mean ± SD, n =10, **a** α≤ 0.05 compared with control, **b** α≤ 0.05 compared with diabetic control, **c** α≤ 0.05 compared with glibenclamide, **d** α≤ 0.05 compared with MLVA, 200mg/kg, **e** α≤ 0.05 compared with MLVA, 400mg/kg.

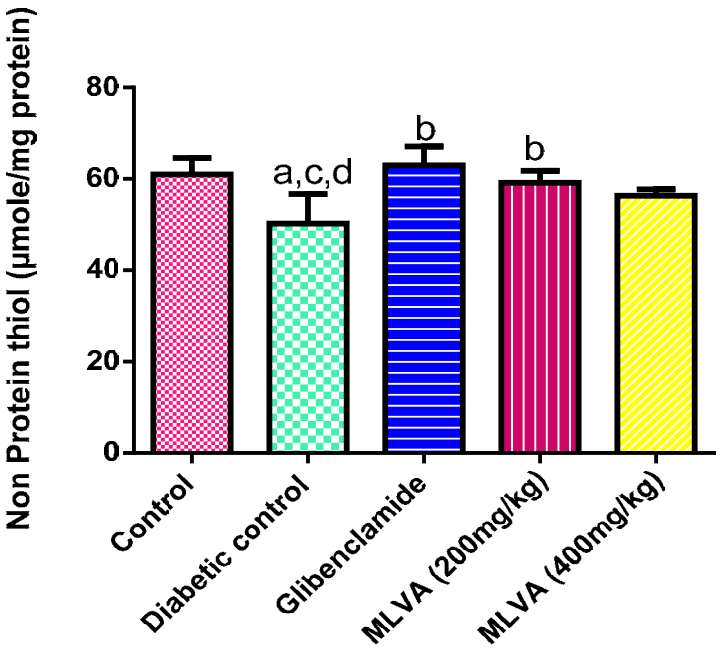

Fig. 7 Effect of MLVA on non protein thiol concentration in cardiac post-mitochondrial fractions (PMFs) of alloxan induced diabetic rats. Values are mean ± SD, n =10, **a** α≤ 0.05 compared with control, **b** α≤ 0.05 compared with diabetic control, **c** α≤ 0.05 compared with glibenclamide, **d** α≤ 0.05 compared with MLVA, 200mg/kg, **e** α≤ 0.05 compared with MLVA, 400mg/kg.

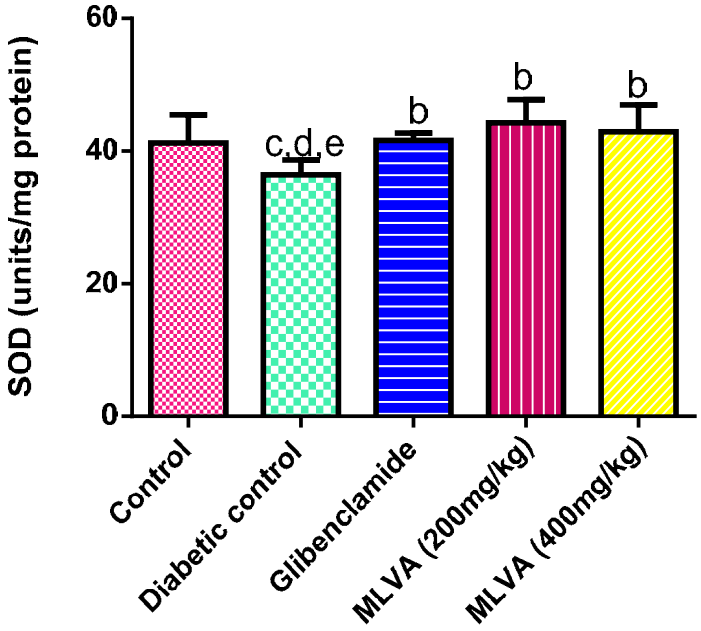

Fig. 8 Effect of MLVA on superoxide dismutase (SOD) activity in cardiac post-mitochondrial fractions (PMFs) of alloxan induced diabetic rats. Values are mean ± SD, n =10, **a** α≤ 0.05 compared with control, **b** α≤ 0.05 compared with diabetic control, **c** α≤ 0.05 compared with glibenclamide, **d** α≤ 0.05 compared with MLVA, 200mg/kg, **e** α≤ 0.05 compared with MLVA, 400mg/kg.

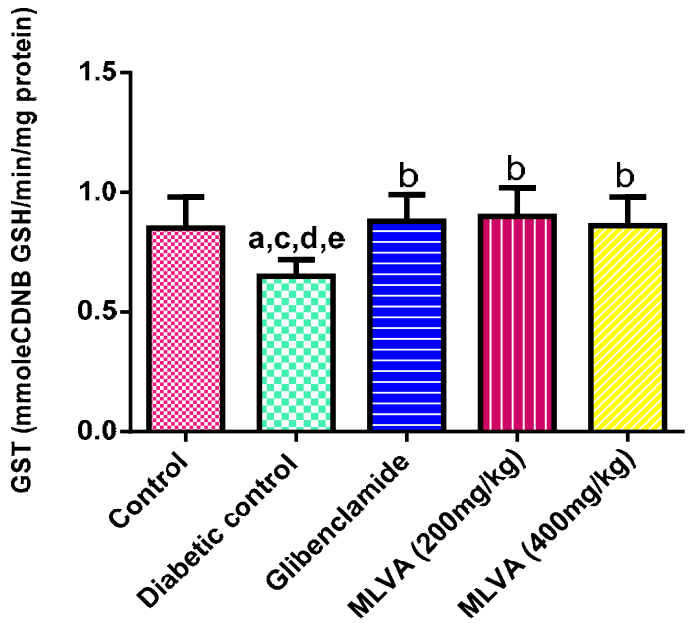

Fig. 9 Effect of MLVA on glutathione-S-transferase (GST) activity in cardiac post-mitochondrial fractions (PMFs) of alloxan induced diabetic rats. Values are mean ± SD, n =10, **a** α≤ 0.05 compared with control, **b** α≤ 0.05 compared with diabetic control, **c** α≤ 0.05 compared with glibenclamide, **d** α≤ 0.05 compared with MLVA, 200mg/kg, **e** α≤ 0.05 compared with MLVA, 400mg/kg.

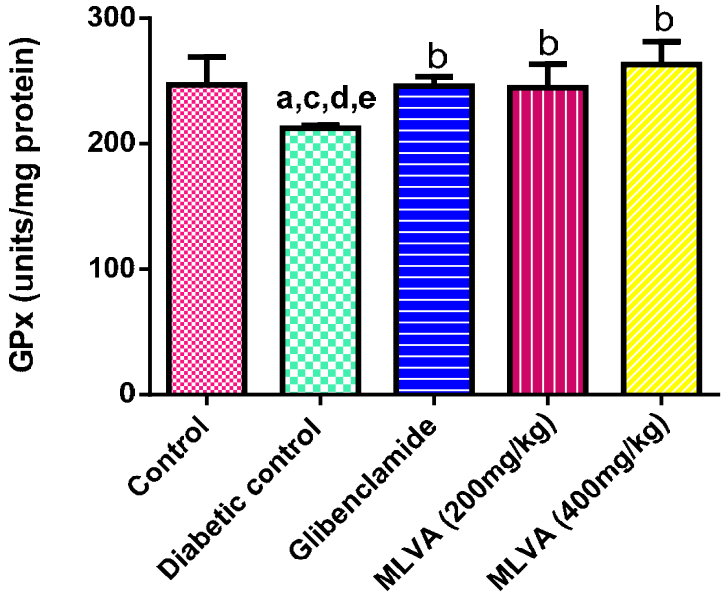

Fig. 10 Effect of MLVA on glutathione peroxidase (GPx) activity in cardiac post-mitochondrial fractions (PMFs) of alloxan induced diabetic rats. Values are mean ± SD, n =10, **a** α≤ 0.05 compared with control, **b** α≤ 0.05 compared with diabetic control, **c** α≤ 0.05 compared with glibenclamide, **d** α≤ 0.05 compared with MLVA, 200mg/kg, **e** α≤ 0.05 compared with MLVA, 400mg/kg.

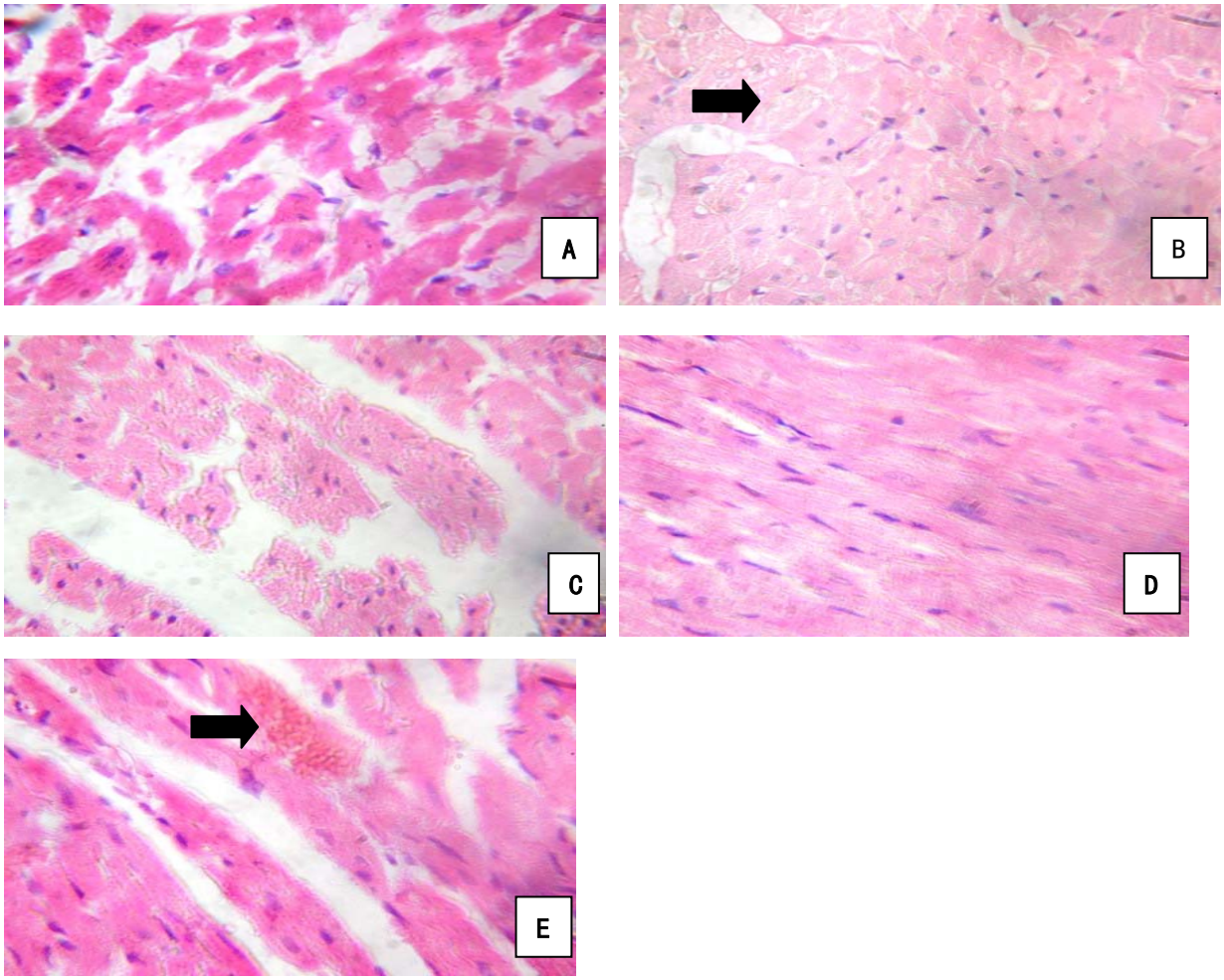

Fig. 11 Photomicrograph showing heart of rats. Rats in control group show no visible lesions (A). The diabetic control rats showed myocardial infarction (B). Treatment with glibenclamide showed no visible lesions (C). Heart of diabetic rats treated with MLVA at 200mg/kg showed focal area of lymphoid aggregate (D). Treatment with MLVA at 400mg/kg showed a mild disseminated haemorrhagic lesion. The tissues were stained with H & E (X400 objectives).

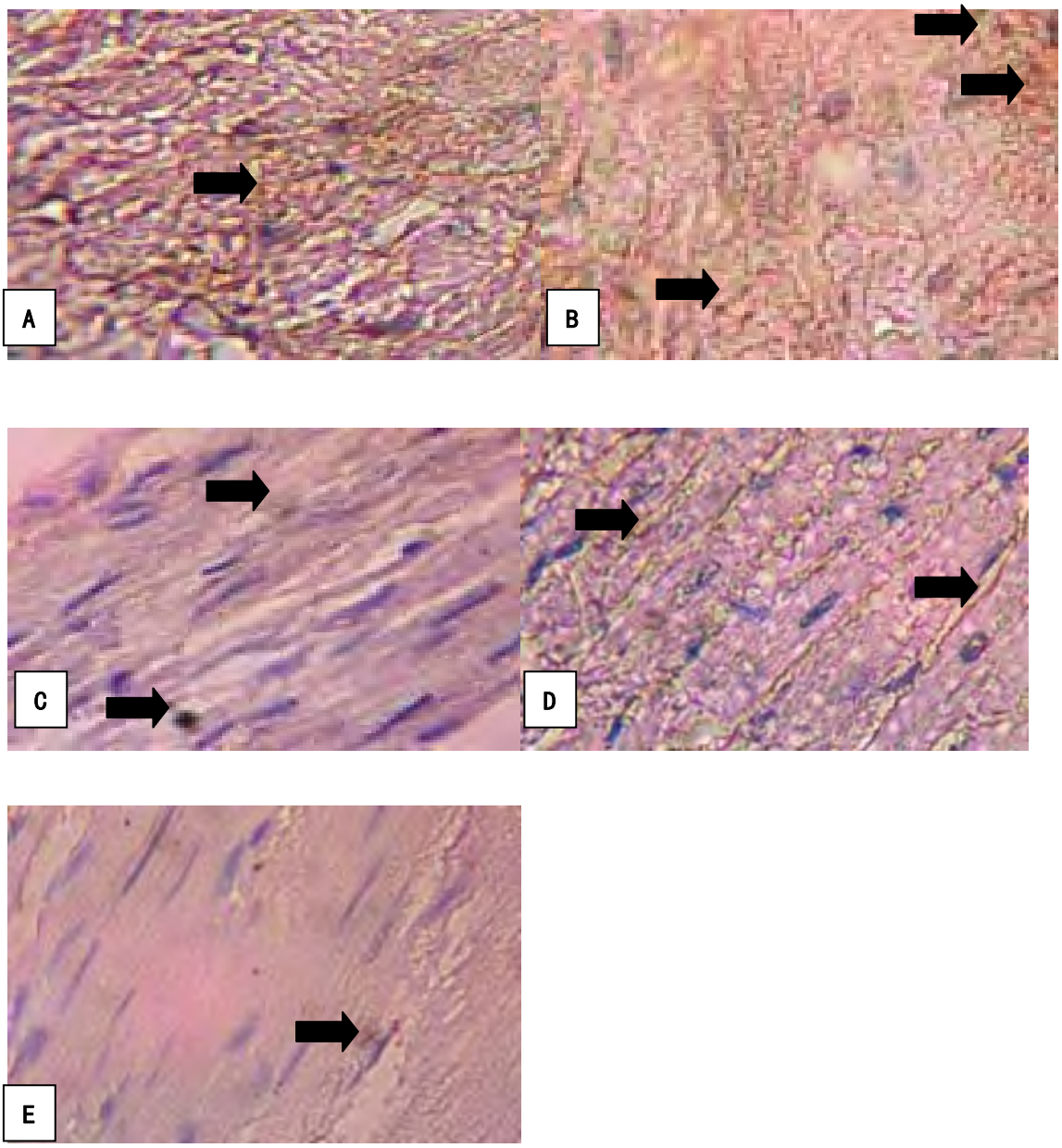

Fig. 12 Effect of MLVA on expression of CRP in cardiac tissue of alloxan-induced diabetic rats. Slides were counterstained with high definition haematoxylin and viewed 400× objectives. A- Normal control, B – Diabetic control, C- glibenclamide treated group, D- 200mg/kg MLVA, E- 400mg/kg MLVA. Note: CRP is C - reactive protein.

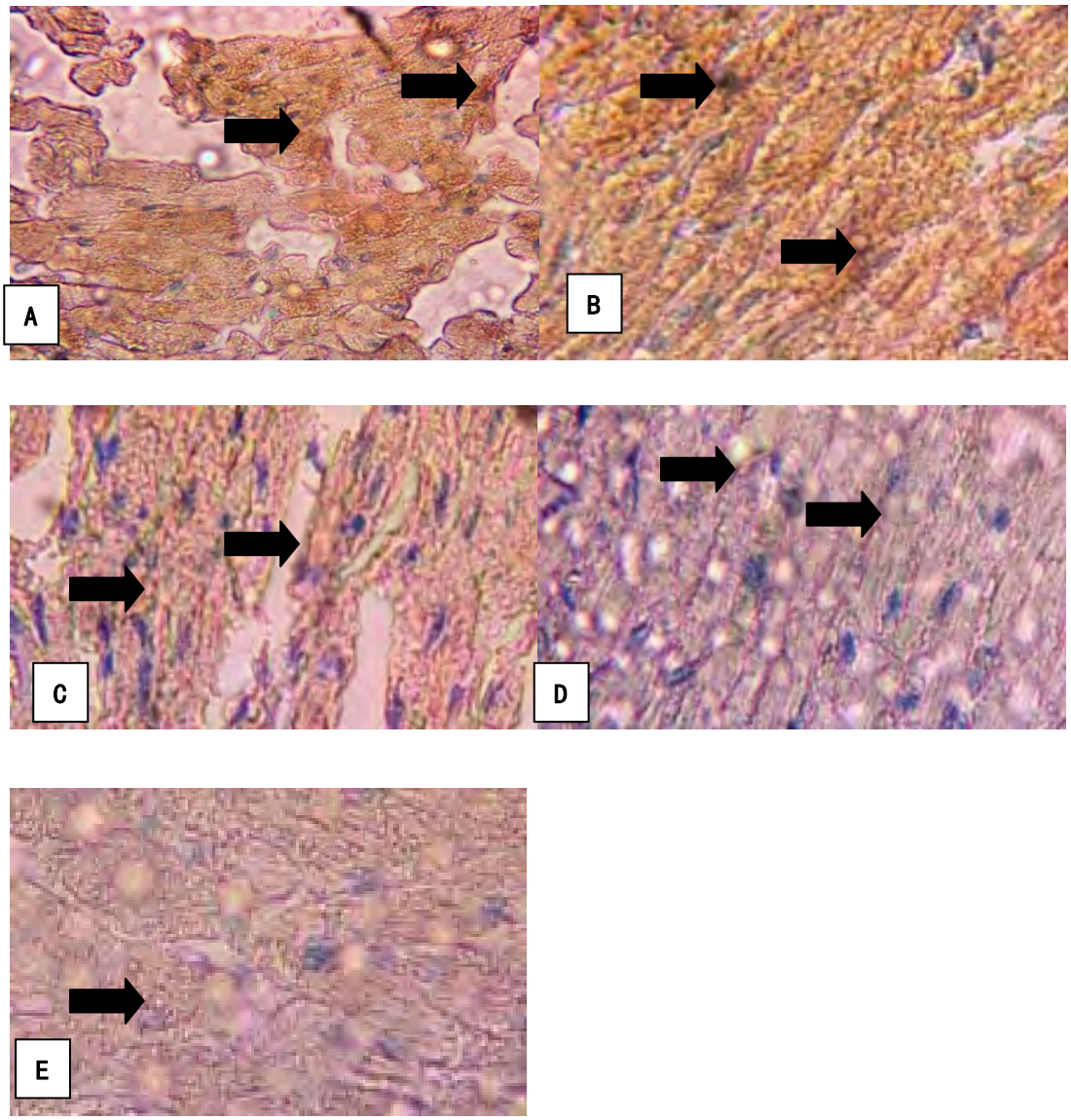

Fig. 13 Effect of MLVA on IL-1B expression in cardiac tissue of alloxan-induced diabetic rats. Slides were counterstained with high definition haematoxylin and viewed 400× objectives. A- Normal control, B – Diabetic control, C- Glibenclamide treated group, D- 200mg/kg MLVA, E- 400mg/kg MLVA.

4 Discussion

Diabetes markedly affects the function of the cardiovascular system, both the microcirculation as well as the large conduit arteries supplying vital organs such as the heart brain and kidney. As a consequence, diabetes and other conditions such as dyslipidaemia and hypertension is a risk predictor for myocardial infarction, stroke, and renal failure (Luscher and Steffel, 2008). Myeloperoxidase (MPO), an abundant haeme protein that is released by activated neutrophils, monocytes and tissue – associated macrophages after inflammatory stimuli (Stenvikel et al., 2006) is an enzyme involved in both inflammation and oxidative stress. Release of MPO by neutrophils and monocytes during inflammation plays an important role in the innate immune response (Klebanoff, 2005) but MPO activity may also lead to tissue damage and precipitate atherogenesis (Nicholls and Hazen, 2005). Patients with type 2 DM are in a state of low-degree chronic inflammation that induces

hypersecretion of inflammatory factors, which results in a constantly elevated neutrophilic granulocyte count (Lou et al., 2015). MPO catalyzes the production of hypochlorous acid and a range of other highly reactive species. However, HOCl and other MPO-derived oxidants participate as mediators of oxidative damage to biomolecules, including proteins, nucleic acids, lipids, and carbohydrates, thus damaging the host tissue. In that way they may initiate and contribute to the development of atherosclerosis, endothelial dysfunction (Rees et al., 2012). These MPO derived reactive substances may damage the arterial wall, thereby reducing its elasticity. In addition MPO reduces the bioavailability of nitric oxide (NO) (which is a powerful vasodilator produced by endothelial NOS which plays a critical role in the regulation of vascular tone) (Nicholls and Hazen, 2009; Schindhelm et al., 2009) by several mechanism. First, NO serves as a substrate for peroxidases, and MPO may thus serve as a catalytic sink for NO (Abu-Soud and Hazen, 2000; Baldus et al., 2004). Second, scavenging of NO by MPO-derived reactive substances may further reduce the bioavailability of NO. Third, hypochlorous acid can react with nitrogen atoms of the NOS substrate arginine to produce chlorinated arginine species that are inhibitors of all isoforms of NOS and have been shown to impair endothelium-dependent relaxation of rat aortic rings (Yang et al., 2006). Finally, it has been demonstrated that hypochlorous acid is a potent inducer of uncoupling of endothelial NOS, thereby turning NOS into a superoxide producing enzyme (Klebanoff, 2005). Together, these mechanisms may lead to an increase in blood pressure. Because hydrogen peroxide is an obligate cosubstrate of MPO, the activity of MPO in the vasculature may be enhanced by increased local production of reactive oxygen species. Vascular production of superoxide and its dismutation product hydrogen peroxide has been shown to be stimulated by high glucose concentrations, resulting in increased activity of MPO (Zhang et al., 2004).

In this present study, myeloperoxidase and blood pressure were observed to be increased significantly with a concurrent reduction in nitric oxide in diabetic control rats when compared to the normal control. This is similar to a report by Van der Zwan et al. (2010) who observed that the concentration of MPO in the circulation was positively associated with both systolic blood pressure and diastolic blood pressure and this association was most prominent on a background of hyperglycemia or oxidative stress suggesting that hyperglycemia induced oxidative stress potentiates the activity of myeloperoxidase in the vascular endothelium resulting in the cascade of events earlier described that results in hypertension.

On the other hand, administration of MLVA to diabetic rats for 28 days resulted in a dose dependent decrease in MPO generated, an increase in NO and as a result a lowering of blood pressure of the treated rats. These observations could be as result of the antioxidant activity of the phytochemicals in the plant which has been reported. Thus, *Vernonia amygdalina* was able to scavenge the free radicals generated as a result of the oxidative stress and thereby preserve the endothelium from oxidative damage.

Persistent hyperglycemia and hyperlipidemia are believed to be the main causes of increased oxidative stress, mitochondrial dysfunctions, fibrosis and apoptosis of cardiomyocytes in diabesity and associated complications (Giacco and Brownlee, 2010; Shen, 2010; Folli et al., 2011). Oxidative stress has been suggested to be involved in the development and progression of diabetes-induced cardiomyopathy (Giugliano et al., 1995). Oxidative damage in various tissues may be controlled or prevented by enzymic and nonenzymic antioxidant defense systems, which include reduced glutathione (GSH), superoxide dismutase, catalase, glutathione peroxidase (Aksoy et al., 2003). Studies have shown antioxidant enzymes activities are decreased in diabetes (Cai and Kang, 2001; Shen et al., 2006). The serious imbalance between ROS production and the decline of antioxidative ability leads to the enhancement of oxidative stress in diabetes. Recently a study also showed that ROS can augment myocardial fibrosis in diabetes (Aragno et al., 2008). In diabetes, these changes can cause myocardial remodeling and lead to the occurrence and development of DCM.

Lipid peroxidation is a characteristic of diabetes. The increase of free radicals in diabetic condition is

suggested to be due to increased lipid peroxidation (Kakkar et al., 1995; Baynes and Thorpe, 1999) and damage of antioxidant defence system. Elevated lipid peroxide commonly based on concentration of TBARS and decreased activity of antioxidant molecules in diabetic rats could probably be associated with oxidative stress and decreased antioxidant defence potential (Koshio et al., 1994; Rosa et al., 2003). Results of the present study is consistent with previous findings (Chis et al., 2009; Bhaskar and Kumar, 2012) demonstrating a significant increase in TBARS level. The drop in the glutathione pool and GSH in the tissues of diabetic animals indicates a greater utilization of GSH to counteract the increased ROS and decreased ability to synthesize glutathione. The lower glutathione synthesis during diabetes has been associated to a decrease in the catalytic and regulative subunits of g-glutamyl cysteinyl ligase (Urata et al., 1996; Catherwood et al., 2002), enzyme that limits GSH synthesis or to a lower availability of the precursor amino acids, especially cysteine (Xu et al., 2002). The increase in GSH levels following treatment with MLVA suggests a total or partial reestablishment of GSH pool and this goes further to explain the observed diminution of TBARS.

There was also an observed decrease in superoxide dismutase (SOD) and glutathione peroxidase (GPx) in diabetic rats compared to control rats. SOD protects tissues against oxygen free radicals by converting the superoxide radical into hydrogen peroxide and molecular oxygen, while GPx catalyses the detoxification of hydrogen peroxide, preventing damage to cell membranes and other biological structures (Chis et al., 2009). A study by Shen et al. (2006) showed that overexpression of manganese superoxide dismutase (MnSOD) to the heart can protect heart from oxidative damage in diabetic mice. In this study, treatment with MLVA resulted in an increase in the enzymatic (SOD, GST and GPx) antioxidant systems of cardiac tissue of treated diabetic rats. These results support several studies demonstrating the antioxidant properties of the *Vernonia amygdalina* (Nwanjo, 2006; Owolabi et al., 2008; Ong et al., 2011), which has been attributed to the presence of flavonoid and polyphenols in the plant known to possess antioxidant activities (Akah and Okafor, 1992; Ong et al., 2011).

Activation of GPx in diabetic rat cardiac tissue could reduce the pool of H_2O_2 in vivo, and hence disturb the balance of metabolism of this stable ROS which might have implications in insulin signaling. There are reports which suggest that over expression of antioxidant enzymes and altered H_2O_2 clearance in obesity may be responsible for the development of insulin resistance and interfere in insulin-dependent signaling (McClung et al., 2004). Activation of GSH metabolism by GST enzyme and recycling of GSH by GSSG-reductase appears to be a defense response to avoid over scavenging of the H_2O_2 (Haider et al., 2012). Also the presence of phytochemicals with antioxidant properties present in *Vernonia amygdalina* might serve to augment the endogenous antioxidant systems.

Heart failure, diabetes, and obesity are recognized as states of chronic inflammation. Inflammatory cytokines may play a role in all three of these conditions. C-reactive protein (CRP) is an acute-phase protein and is rapidly synthesized and released in response to inflammation and tissue damage (Szalai and McCrory, 2002). Many recent studies have examined the association between circulating CRP concentrations and increased cardiovascular disease risk. CRP is a classical plasma protein marker that is elevated during acute phase of inflammation, infection, and tissue injury (Black et al., 2004; Sun et al., 2005). Although, CRP is mainly produced by hepatocytes, there is some recent evidence suggesting that CRP may possibly be produced by macrophages (Dong and Wright, 1996), smooth muscle cells (Sun et al., 2005) or adipose tissue (Ouchi et al., 2003). Joshi et al. (2012) demonstrated the presence of CRP in the cardiac tissues and for the first time established that there is a measurable level of CRP in cardiac tissue from human left ventricular samples with evidence of cardiovascular disease. These observations suggest that CRP might be produced locally in cardiomyocytes. Previous studies indicated that CRP might promote apoptosis in endothelial progenitor cells (Fujii et al., 2006) and cardiac myocytes (Yang et al., 2008).

In this present study, an upregulation of cardiac CRP was observed in the diabetic control when compared with the normal, this is in consonant with other findings in this study such as the myocardial infarction observed in the histology of the diabetic control and also the increase in markers of oxidative stress and hypertension. These observations are in agreement with a study that observed that overexpression of human CRP exacerbates cardiac dysfunction in diabetes and that CRP augmented inflammation and RAS, which leads to increased oxidative stress, promotion of cardiac apoptosis, and fibrosis in diabetic cardiomyopathy (Yoshinori Mano et al., 2011). Administration of MLVA resulted in a down regulation of the expression of CRP in the treated diabetic groups in a dose dependent manner. This could be as a result of the antioxidant effects of *Vernonia amygdalina.* These observations are consistent with clinical reports suggesting that vitamin C supplementation yielded a 24.0% reduction in plasma CRP levels of healthy individuals exposed to active or passive smoking (Block et al., 2004). Although further investigations are needed, *Vernonia amygdalina* might serve as an agent to target CRP in diabetic cardiomyopathy.

Diabetes mellitus is nowadays acknowledged as a low-grade chronic inflammatory condition characterized by the over-secretion of pro-inflammatory cytokines. A growing body of evidence currently points at interleukin-1β (IL-1β), which is a major player in a wide array of auto-inflammatory diseases, to also act as key promoter of systemic and tissue inflammation in DM (Dinarello et al., 2010; Sumpter et al., 2011). Indeed, an enhanced expression of IL-1β in a high glucose milieu has been described in human monocytes and macrophages (Shashkin et al., 2006; Dasu et al., 2007), pancreatic islets (Maedler et al., 2004), myocardium (Niu et al., 2014), and aortic endothelium (Asakawa et al., 1997), while the upregulated IL-1β levels have been described in the heart and the retina and retinal vessels from diabetic rats, amongst others (Ares-Carrasco et al., 2009; Liu et al., 2012). Although the IL-1 pathway is considered at present a critical player in the pathophysiology of both T1DM and T2DM (Donath and Shoelson, 2011; Herder et al., 2015), the evidence on the impact of IL-1β on vascular function is still limited.

In this study expression of IL-1β was found to be upregulated in myocardiac tissue of the diabetic control when compared with the normal control. This has also been described by Niu et al. (2014). However treatment with MLVA resulted in a downregulation of the expression of IL-1β in the treated rats suggesting an anti-inflammatory activity of *Vernonia amygdalina* in diabetic cardiomyopathy thus resulting in protection of the diabetic heart from further damage. This study concludes that *Vernonia amygdalina* possesses antioxidant and anti-inflammatory properties in diabetes-induced cardiovascular events and can protect the cardiomyocyte from oxidative damage due to high blood glucose. Further studies howbeit should be carried out to describe the pathway by which it does this.

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