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Cardioprotective Activities of *Pterocarpus mildbraedii* Leaves on Isoproterenol-induced Myocardial Infarction in Rats

A. O. Fajobi[1*], B. O. Emma-Okon[2], O. O. Oyedapo[1], C. A. Dare[3], A. O. Akinlalu[4], I. J. Komolafe[5] and M. Ogunsusi[1]

[1]Department of Biochemistry and Molecular Biology, Obafemi Awolowo University, Ile-Ife, Nigeria.
[2]Department of Medical Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria.
[3]Department of Biochemistry, Osun State University, Osogbo, Nigeria.
[4]Department of Pharmaceutical Chemistry, Obafemi Awolowo University, Ile-Ife, Nigeria.
[5]Department of Biochemistry, Hallmark University, Ijebu-Itele, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Authors AOF, BOEO and OOO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AOF, CAD, AOA, IJK and MO managed the analyses of the study. Author AOF managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The study evaluated the effects of *Pterocarpus mildbraedii* leaf extracts on myocardial infarction induced by isoproterenol (ISO) in Wistar albino rats with a view to ascertain the value of its use in the management of heart-related diseases. Fresh plant leaves were collected, identified, extracted, fractionated and the aqueous layer partitioned with ethyl acetate. GC-MS was carried out on the ethyl acetate fraction (EAF) and unknown compounds were identified by comparing measured mass spectral data with those in NIST 14 Mass Spectral Library. Twenty-five adult rats were divided into five groups of 5 rats each. Groups I &II were the control groups. Rats in groups III-V were pretreated with 50 mg/kg and 100 mg/kg of EAF and 1.8 mg/kg of propranolol respectively for

**Corresponding author: E-mail: fajobi2009@gmail.com;*

21 days. Myocardial infarction was then induced in all the rats (except those in Group I) with the intraperitoneal injection of ISO (85 mg/kg) for 2 days. Afterwards, the rats were sacrificed and blood samples, heart homogenate and samples for histological studies were aseptically collected. Activities of cardiac biomarkers, lipid profile, enzymatic and non-enzymatic antioxidants were evaluated using standard methods. GC-MS analysis showed that the most abundant components of the plant are propionic acid, 2,3-dimethylphenyl ester, catechol, octyl-β-D-glucopyranoside, phenol and n-Hexadecanoic acid. Administration of ISO caused significant elevation of the activities of cardiac biomarkers (troponin-T concentrations, creatine kinase-MB and lactate dehydrogenase) while rats pretreated with EAF had significantly lower levels of the biomarkers. Moreover, alterations in lipid profile, enzymatic and non-enzymatic antioxidants brought about by the administration of ISO were ameliorated. Histological examinations revealed lesser degree of myocardial injury in pre-treated rats.

Keywords: Pterocarpus mildbraedii leaves; isoproterenol; myocardial infarction; GC-MS; Troponin-T; CK-MB; LDH; lipid profile.

ABBREVIATIONS

PMME : Pterocarpus mildbraedii Methanol Extract;
EAF : Ethyl Acetate Fraction;
HF : Hexane Fraction.

1. INTRODUCTION

Cardiovascular diseases (CVDs) can be defined as a group of derangements associated with the heart and blood vessels. These include atherosclerosis, rheumatic heart disease and vascular inflammation. These diseases constitute the principal cause of death globally (31% of the world deaths between 2012 and 2017) and an estimated 23.6 million people will die from CVDs by 2030 with more than three quarter of these deaths taking place in low and middle-par capital countries due to the high cost of medical care and unavailability and/or inability to assess quality medical centers [1]. Eighty percent of CVD associated deaths are caused by myocardial infarction (commonly called heart attacks) and strokes [1], believed to be primarily caused by arteriosclerosis, a disease of the arteries characterized by the deposition of plaques of fatty material on their inner walls [2].

Myocardial Infarction (MI), a medical condition that arises when a blood clot completely obstructs the coronary artery (coronary thrombosis), leading to death of tissue in that part of the heart [3], is a manifestation which arises due to unbalanced build-up of leukocytes, triacylglycerol and cholesterol [4]. Clinical symptoms of acute myocardial infarction (AMI) include sudden chest pain, nausea, shortness of breath, vomiting, weakness, diaphoresis, generalized sweating, anxiety/sense of impending doom and palpitations [4,5]. Drugs frequently prescribed for the management and prevention of MI include clopidogrel, a beta-blocker drug, aspirin (involved in reducing the clustering of blood platelets), statins (lowering cholesterol level in the blood), and angiotensin converting enzyme (ACE) inhibitor (protecting the heart) [6]. Many studies have reported a direct link between low risk of cardiovascular diseases and the consumption of fresh fruits, vegetables, or natural antioxidants-rich plants. Studies have indicated that fruits and leafy vegetables possess as well as exhibit potent and appreciable cardio protective potentials due to their antioxidant, anti-inflammatory and thrombolytic properties [7,8].

Isoproterenol (ISO), a β-adrenergic agonist is a synthetic catecholamine derivative which when present in high concentrations exerts severe stress in the myocardium leading to infarct-like necrosis. It is also known to stimulate lipid peroxidation, which might be a causative factor in the irreversible damage to the myocardial membrane in experimental myocardial infarction models. Generally, catecholamines undergo auto-oxidation to generate free radicals that are responsible for myocardial changes [9,10].

Pterocarpus mildbraedii, locally called *oha/ora* is widely consumed in the Southeastern part of Nigeria, for its therapeutic and medicinal values. The plant grows in the lowland rainforest, dry evergreen and riverine forests, and has a smooth, gray or pale brown bark, exuding red gum when cut [11,12]. The young tender leaf of *P. mildbraedii* is one of the vegetables widely consumed for its therapeutic and medicinal values and some of its folklore uses include prevention of diabetes and treatment of headaches, pains, fever and convulsions. It has

been reported to contain phytochemicals that possess and exhibit antioxidant, hypocholesterolemic, anti-microbial and chemoprotective properties [12,13]. Fresh leaves of *P. mildbraedii* are used in the preparation of soup especially by the Eastern Nigerian people with the claim that it prevents heart-related diseases. However, there are yet no scientific reports or findings to support such claims. This study therefore investigates the effect of extracts of the plant on cardiac parameters in rats induced with myocardial infarction in order to ascertain the validity or otherwise of such claims.

2. MATERIALS AND METHODS

2.1 Preparation of Methanolic Extract of *P. mildbraedii*

Fresh leaves of *P. mildbraedii* were collected from Ajebandele, Ile-Ife, Nigeria. The plant was identified and authenticated at IFE Herbarium, Department of Botany, Obafemi Awolowo University (OAU), Ile-Ife. The leaves were cut into bits, air-dried and pulverized. The pulverized leaves were suspended in 80% (v/v) methanol for 72 hours at room temperature with occasional stirring and then filtered using cotton wool. The residue was further re-extracted with the same solvent until the extract became colourless. The extracts were pooled together, filtered and concentrated *in vacuo* to slurry under reduced pressure at 40°C on rotary evaporator (Rotavapor RII, BUCHI Switzerland) according to method of Oyedapo & Amos [14]. The slurry was dispensed into clean petri dishes and exposed to air to obtain dark brown residue termed *P. mildbraedii* methanol extract (PMME) which was then kept in the desiccator for further analysis.

2.2 Fractionation of Methanol Extract Using Solvent Partition Technique

The methanol extract was partitioned according to previously described methodologies [15,16] using solvents of increasing polarities. PMME (21 g) was suspended in hot distilled water (100 ml) and allowed to dissolve, filtered and partitioned sequentially in a separating funnel with n-hexane (500 ml x 6). The hexane fractions were pooled together and concentrated to dryness under reduced pressure to yield hexane fraction (HF). The aqueous layer was partitioned with ethyl acetate (500 ml x 13). The ethyl acetate fractions were pooled together and concentrated to dryness under reduced pressure to yield ethyl acetate fraction (EAF).

2.3 Gas Chromatography-Mass Spectrometry Analysis of Ethyl Acetate Fraction

Gas chromatography-mass spectroscopy (GC-MS) was carried out using 7820A gas chromatograph coupled to 5975C inert mass spectrometer (with triple axis detector) and electron impact source (Agilent Technologies). The separation of the compounds was carried out on HP-5 Capillary Column coated with 5% of Phenyl Methyl Siloxane (30 m length x 0.32 mm diameter x 0.25 µm film thickness) (Agilent Technologies) with helium as carrier gas used at a constant flow rate of 1.573 ml/min, an initial nominal pressure of 1.9514 psi and at an average velocity of 46 cm/s. The relative percentage (%) amount of each component was calculated by comparing its average peak area to the total areas and interpretation on mass spectrum of GC-MS using the database of National Institute of Standard and Technology (NIST) having more than 62,000 patterns. The unknown compounds were identified by comparing the measured mass spectral data with those in NIST 14 Mass Spectral Library.

2.4 Animal Study

Male albino Wistar rats (176.4 ± 5.2 g) were procured from the Faculty of Pharmacy, Obafemi Awolowo University (OAU), Ile-Ife and housed in the Animal House, Department of Biochemistry and Molecular Biology, OAU Ile-Ife under standard conditions. The animals were left for 3 weeks to acclimatize before the commencement of treatment.

2.5 Grouping and Treatment of Experimental Animals

The experimental rats were divided randomly into five groups of five animals each and treated as follows:

Group I: Rats (control, Normal saline);
Group II: Rats + ISO (ISO, 85 mg/kg);
Group III: Rats + EAF (50 mg/kg) + ISO;
Group IV: Rats + EAF (100 mg/kg) + ISO;
Group V: Rats + Propranolol (1.8 mg/kg) + ISO.

Animals were pre-treated regularly with normal saline, EAF and propranolol for 21 days before the induction of myocardial infarction. On days 22 and 23 at an interval of 24 hrs, animals in Groups II-V were induced with ISO (85 mg/kg). On day 24, after overnight fast, the rats were anaesthesized using diethyl ether, sacrificed,

dissected and blood was collected by cardiac puncture into heparinized tubes. Heart tissue was removed, washed free of blood in normal saline and blotted with tissue paper for the preparation of homogenate. The left ventricle of the heart was fixed in 10% (v/v) formal-saline for histological study.

2.6 Preparation of Plasma and Heart Homogenate

Heparinized blood was centrifuged on Bench Centrifuge (90-2 Microfield Instrument, Essex, England) at 3000 rpm for 20 minutes. The supernatant (plasma) was carefully removed with clean Pasteur pipette into sterile vial and kept at -20°C for biochemical analyses.

The heart homogenate (10% w/v) was prepared according to the method of Bode & Oyedapo [17]. Blood-free heart tissue (1 g) was cut into bits, homogenized in 10 ml of 100 mM phosphate buffer, pH 6.8 and centrifuged on TGL16A High-Speed Refrigerated Centrifuge at 4000 rpm for 15 min. The supernatant was carefully transferred into sterile vials and kept frozen for biochemical analyses.

2.7 Biochemical Analyses

Diagnostic kits for the assays of Creatine kinase-MB (CK-MB), Lactate dehydrogenase (LDH), Total Protein (TP), Triacylglycerol (TG), Total Cholesterol (TC), High-density lipoproteins cholesterol (HDL-c) and Low-density lipoproteins cholesterol (LDL-c) were obtained from Randox Laboratories Ltd, United Kingdom. Isoproterenol (Isoprenaline hydrochloride) was obtained from Shangai Aladdin Biochemical Technology Company Limited, Shangai China. Alpha (α)-lipoic acid, 3-methyl-2-benzothiazolinone hydrazine (MBTH) were obtained from AK Scientific, Union City, C.A. USA and Rat Cardiac Troponin T ELISA kit was obtained from Adel Crystal Lake Limited, Lagos, Nigeria.

2.8 Assay of Cardiac Troponin T (cTnT), Creatine Kinase (CK-MB) and Lactate Dehydrogenase (LDH) and Concentrations

Plasma troponin T (cTnT), Creatine Kinase (CK-MB) and Lactate Dehydrogenase (LDH) and Concentrations were measured with highly specific enzyme-linked immunosorbent (ELISA) kit according to the procedure provided in the kit manuals.

2.9 Evaluation of Lipid Profile

Assays of Total cholesterol, Triacylglycerol and HDL were carried out according to instructions specified in the respective Randox Diagnostic kit manuals. Low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) concentrations were calculated using Friedewald equation with the following expression:

LDL-C = TC – ($\frac{TG}{5}$ + HDL-c) mg/dl

VLDL-C = ($\frac{TG}{5}$) mg/dl

Where, TC = Total cholesterol and TG = Triacyglycerol.

2.10 Estimation of Total Protein Concentration

Estimation of plasma and heart homogenate total protein concentrations were carried out using biuret reaction method as described in the Randox Diagnostic kit manual and bovine serum albumin (5.95 g/dl) as standard. The assay mixture comprised of plasma (20 µl) or heart homogenate (20 µl) or BSA (20 µl) and distilled water (980 µl). Biuret reagent (1 ml) was added to the mixture, all components were mixed and incubated at room temperature for 30 min. The absorbance was read at 540 nm against the reagent blank. The concentration of total protein was calculated as:

Concentration of Total protein (g/dL) = $\frac{Abs_{sample}}{Abs_{standard}}$ x 5.95 g/dL

2.11 Assay of Heart Superoxide Dismutase (SOD) Activity

Assay of superoxide dismutase activity in heart homogenate was carried out according to the method of Maklund & Maklund [18] based on the ability of superoxide dismutase to inhibit the auto-oxidation of pyrogallol at alkaline pH. The assay mixture contained 2.8 ml buffer [0.1 M Tris-HCl buffer pH 8.20 containing 2 mM ethylenediamine tetraamine (EDTA)] (0.1 ml) and 0.1 ml pyrogallol (4.5 mM in 10 mM HCl).

The mixture was transferred into a cuvette and the absorbance readings were recorded at 420 nm at 30 sec interval for a period of 3 min. Blank contained all reagents except the homogenate was replaced with distilled water. The increase in absorbance was calculated as:

Increase in absorbance per minute = $\frac{A_6 - A_1}{2.5\ min}$

Where,

A_1 = absorbance after 30 sec and A_6 = absorbance after 180 sec

Percentage inhibition = $\frac{Increase\ in\ Absorbance\ of\ substrate}{Increase\ of\ Absorbance\ of\ blank}$ x 100

The activity of SOD was expressed as U/min/mg protein. One unit of SOD was defined as the amount of SOD necessary to cause 50% inhibition of the oxidation of pyrogallol.

2.12 Assay of Heart Catalase Activity

Assay of catalase enzyme activity in heart homogenate was carried out using the method of Sinha [19]. The reaction mixture contained homogenate (0.1 ml) and 1 ml of hydrogen peroxide (70 mM) in 0.05 M phosphate buffer pH 7.4. The reaction mixture was well mixed and incubated at 37°C for 3 min, after which 2 ml of 5% potassium dichromate in acetic acid (1:3) was added and the mixture was incubated at 100°C for 10 min. The test tube was cooled under running water and then the decrease in absorbance was monitored at 570 nm for 2 min. at the interval of 30 sec against reagent blank. The activity of catalase was expressed as U/min/mg protein. One unit of catalase activity was defined as the amount of catalase that converted 1 µmol of H_2O_2 to H_2O.

Catalase Activity (U/min) = $\frac{\Delta Abs/x\ TV\ x\ df}{\varepsilon\ x\ SV}$

Where,

ΔAbs = change in absorbance; TV = total assay volume; df = dilution factor;

ε = molar extinction = 40 $M^{-1}cm^{-1}$; SV = sample volume

2.13 Assay of Heart Glutathione Peroxidase (GPx) Activity

The assay of GPx activity was carried out according to the method of Rotruck et al. [20]. The GPx activity was estimated using the expression:

GPx activity (µmol/mg protein) = $\frac{Abs_{412}\ x\ TV\ x\ df}{\varepsilon\ x\ SV}$

Where,

Abs_{412} = Absorbance at 412 nm; TV = total assay volume; df = dilution factor; ε = Extinction coefficient (6.22 x 10^3 $M^{-1}min^{-1}$); SV = sample volume

2.14 Estimation of Heart Glutathione (GSH)

The concentration of glutathione in heart homogenate was carried out using the method of Moron et al. [21]. The absorbance of reaction mixture was read at 412 nm against reagent blank and the concentration of GSH in the heart homogenate was expressed as µgGSH/g sample.

2.15 Estimation of Vitamin C Concentration

The vitamin C concentration in plasma and heart homogenate was quantified using Folin-Ciocalteu's phenol reagent according to procedure that was based on the modified method of Omaye et al. [22]. The reaction mixture contained 0.5 ml of deproteinized plasma and heart supernatant, 1.5 ml of 10% (v/v) acetic acid and 0.5 ml of Folin-Ciocalteau's reagent (10%). The mixture was incubated at room temperature for 10 min. and absorbance was read at 760 nm against the reagent blank. The concentration of vitamin C was obtained from the standard calibration curve.

2.16 Estimation of Vitamin E Concentration

The total vitamin E concentration in plasma and heart homogenate was determined based on modified method of Baker and Frank [23]. The analysis was based on the reduction of ferric to ferrous ion by vitamin E and the formation of red coloured complex with 2, 2'-bipyridyl. Vitamin E (1 ml) extracted in heptane was mixed with 1 ml of 2, 2'-bipyridyl reagent (0.12% w/v) and 1 ml of ferric chloride (0.12% w/v). The concentration of vitamin E was obtained from the standard calibration curve.

2.17 Estimation of Alpha (α)-Lipoic Acid Concentration

The concentration of α-lipoic acid in plasma and heart homogenate was determined using the procedure that was based on the method of Deepthi et al. [24] using 3-methyl-2-benzothiazolinone hydrazine hydrochloride (MBTH) and ferric chloride.

2.18 Histopathological Study

The myocardial tissues were fixed in 10% (w/v) formal-saline solution immediately after the

animals were sacrificed. The fixed tissues were washed, dehydrated with alcohol embedded in paraffin and serial sections were cut using a rotary microtome and each section stained with hematoxylin and eosin (H & E) [25]. The preparation of slides, examination and interpretation of micro-photographs of slides were carried out in the Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife under digital microscope camera Leica ICC50HD and photograph captured at X400.

2.19 Statistical Analysis

Data were analysed using Microsoft Excel 2010 and expressed as Mean ± SEM, n=5. Differences between the values of control and treated groups were determined by One-Way Analysis of Variance (ANOVA) followed by Tukey's Post-Test, using GraphPad Prism 5. Differences were considered to be significant if $\rho < 0.05$.

3. RESULTS

3.1 GC-MS Profiles of Ethyl Acetate Fraction of *P. mildbraedii* Leaf

Fig. 1 shows that the ethyl acetate fraction of *P. mildbraedii* leaf as revealed by GC-MS analysis consists of eighty-eight compounds. The most abundant constituents were found to be propionic acid, 2,3-dimethylphenyl ester (17.40%), catechol (8.54%), octyl-β-D-glucopyranoside (6.52%), phenol (3.70%) and n-Hexadecanoic acid (3.46%) (Table 1).

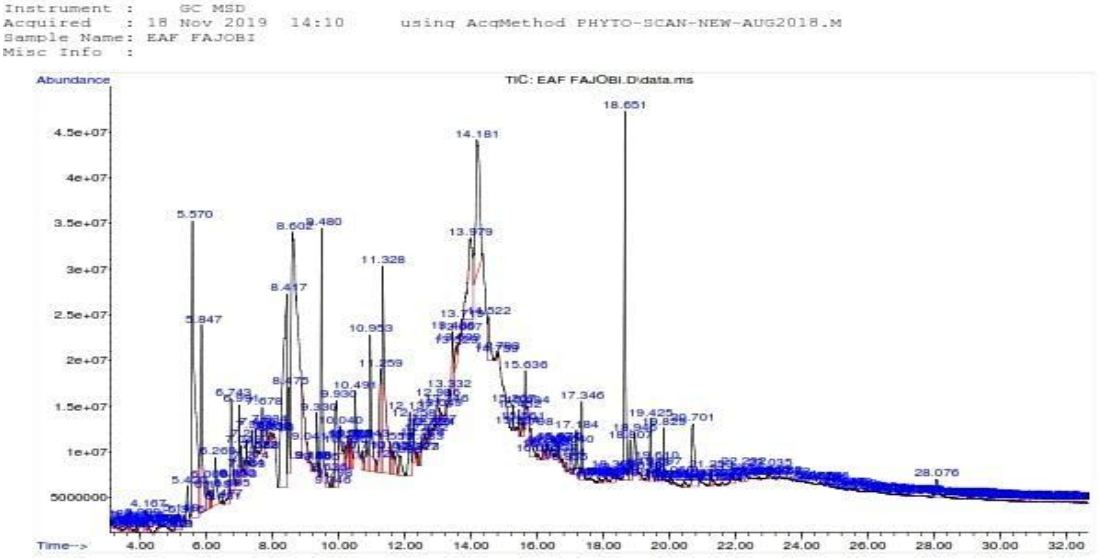

Fig. 1. GC-MS spectrum of ethyl acetate fraction of *P. mildbraedii* leaf

Table 1. Most abundant constituents in ethyl acetate fraction of *P. mildbraedii* leaf

S/NO	Compound name	Retention time	Percentage (%)
1	Phenol	5.5701	3.70
2	3-Aminoacrylonitrile	5.8473	1.51
3	Thiophene	8.4174	3.77
4	Catechol	8.6022	8.54
5	2-Methoxy-4-vinylphenol	9.48	1.36
6	Benzenamine	11.3281	3.32
7	α-D-Galactopyranoside, methyl	13.3321	2.18
8	Propionic acid, 2,3-dimethylphenyl ester	14.1811	17.40
9	Octyl-β-D-glucopyranoside	14.7933	6.52
10	Benzenepropanoic acid	15.4517	1.09
11	n-Hexadecanoic acid	15.6365	3.46
12	Lactose	16.6241	1.07
13	9-Octadecenamide	18.6512	2.26

Table 2. Effect of Ethyl Acetate Fraction (EAF) of *P. mildbraedii* leaf on the rat body and heart weights

Groups	Initial body weight (g)	Final body weight (g)	Change in body weight (%)	Weight of heart (g)	Ratio of body to heart (%)
I	167.00 ± 5.39	185.00 ± 4.47	9.74 ± 1.84	0.61 ± 0.05	0.33 ± 0.01
II	190.00 ± 6.32	209.00 ± 11.87	8.55 ± 2.54 (12.22%)[a] ↓	0.87 ± 0.12	0.42 ± 0.01 (21.43%)[a] ↑
III	169.00 ± 6.00	185.00 ± 6.32	8.46 ± 3.18 (1.05%) ↓	0.77 ± 0.14	0.40 ± 0.02 (4.76%) ↓
IV	188.00 ± 11.58	198.00 ± 15.94	4.43 ± 1.95 (48.19%) ↓	0.78 ± 0.10	0.39 ± 0.01 (7.14%) ↓
V	168.00 ± 2.81	180.00 ± 6.77	5.98 ± 2.81 (30.06%) ↓	0.73 ± 0.06	0.41 ± 0.01 (2.38%) ↓

Each value represented the Mean ± SEM of 5 readings

[a]p< 0.05 statistically significant when compared with control (group I)

Change in body weight (Bwt) (%) = [(final bwt-initial bwt)/final bwt] x 100

Ratio of body to heart (%) = (Weight of heart/Final body weight) x 100

Grp I = Control; Grp II = ISO treated; Grp III = EAF (50 mg/kg bwt + ISO; Grp IV = EAF (100 mg/kg bwt + ISO; Grp V = Propranolol (1.8 mg/kg bwt + ISO)

Table 2 presents a summary of the body weights of the control and experimental rats. The body to heart ratio of ISO-treated rats increased significantly ($\rho < 0.05$ when compared with the control (group I). However, pre-treatment with EAF reduced the ratio when compared with the ISO-treated group (group II).

Fig. 2 shows a summary of the levels of cardiac biomarkers, Troponin T, CK-MB and LDH of experimental groups of rats. The induction of myocardial infarction caused significant increase in the biomarkers as seen between Groups I&II. However, levels of these biomarkers were significantly reduced in the groups of rats pre-treated with EAF when compared with the induced group (group II) and compared favourably with group pre-treated with the standard drug (group V).

The effect of EAF on lipid profile is presented in Fig. 3. There was a significant increase in the lipid profile (except the HDL-C which significantly decreased) of the induced group (Group II) when compared with the control group. However, there were reversals in the lipid profile in the groups of rats pre-treated with EAF at both concentrations when compared with the induced group.

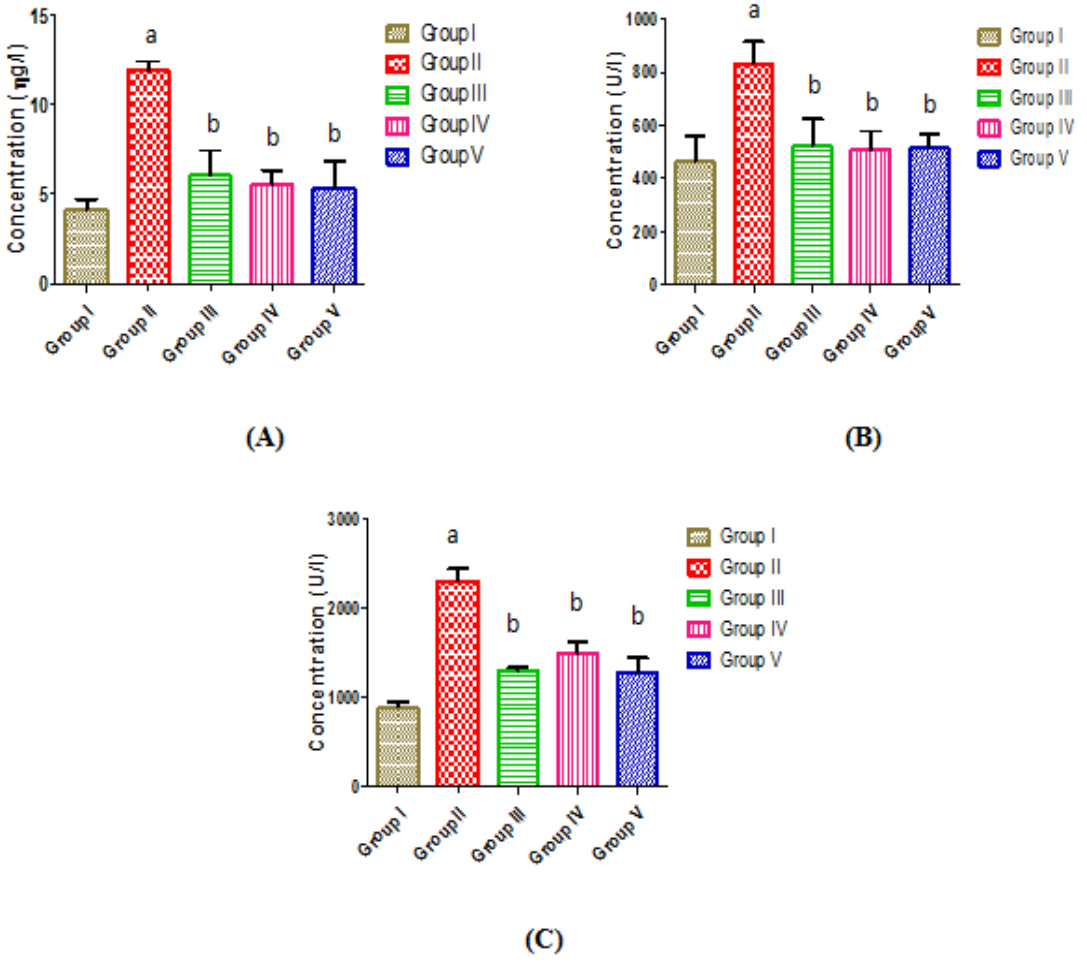

Fig. 2. Effect of ethyl acetate fraction on (A) Troponin T concentration, (B) CK-MB and (C) LDH activities

Each value represented the Mean ± SEM of 5 readings. [a]p< 0.05 statistically significant when compared with control (Group I), [b]p< 0.05 statistically significant when compared with ISO-induced group (Group II), Grp I = Control; Grp II = ISO treated; Grp III = EAF (50 mg/kg bwt) + ISO; Grp IV = EAF (100 mg/kg bwt) + ISO; Grp V = Propranolol (1.8 mg/kg bwt) + ISO

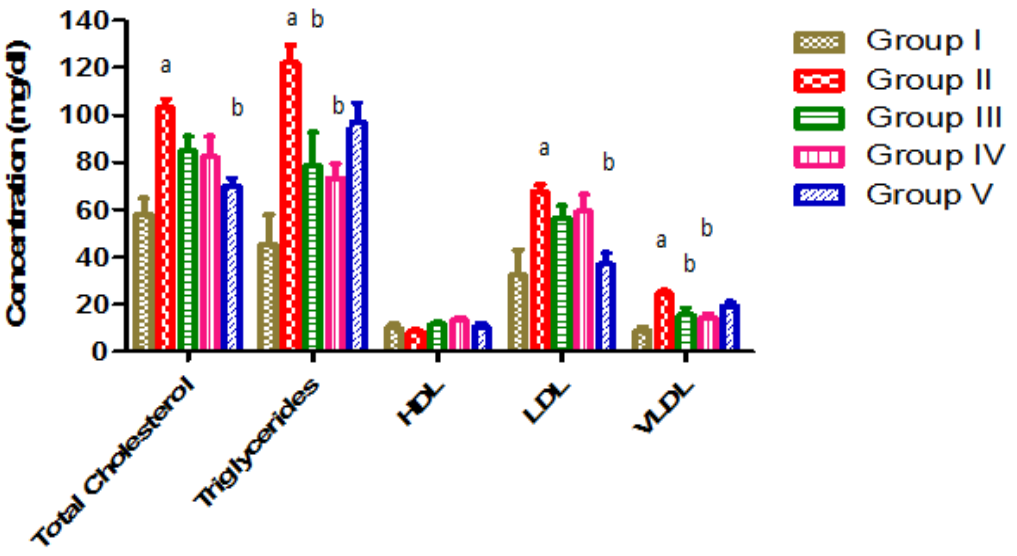

Fig. 3. Effect of ethyl acetate fraction of *P. mildbraedii* leaf on lipid profile

Each value represents the Mean ± SEM of 5 readings, [a]p< 0.05 statistically significant when compared with control (Group I), [b]p< 0.05 statistically significant when compared with ISO-induced group (Group II), Grp I = Control; Grp II = ISO treated; Grp III = EAF (50 mg/kg bwt) + ISO; Grp IV = EAF (100 mg/kg bwt) + ISO; Grp V = Propranolol (1.8 mg/kg bwt) + ISO

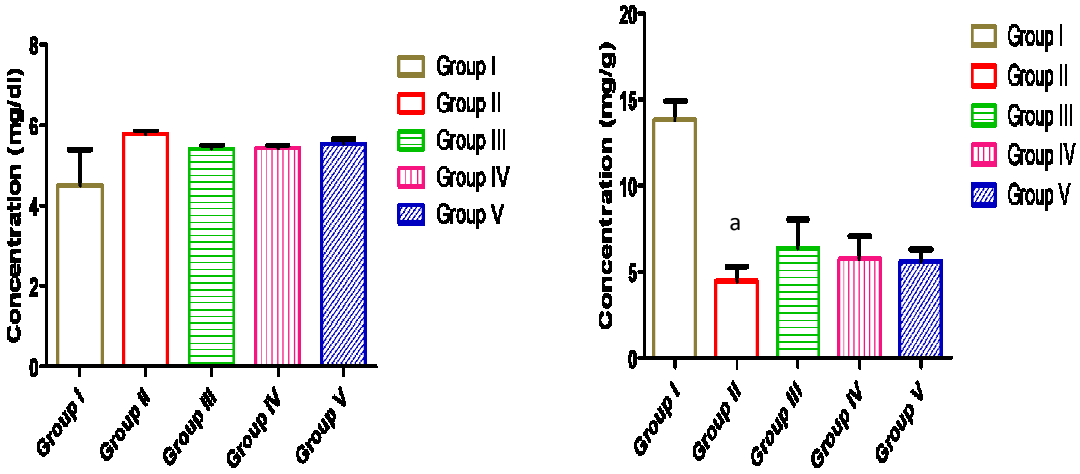

Fig. 4. Effect of EAF on the (a) Plasma and (b) Heart total protein

Each value represented the Mean ± SEM of 5 readings, [a]p< 0.05 statistically significant when compared with control (group I), Grp I = Control; Grp II = ISO treated; Grp III = EAF (50 mg/kg + ISO; Grp IV = EAF (100 mg/kg + ISO; Grp V = Propranolol (1.8 mg/kg + ISO)

There was an increase (22.05%) in the plasma total protein and a significant decrease (67.95%) in the heart total protein in the induced group (Group II) when compared with the control (Group I) (Fig. 4). Pre-treatment with EAF at 50 and 100 mg/kg as well as propranolol showed

decrease in the plasma total protein by 6.08%, 5.73% and 3.99% respectively when compared with the induced group. In addition, the pre-treatment with EAF and propranolol showed increase in the heart total protein by 30.06% (50 mg/kg), 22.59% (100 mg/kg) and 20.79% (propranolol) when compared with the induced group.

Fig. 5 is the summary of the treatment of rats with both EAF and the reference drug on the activities of enzymatic antioxidants (SOD, GPx and Catalase) in the heart. There was an increase in the activities of the enzymatic antioxidants in ISO-treated rats when compared with the control (group II). However, pre-treatment with EAF caused a reduction in the activities of these enzymes at different concentrations.

Figs. 6-9 present the summary of the levels of the non-enzymatic antioxidants in the control and experimental rats (reduced glutathione, α-liopic acid, vitamins C and E). The administration of ISO altered the levels of these antioxidants when compared with the control (group I). However, pre-treatment with EAF ameliorated the changes in the levels of plasma and heart non-enzymatic antioxidants.

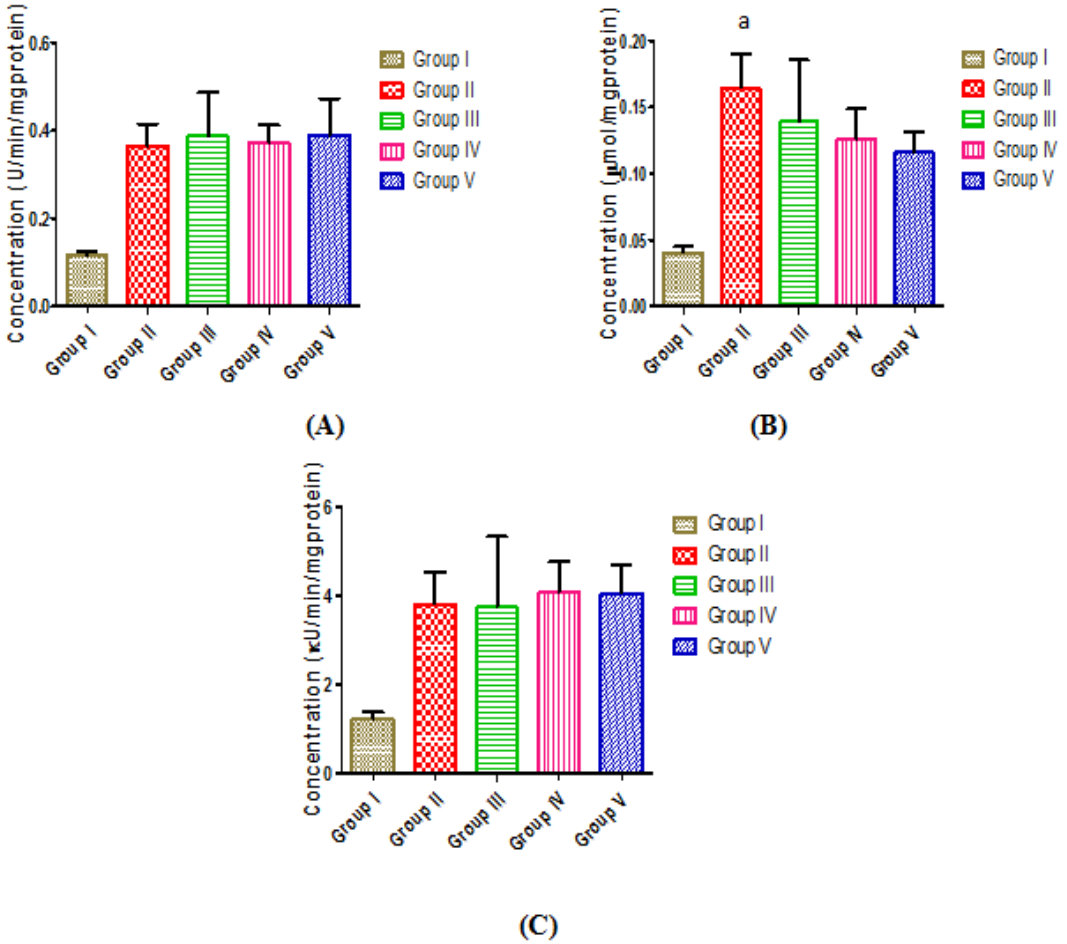

Fig. 5. Effect of EAF on (a) Superoxide dismutase, (b) Glutathione peroxidase and (c) Catalase activities

Each value represented the Mean ± SEM of 5 readings, [a]p< 0.05 statistically significant when compared with control (group I), Grp I = Control; Grp II = ISO treated; Grp III = EAF (50 mg/kg bwt + ISO; Grp IV = EAF (100 mg/kg bwt + ISO; Grp V = Propranolol (1.8 mg/kg bwt + ISO)

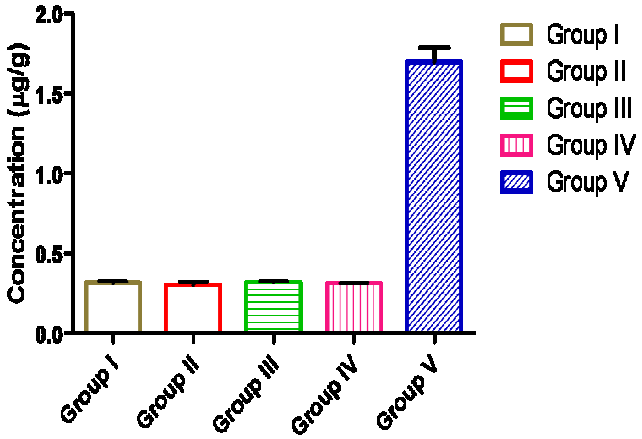

Fig. 6. Effect of EAF on Heart Reduced Glutathione (GSH)

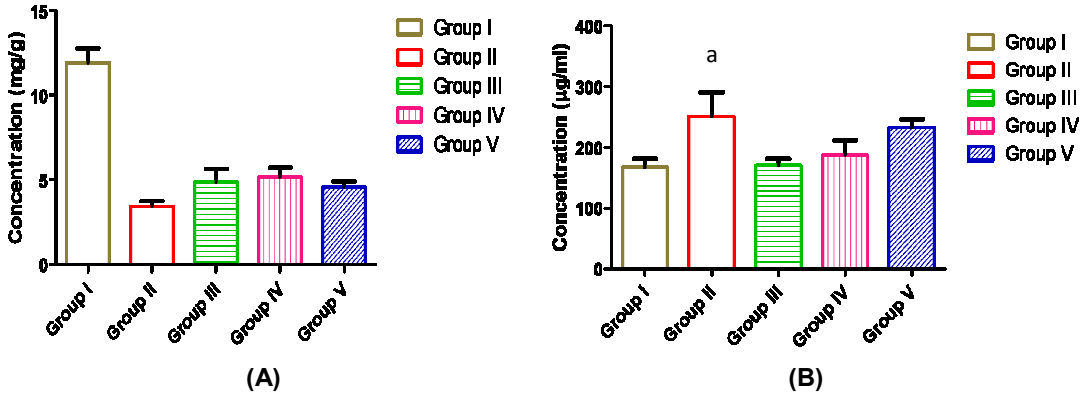

Fig. 7. Effect of EAF on (a) Heart and (b) Plasma α-Lipoic acid

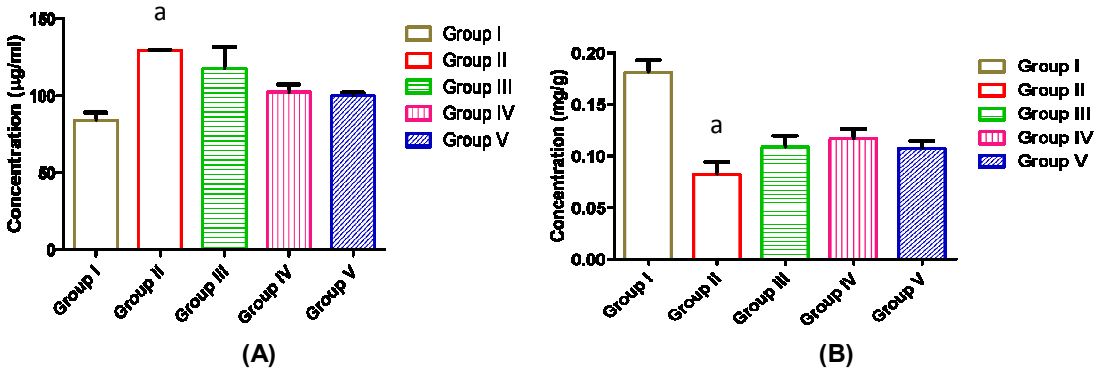

Fig. 8. Effect of EAF on (a) Plasma and (b) Heart vitamin C

Plate 1(a-e) shows the microphotographs of a section of the heart of control and experimental rats. The microscopic observations of myocardial histoarchitecture were graded quantitatively on the basis of formation of myocardium necrosis, oedema and infiltration of inflammatory cells. The

results of the histological examinations revealed the normal histoarchitecture of myocardium in control rats (Plate 1a). ISO treatment caused changes of the heart architecture as indicated by the marked necrosis of myofibres with oedema (red arrows) and infiltration of inflammatory cells (black arrows) (Plate 1b). Rats pre-treated with EAF and reference drug showed reduced degree of necrosis and infiltration of inflammatory cells (Plate 1c-e).

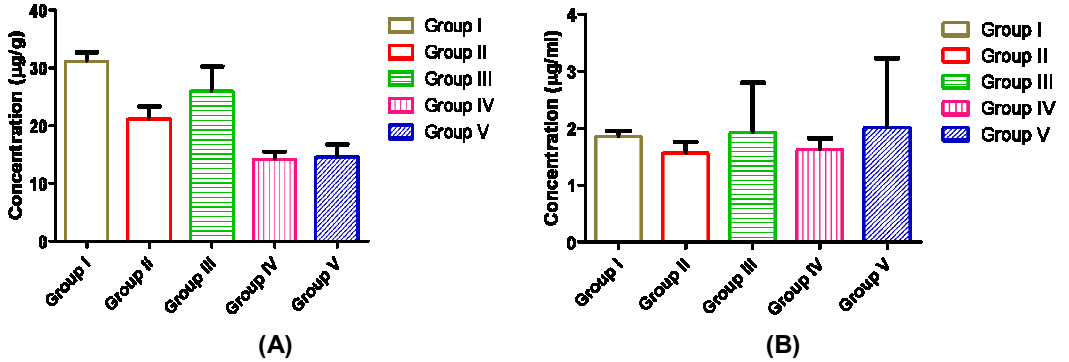

Fig. 9. Effect of EAF on (a) Heart and (b) Plasma vitamin E

Each value represented the Mean ± SEM of 5 readings, [a]p< 0.05 statistically significant when compared with control (group I), Grp I = Control; Grp II = ISO treated; Grp III = EAF (50 mg/kg bwt + ISO; Grp IV = EAF (100 mg/kg bwt + ISO; Grp V = Propranolol (1.8 mg/kg bwt + ISO)

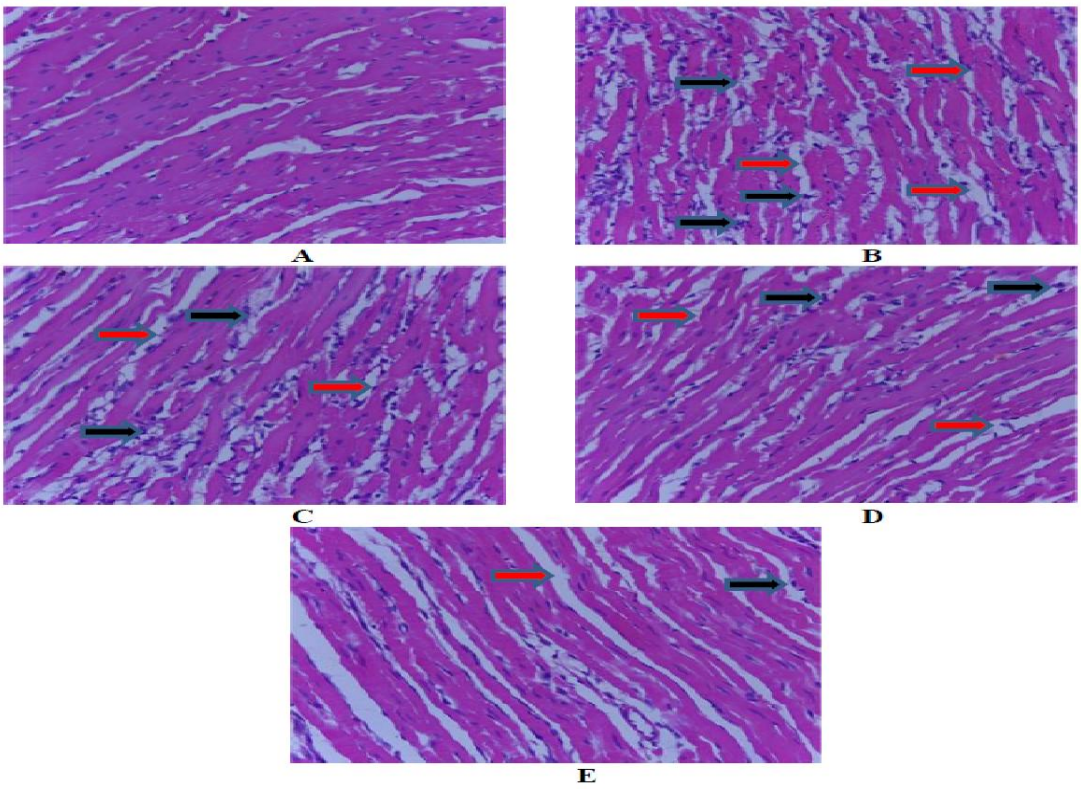

Plate 1. Microscopic section of heart tissues (H & E, Magnification X400)

A: Section of the heart of the control group (group 1); B: Section of the heart of rats + Isoproterenol (ISO) (group II); C: Section of the heart of rats + EAF (50 mg/kg) + ISO (group III); D: Section of the heart of rats + EAF (100 mg/kg) + ISO (group IV); E: Section of the heart of rats + Propranolol (1.8 mg/kg) + ISO (group V)

4. DISCUSSION

Fruits and vegetables have been reported to possess significant levels of biologically active components that elicit effects that are beneficial to health. Consumption of fruit and vegetables has been associated with lower incidence and lower mortality rates caused by cancer, cardiovascular and cerebrovascular diseases. They serve as a major source of dietary antioxidants that increase the plasma antioxidant capacity resulting in the reduction of atherosclerosis related diseases in humans [26]. Dietary components in vegetables and fruits acting as antioxidants include polyphenols, flavonoids, dietary glutathione, carotenoids and vitamins A, B, C and E [26,27]. These compounds may act independently or in combination as cardio-protective or anti-cancer agents by a variety of mechanisms such as free radical scavenging, singlet and triplet oxygen quenching and peroxide decomposing [27].

GC-MS analysis carried out in this study reveals that leaves of *P. mildraedii* contain propionic acid, 2,3-dimethylphenyl ester, catechol, phenol, n-hexadecanoic acid and octyl-β-D-glucopyranoside as the most abundant. These phytoconstituents have been reported to possess antioxidant, anti-inflammatory, hypocholesterolemic and anti-microbial activities [28-32]. Other plants which contain these phytochemicals include *Sida cordata* which is reported to possess diuretic properties and recommended for the treatment of colic gonorrhea and piles [31], *Calliandra portoriscensis*, well known for its antispasmodic, analgesic, antimicrobial, anti-ulcer and antipyretic activities [28], *Adiantum capillus-veneris* known for antimicrobial activity [29], *Dipterygium glaucum* reported for its significant antioxidant and cytotoxic activity aside its use in the treatment of chronic fever as well as a bronchodilator [32] and *Barleria acuminata* reported to exhibit antioxidant, hypocholesterolemia, anti-inflammatory and anti-microbial activities [30].

In this study, the weight of heart tissue was observed to increase in the ISO-induced group, with concomitant decrease in the body weight when compared with the control. The significant increase in the ratio of body to heart weights of the ISO-induced group might be attributed to increased water accumulation (due to increased membrane permeability following catecholamine toxicity) and oedematous intramuscular space in the heart tissue [7,10] which was also confirmed by histopathological examination. An increase of 1% in myocardial water content may reduce myocardial function by approximately 10% [10]. This observation is consistent with previous reports on ISO-induced myocardial infarction of [7,10] in which increase weights of body and heart were observed as a result of the ISO-induction of myocardial infarction. However, pre-treatment with EAF at both concentrations helped to maintain the absolute and relative weights of hearts to near normal and was comparable with the effect of propranolol which was used as the reference drug. This demonstrates a protective role of the fraction on the myocardium.

Cardiac troponin, a low molecular weight protein, is a constituent of the myofibrillary contractile apparatus of the cardiac muscle and serves as a very sensitive and specific biomarker for tissue injury of the heart [10,33]. In this study, administration of ISO to the rats caused a significant increase (64.96%) in the level of plasma cTnT when compared with the control. This can be attributed to ISO-induced myocardial damage which causes the cardiac membrane to become permeable or ruptured due to deficient oxygen supply thereby resulting in the leakage of cTnT and cardiac biomarker enzymes. This observation is consistent with previous reports [7,10,34] that reported significant elevation of cTnT in serum of ISO-induced rats when compared with the control. However, pre-treatment with EAF significantly decreased the level of cTnT in the plasma when compared with the ISO-induced group, and the protection was concentration dependent. It could be inferred that EAF was able to preserve the structural and functional integrities of the contractile apparatus, preventing cardiac damage and troponin leakage from the heart [7].

When cardiac membrane ruptures or becomes permeable, cytosolic enzymes which serve as diagnostic biomarkers such as creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) leak out of the damaged heart tissue and the level of these cellular enzymes in the serum/plasma reflects the level of alterations in the plasma membrane integrity as a response to β-adrenergic stimulations [7,10]. In the present study, group of rats administered with ISO showed significant increase in the levels of these enzymes in plasma when compared with the control, indicating ISO-induced necrotic damage

of the myocardium and leakage of the plasma membrane. These observations are in agreement with previous reports of [35,36] that CK-MB, LDH, AST and ALT were elevated when myocardial infarction was induced. Treatment of rats with EAF before induction, however, decreased the levels of the cytosolic enzymes in a concentration dependent manner and compared favourably with the standard reference drug. It is plausible to postulate that antioxidant compounds such as phenolic acids and flavonoids protect against oxidative and cardiac injury, resulting in prevention of leakage of theses enzymes from the myocardium [10,35,36].

Lipids play significant roles in cardiovascular diseases, not only by contributing to the development of atherosclerosis but also in the modification of the composition, structure and stability of the cellular membrane [10]. Lipolysis is rapidly promoted by the actions of lipolytic hormones which activate adenylate cyclase that converts ATP to cAMP [37]. cAMP promotes lipolytic activity by activating cAMP-dependent protein kinase, which phosphorylates hormone-sensitive lipase and results in the hydrolysis of stored triacylglycerol, thereby contributing significantly to hyperlipidaemia. High levels of LDL-c have been closely related with incidence of atherosclerosis, while high levels of HDL-c which facilitates the transport of cholesterol from the peripheral tissues to the liver, where it is catabolized and excreted form the body [38,39] are thought to signal protection of the arteries. In the present study, rats induced with ISO showed significant increase in the levels of total cholesterol (TC), triacylglycerols (TRIGS), LDL-C and VLDL-C when compared with the control while levels of HDL- C in the ISO-induced rats decreased significantly when compared with the control. Pre-treatment with EAF decreased the levels of total cholesterol, triacylglycerol, LDL-C and VLDL-C in a concentration dependent manner. The levels of the HDL-c in the pre-treated rats were increased and compared favourably with the standard reference drug. The restoration of these alterations could be said to maintain the normal fluidity and function of the myocardium which might be due to the reduction in catecholamine-stimulated lipolysis mainly by decreasing the inhibition of phosphodiesterase, leading to a decrease in cAMP [7]. It is possible that that the reduction in the cholesterol by EAF could be via antioxidants present especially polyphenols,as they inhibit cholesterol esterase [7,10,38].

Proteins are synthesized in the liver and involved in the maintenance of cellular shape and physical integrity among a host of important roles. These functions are affected by the rate of their synthesis and degradation [40]. The total protein in the heart is significantly reduced due to peroxidation of the heart membrane by accumulation of free radicals resulting in the increase of plasma total protein. However, pre-treatment with EAF increased the total protein in the heart, suggesting some protection from peroxidation. Also, the protection exerted by EAF might be due to the presence of alkaloids which inhibit cAMP phosphodiesterase [41].

Generation of highly cytotoxic free radicals due to the auto-oxidation of catecholamines is one of the mechanisms known to be involved in ISO-induced myocardial injury. The endogenous antioxidant enzymatic defense system is of paramount importance in neutralizing and scavenging oxygen free radical-mediated tissue injury [35]. Glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) are the primary free radical scavenging enzymes involved in the first line of cellular defense against oxidative injury, removing O_2 and H_2O_2 before they can interact to form more reactive hydroxyl radicals [10,35]. In this study, the activity of SOD was elevated as a result of the administration of ISO on the induced rats when compared with the control. The increase in the endogenous antioxidant enzymes might be to compensate for the presence of oxidants accumulating in the system [38,42]. Pre-treatment with EAF caused perturbation in the activity of this enzyme and compared favourably with the reference standard (propranolol). This may be due to enhanced antioxidant activity.

Reduced glutathione (GSH) is a tripeptide which exerts its antioxidant property by reacting with superoxide radicals, peroxy radicals and singlet oxygen which is followed by the formation of oxidized glutathione (GSSG) and other disulfides [34]. It plays a critical role in the regulation of various cell functions and protects cells from oxidative stress. Thus a reduction in cellular GSH content would impair protection of cells [34,43]. In this study, the administration of ISO caused decrease in the content of GSH in the induced group when compared with the control which is in agreement with earlier report [44] which observed a reduction in GSH concentration in acetaminophen-induced hepatotoxicity. It has been postulated that reduction in GSH levels might be due to increased utilization in protecting

thiol (SH) containing proteins from lipid peroxides [34]. Pre-treatment with EAF however, restored the level of GSH when compared with the induced group. This may be attributable to the presence of phytochemicals present in the ethyl acetate fraction [45].

Vitamin C prevents oxidation of lipids by trapping water soluble peroxyl radicals before diffusing into lipid membranes while vitamin E protects against lipid peroxidation, and these vitamins have been reported to be linked with the prevention of cardiovascular diseases and cancers [45]. In the present study, the level of plasma vitamin C significantly increased and the level in the heart significantly decreased when compared with the control. The increase in plasma vitamin C as a result of its release from the heart might be in response to the oxidative stress overload on the heart due to ISO administration. However, the pre-treatment with EAF restored the level of vitamin C in a concentration dependent manner and compared favourably with propranolol. The result is in agreement with earlier studies of [45,46] that plasma vitamin C and E levels increased by the induction of ISO were restored by the treatment of *Bryophyllum pinnatum* and *Piper guineense* seed extract respectively.

The level of vitamin E in the plasma and heart decreased in ISO-induced rats when compared with the control. This depletion could be due to high levels of free radicals and peroxidation of the membrane by free radicals generated from auto-oxidation of ISO [7,10]. The level of vitamin E in the plasma and heart was restored by the pre-treatment with EAF except at 100 mg/kgbwt, and compared favourably with propranolol. The restoration of vitamin E in the plasma might be due to the protection against lipid peroxidation [7,10].

Alpha lipoic acid, an organic compound produced by the body has been shown to possess and exhibit various biological activities such as antioxidant, detoxifying, neuroprotective, cardiovascular, anti-ageing and anti-inflammatory properties [46]. The reduced form (dihydrolipoic acid, DHLA) has the ability to exert antioxidant effect by directly donating electrons to pro-oxidant or an oxidized molecule, chelating metals and regenerating glutathione, vitamins C and E [46,47].

In this study, the plasma level of α-lipoic acid increased significantly and the level in the heart significantly decreased in the induced rats when compared with the control. The increase in plasma α-lipoic acid could be a result of its release from the heart in response to oxidative stress due to ISO administration [47,48]. However, pre-treatment with EAF restored the level of α-lipoic acid in a concentration dependent manner and compared favourably with propranolol. This is in agreement with the report of [47] that lipoic acid counteracts the damage associated with myocardial infarction, provides protection by inhibiting ROS generation, blocks inflammation and reduces myocardium apoptosis.

Histopathological investigation of the left ventricle of the heart of control showed a clear, intact homogenous structure of myocardium without sign of necrosis, oedema or infiltration of inflammatory cells. The ISO-induced group showed significant necrosis (which could be as a result of anoxia) with oedema (due to accumulation of water) and infiltration of inflammatory cells. However, pre-treatment with EAF decreased the infiltration of inflammatory cells and formation of necrosis. This observation is in agreement with earlier reports [10,49]. It observed that *Withania somnifera* leaf extract protection against myocardial damage was due to its antioxidant properties [10], an observation corroborated by previous reports [49,50] that Genistin (a flavonoid) and Malvidin (a polyphenol) reduced myonecrosis, oedema and infiltration of inflammatory cells.

5. CONCLUSION

The study demonstrates the protective effect of ethyl acetate fraction of *P. mildbraedii* leaf in ISO-induced myocardial infarction in rats. The cardioprotective potential might be due to the presence of vital constituents which have been reported to possess antioxidant and hypocholesterolemic activities. Further studies on the plant will include quantitative NMR analysis, isolation of active phytochemicals and clinical trials.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Ethical clearance was obtained from the Health Research Ethics Committee (HREC) of the Institute of Public Health (IPH), Obafemi Awolowo University, Ile-Ife.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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