CARDIAC AND RENAL PROTECTIVE EFFECTS OF *Andrographis paniculata* LEAF EXTRACT IN MALE WISTAR RATS

BY

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ABSTRACT

Cardiovascular and renal diseases are one of the leading causes of morbidity and mortality in humans. However, conventional drugs used for treatment of these diseases have adverse effects and are not readily affordable. Botanicals such as *Andrographis paniculata* rich in antioxidants are promising alternatives. Hence, protective effect of Ethanol Leaf Extract of *Andrographis paniculata* (EEAP) in isoproterenol-induced myocardial infarction and cisplatin-induced renal injury in rats were investigated.

Fresh leaves of *Andrographis paniculata* (Voucher No:UIH-2846) was extracted using ethanol. Cardioprotective effects of EEAP were evaluated in male wistar rats (n=49; 100-160 g) equally divided into seven groups. Group A (control) was administered normal saline, group B; isoproterenol at 85 mg/kg, groups C, D, E and F were pretreated with enalapril 10 mg/kg, EEAP 100, 200 or 400 mg/kg, respectively, for 7 days and thereafter administered isoproterenol on days 8 and 9. Group G was administered isoproterenol on days 1 and 2, thereafter treated with 200 mg/kg of EEAP for 7 days. Administration of isoproterenol was subcutaneous, while enalapril and EEAP were oral. Electrocardiogram and blood pressure (BP) parameters were done on day 10 and animals were sacrificed 24 hours later. Cardiac tissues were assayed for markers of oxidative stress (malondialdehyde, H_2O_2), and antioxidant defence system (SOD, GPx, GST). Histopathology and immunohistochemistry (Cardiac troponin-I, C-reactive protein, Interleukin-10) were evaluated. Renoprotective effects of EEAP were evaluated in another 49 wistar rats (100-150 g) divided into seven equal groups. Group A1 (control), group B1 was treated with cisplatin (10 mg/kg) only on day 8, groups C1 and D1 were pre-treated with EEAP (200 and 400 mg/kg, respectively), for seven days and cisplatin was administered on day 8. Group E1 received cisplatin only on day 1, groups F1 and G1 received cisplatin on day 1; 72 hours after EEAP (200 and 400 mg/kg) were administered, respectively, for 7 days. Administration of cisplatin was intraperitoneal, while EEAP was oral. Nephroprotective effects were evaluated using markers of oxidative stress (protein carbonyl, H_2O_2), histopathology and immunohistochemistry [Kidney injury molecule-1, Nuclear factor (erythroid-derived 2)-like 2]. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

Reduced BP caused by isoproterenol was restored to near normal values in group F (systolic BP 102.33±2.31 to 131.50±2.1 mmHg, diastolic BP 82.67±1.80 to

101.70±1.3 mmHg). Treatment in group G restored prolonged QT interval (140.21±4.10 to 75.55±3.01 ms), significantly reduced Malondialdehyde (5.98±0.44 to 4.24±0.39 μmol/mgprotein) and H_2O_2 (13.73±1.30 to 11.44±0.49 μmol/mgprotein), but increased SOD (1.74±0.05 to 1.98±0.14 U/mgprotein), and GST (19.99±0.68 to 23.99±1.38 units/mg tissue). Groups E and F had reduced cellular infiltration, downregulated CTnI and CRP, but upregulated IL-10 expressions. Treatment in group D1 significantly decreased protein carbonyl (40.12±5.93 to 23.85±6.45 nmoles/mgprotein), H_2O_2 (29.93±0.87 to 26.65±0.74 μmol/mgprotein), increased activities of SOD (48.28±1.24 to 52.94±2.17 U/mgprotein), GPx (52.95±2.00 to 55.92±1.92 mmole/GSH complex formed/min/mg protein) and downregulated Kim-1 but upregulated Nrf2 expressions.

Andrographis paniculata at 200 mg/kg exhibited cardiac and renal protective activities through its antioxidant and anti-inflammatory properties. Therefore, it is a potential candidate in treatment of cardiovascular and renal diseases.

Keywords: A*ndrographis paniculata*, Phyto-antioxidant, Myocardial infarction, Renal injury
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Finally, He raiseth up the beggar from the dunghill, to set them among princes, and to make them inherit the throne of glory: for the pillars of the earth are the Lord's, and he hath set the world upon them. 1Sam 2:8. The God I serve hath made everything in his time beautiful.

CERTIFICATION

I certify that this work was carried out by Dr B. O. ADEOYE in the Department of Veterinary Pharmacology and Toxicology, University of Ibadan

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DEDICATION

To God Almighty, the ancient of days and to all helpers of destiny.

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GENERAL INTRODUCTION

1.1   Background

1.1.1   Cardiovascular disease

Cardiovascular diseases (CVD) pose serious problems to human health. In fact, CVD is the foremost cause of demise throughout the world (Gatica *et al.,* 2015). Not only the underdeveloped but also the developed countries have not been able to control this disease. Several increase rates of cardiovascular diseases amidst South Asian living in Western countries has been established, but the vast cardiovascular diseases problem within the Indian subcontinent itself has been given less attention. At present it is the highest killer disease in the US (Hina *et al.,* 2010).

Universally, myocardial infarction is known to be a foremost cause of death-rate (Go *et al.*, 2014) followed by cardiac failure and left ventricular dysfunction and various drugs are used for management of these conditions (Cleland *et al*., 2005). In the management of Myocardial infarction herbal medicine as a supportive remedy is gaining more popularity (Chung *et al*., 2013). Many susbstantial epidemiological reasons reported that consumption of fresh fruits and vegetable are linked with reduction of cardiovascular diseases (Segasothy and Phillips, 1999; Bhupathiraju *et al.,* 2013).

1.1.2   Cisplatin/Isoproterenol

Abnormality in cell development with the ability to proliferate to other parts of the body is known as cancer. It is well informed that new cases of cancer, about 14.1 million occurs worldwide annually causing up to 8.2 million deaths (WHO, 2014). Palliative care, surgery, use of hormone as a therapy, chemotherapy, targeted therapy and actinotherapy are remedies use for cancer treatment.   Cancer chemotherapy

involves the treatment of cancer with one or more cytotoxic antineoplastic drugs such as antimetabolites and alkylating agents (Lind, 2008).

Cisplatin is effective for different categories of cancer. The first member of platinum-based group of chemotherapeutic agents was cisplatin; followed by oxaliplatin and carboplatin (Apps *et al.*, 2015). Nevertheless, the major side effect of this drug is nephrotoxicity which is due to buildup of cisplatin in the renal tissue. The renal toxicity of cisplatin is associated with reactive oxygen species (Sreedevi and Bharanthi, 2011).

Isoproterenol is a β-adrenergic agonist involved in regulation of myocardial contractility and metabolism, hence serving as an important element of a good model for the study of pharmacology activities of many compounds on heart function (Khalil *et al.*, 2015).

Lately, there is great focus on medicinal plants because of many ways of protective mechanisms apart from antioxidant activity (Basile *et al.*, 1999). Many bioactive chemical compounds like saponins, tannins and flavonoids are found in Medicinal plants.

Antioxidant properties could be responsible for the medicinal effects of fruits and vegetable. In the pathophysiology of heart and kidney disease, reactive oxygen intermediate is implicated (Forbes *et al*., 2008). Hence, by focusing on oxidative stress the prevention and treatment of heart and kidney damage can be greatly improved. As a matter of fact, production of highly cytotoxic reactive oxygen has been associated with the acute phase of myocardial necrosis induced by isoproterenol and kidney damage caused by cisplatin (Khalil *et al.,* 2015).

Therefore, this study was done to assess cardiac and renal protective ability of *Andrographis paniculata* leaf against myocardial damage induced by isoproterenol-induced and cisplatin-induced renal injury in male wistar rats.

1.1.3 *Andrographis paniculata*

Andrographis paniculata belong to the family of Acanthaceae, commonly called Kalmegh is a dietary component in Indian. Also, for several decades Indian and Chinese have been using this plant for traditional remedies. It is use in Nigeria traditional medicine for treatment of heart diseases (Mshelbwala *et al*., 2013).

Pharmacological studies have reported hepatoprotective (Nagalekshmi *et al*, 2011), antiinflammatory (Adedapo *et al.,* 2015), antihyperglycaemic (Premanath *et al.*, 2015). Active component of *Andrographis paniculata* such as diterpenoids compounds (andrographolide, 14-de-oxy-11, 12-didehydro andrographolide and neo-andrographolide), collectively termed as andrographolides have shown several therapeutic effect including antioxidant, antiplatelet, hypotensive and antiinflammatory activities Andrographolide is the active component of *Andrographis paniculata* is a well-recognized medicinal herb. It has a broad range of beneficial effects, including immunosuppressant, antithrombotic, anti-inflammatory, antineoplastic, anti-viral, anti-bacterial, anti-oxidative stress, antipyretic, anti-oedematogenic, and anti-nociceptive activities (Jarukamjorn and Nemoto, 2008).

1.2   Statement of problem

Conventional drugs are becoming expensive, not readily available and are not safe due to different toxicity effects. There is a need to study natural products that are readily available in our environment, cheaper and less safe in terms of toxicity. Plants are natural products that are assumed to be effective in protection and management of various illnesses. Cardiovascular diseases and cancer pose serious problems to both man and animal health and generation of reactive oxygen species is connected with their pathophysiology.

1.3   Justification

Reactive oxygen species are implicated in the pathogenesis of myocardial infarction causing tissue necrosis. Also nephrotoxicity is one of the major limitations of cisplatin in treatment of cancer and reactive oxygen species has been implicated. Botanicals are known to have phytochemical constituents that can reduce or remove reactive oxygen species. These phytochemical constituents are important in prevention and treatment of heart and kidney diseases. Therefore there is need to study these botanicals especially in Nigeria where up to 61% of its native are reduce to penury and cannot readily afford conventional drugs.

1.4   Aims and Objectives

1.   To investigate potential of leaf extract of *Andrographis paniculata* (EEAP) in myocardial infarction mediated by isoproterenol.

2.   To investigate effect of EEAP in renal injury caused by cisplatin.

3.   To evaluate *in-vivo* and *in-vitro* antioxidant potentials of EEAP

4.   Phytochemical analysis (quantitative) of EEAP

5.   GC-MS analysis of Essential oil of *Andrographis paniculata*

1.5   Study Hypotheses

Ho1: EEAP cannot protect or ameliorate isoproterenol-induced myocardial infarction.

Ho2: EEAP cannot protect or ameliorate cisplatin-induced kidney damage

Ho3: EEAP do not have any antioxidant property

CHAPTER TWO
LITERATURE REVIEW

2.1 *Andrographis paniculata*

2.1.1 Plant Description

Kingdom: Plantae

Family: Acanthaceae

Genus: Andrographis

Andrographis paniculata (Burm.f.), popularly called Kalmegh (India), king of bitters (English), Meje-meje (Yoruba), Chuan xin lian (Chinese) is an annual plant, branching to 1 meter in height and it active phytochemical components have been extracted from both leaves and stems. Normally germinated from seeds, *Andrographis paniculata* is found everywhere in its native area. Because of its used in traditional medicine, *Andrographis paniculata* can be easily grown and it also grows in all types of soil where virtually no other plant can be planted which account for its extensive distribution (Latto *et al*., 2006). The use of the plant in Nigeria for treatment of diseases is based on folklore. The medicinally active phytochemical in the plant (andrographolide) are found more in the leaves (Sareer *et al.,* 2014).

Figure 2.1: *Andrographis paniculata* (source Wikipedia)

2.2   Myocardial infarction

Myocardial infarction is a frequent presentation of heart condition. In the advanced and developing countries, myocardial infarction is the prominent origin of mortality and main pathological concern globally regardless of rapid improvement made in science (Boudina *et al.*, 2002).   The occurrence is of increase in Developing country like Nigeria due to rapid urbanization and Anjorin *et al.,* (2005) reported 39 % mortality in patients suffering from myocardial infarction. World Health Organization reported that by year 2020 myocardial infarction will be a foremost cause of mortality across the globe (Farvin *et al.*, 2006). It occurred when there is imbalance between heart metabolic demand and supply of nutrients and oxygen through the coronary circulation to the myocardium. Biochemical alterations including free radical damage and lipid peroxidation always accompany myocardial infarction resulting to qualitative and quantitative alterations of heart tissue (Kukreja and Hess, 1992).

2.2.1   Isoproterenol (ISO)

Isoproterenol is a β-adrenergic agonist involved in regulation of myocardial contractility and metabolism, hence serving as an important element of a good model for the study of pharmacology activities of many compounds on heart function (Khalil *et al.*, 2015). Myocardial infarction is an acute disease of heart causing cardiac dysfunctions; stimulate free radical production. This pathophysiological and morphological shown in animal model is related to what is witnessed in human patient suffering from myocardial infarction (Nirmala *et al.,* 1996).

According to Rona (1985) similar infarct in human has been induced in animal models using isoproterernol. Isoproterenol-induced myocardial infarction occurred when heart oxidative metabolism overwhelmed the available oxygen to heart cells due to blockage of coronary circulation. In the pathogenesis of the heart cell damage lack of oxygen accompany by altered biochemical activities and structural changes aggravate the cell damage (Moris *et al*., 2017). Isoproterenol-induced myocardial infarction also changes iNOS expression (Huang *et al.,* 2018). Apoptosis and necrosis are seen in isoproterenol induced cardiac toxicity.

Production of cytotoxic free radical due to autooxidation of isoproterenol is one of mechanism suggested to explain isoproterenol-induced heart damage (Rona, 1985).

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When catecholamine is modified, it generates quinoid compounds that result to the generation of superoxide anions. Furthermore, accumulation of free radicals can cause loss of function and structural integrity of heart tissue. The lipids within the membranes may be attacked by the formed free radicals, changing to peroxyl radicals that can then destroyed adjoining fatty acids, instigating a chain reaction of oxidative degradation of lipid. Further tissue and organ damage may be contributed by harmful lipid hydroperoxide end products (Priscilla and Prince, 2009).

In recent years, botanicals, fruits and vegetables rich in antioxidant enzymes are consumed for long-term prevention of cardiovascular disease. Consequently, there has been significant interest in research on natural bioactive compounds, with a widely accepted view that natural products are better in terms of efficacy and safety when compared to their synthetic analogs. Medicinal plants serve as the major source of active natural products that protect against many diseases (Saravanan *et al.,* 2013).

2.3   Angiotensin converting enzyme inhibitor

They are basically produced for management of elevated blood burden and can be used in mixture with other drugs used in the management of high blood pressure. It was later discovered that this drug is beneficial in the management of various cardiac and kidney diseases (Jackson, 2006).

2.3.1   Effects of Angiotensin Converting Enzyme Inhibitors (ACE inhibitors)

ACE is the enzyme the body use to convert angiotensin I to angiotensin II. The function of ACE inhibitors is to deter the transformation of angiotensin I to angiotensin II. Hence, ACE inhibitors reduced arteriolar resistance, increase venous capacity, lower resistance in renal blood vessels and caused hyper natriuresis. The negative feedback of angiotensin I to angiotensin II resulted into accumulation of renin in the blood. For this reason there is increase in angiotensin I, decrease in angiotensin II and aldosterone (Caballero and Pedrosa, 2016).

Physiologically, angiotensin II causes vasoconstriction and hypertrophy of tissue which can result to elevated blood pressure. Additional narrowing of the renal efferent arterioles, results to elevated perfusion pressure in the renal glomeruli. Thus, contributing to alteration and enlargement of the heart ventricles via excessive formation of connective tissue and programmed cell death. In adrenal cortex

angiotensin II activate the release of a hormone known as aldosterone. Aldosterone retains sodium and chloride ions and release potassium. When there is increase in sodium retention, there is increase in water retention in the body resulting to more blood concentration, thus blood pressure is elevated. Increase release of vasopressin in heart condition may result to low level of sodium, thereby amplifying mortality rate in patients. What angiotensin conversion inhibitor does is to reduce activation of angiotensin II thereby reducing blood pressure (Nancy 2003).

2.3.2 Adverse effects of angiotensin converting enzyme inhibitors

This include: cough, hyperkalemia, hypotension, headache, dizziness, nausea, and kidney impairment. Accumulation of bradykinin increase inflammation and this is associated to increase pain experience with the use of ACE inhibitors. ACE inhibitor caused hepatotoxicity and also has effect on the fetus like neonatal kidney failure. The mechanism of action of angiotensin converting enzyme inhibitor is linked with the cause of renal impairment associated with the use of this drug. Glomerular filtration rate is reduced in patient using ACE inhibitors for the first time but this is stabilized after several days of using the drug. In addition, the reduction in glomerular filtration rate may be important in patient with renal and heart condition. Therefore, kidney function test must be done in the patients receiving ACE inhibitor. Concurrent use of non-steroidal anti-inflammatory drug, diuretic and angiotensin converting enzyme inhibitor increase the risk of developing renal failure (Thomas, 2000).

ACE inhibitor may cause hyperkalemia due to its effect on aldosterone hormone that is responsible for the elimination of potassium from the body. Genetic make up of some people allow them to lose potassium while on ACE inhibitor (Cohn *et al.*, 2000). Hyperkalemia may reduce speed of signal pathway in the nerves and muscles. When this occurred in the heart muscle there is cardiac dysfunction and neuromuscular consequences (Sidorenkov and Navis, 2014).

ACE inhibitors caused an elevated in bradykinin level resulting to swelling of the body, dry cough and pain. Neutropenia, agranulocytosis and other hematologic disorder may occur during ACE inhibitors treatment. Fetal abnormalities have been reported in pregnant women using angiotensin converting enzyme inhibitors. Adverse effect occurs in 50 % of fetus exposed to ACE inhibitors (Bullo *et al.*, 2012).

2.4   Function of oxygen in the heart

Oxygen is important in life and in the expression of gene in the heart. Lack or reduce level of oxygen in the heart altered expression of the myocardial gene (Huang *et al.,* 2004). Nitric oxide maintains contractility of the heart, vascular tone and oxygen has been implicated in the production of this oxide. Metabolism of oxygen produce reactive oxygen species which helps in signaling pathway and when in excess may cause cellular injury that cannot be reversed, result to death of beneficial molecules. Hence effect of oxygen may be beneficial or detrimental to health. Under normal condition lipids are the major source (about 90%) and the most preferred source of metabolic energy for the heart leading to production of acetyl-CoA (an important molecule in cellular respiration) which enter the Krebs cycle. Other sources are protein and carbohydrate (Jafri *et al.*, 2001). β-oxidation of lipids gives acetyl-CoA and naturally occurring coenzymes such as $FADH_2$ and NADH. When acetyl-CoA enters the Krebs cycle it promotes production of more NADH and $FADH_2$. By glycolysis using carbohydrate, little amount of energy is produced and this is not sufficient for heart metabolism.

2.5   Cancer

Unusual cell development with the ability to metastize into various organs of the body is known as cancer disease. In 2014 World Health Organisation reported occurrence of up to 14.1 m fresh issues of cancer annually, causing up to 8.2 m mortality worldwide.

2.5.1   Cancer treatments

There are many Treatments method for cancer patients. These include palliative care, surgery, used of radiation, use of hormone, targeted therapy and chemotherapy. Cancer chemotherapy involves the treatment of cancer with one or more cytotoxic antineoplastic drugs such as antimetabolites and alkylating agents (Lind, 2008).

2.5.2   Platinum-based antineoplastic agents

These are chemotherapeutic agents used for treatment of cancer. They are coordination complexes of platinum. Platinum-based antineoplastic agents are used to treat almost 50% of cancer patients. Drugs that belong to this family include cisplatin, carboplatin

and oxaliplatin, but several have been proposed or are under development (Johnstone *et al*., 2014).

2.5.3 Cisplatin

It is the first of the platinum coordination complex with alkylating activity introduced into clinical medicine. Cisplatin is active for the management of different types of cancer (Wang and Lippard, 2005). As effective as cisplatin is, in treating various cancer diseases, its toxicity can result in acute renal failure and direct tubular toxicity, inflammation, vascular factors, and oxidative stress are the resultant outcome of cisplatin-induced nephrotoxicity (Pabla and Dong, 2008).

2.5.4 Cisplatin-induced nephrotoxicity mechanism of action

The mechanism of action of kidney toxicity induced by cisplatin is not yet agreed nevertheless many hypotheses have been suggested and these include monoadduct crosslinking, intra or interstrand crosslinks of deoxyribonucleic acid. Cisplatin prevents the synthesis or repair of deoxyribonucleic acid in cancer cells (Poklar *et al*., 1996). Stimulation of tumor necrosis factor-α mediated programed cell death and pressure on endoplasmic reticulum pathway have also been proposed as pathophysiological mechanisms in kidney toxicity caused by cisplatin (Benedetti *et al*., 2013).

2.6 Reactive oxygen species

Compound containing oxygen (e.g, singlet oxygen and peroxides) is known as reactive oxygen species. In a normal physiological condition reactive oxygen species are produced from cellular metabolism of oxygen that is essential for homeostasis and cellular respiration (Brown and Griendling, 2015).   Nonetheless, excessive accumulation of reactive oxygen species injures cell structures and this may occur when there is oxidative stress.

2.7 Free radical

Mutual properties that are shared by radicals are caused by the existence of unpaired electron in the outer layer making them highly reactive and unstable. They can behave as a reductants or oxidants via accepting or donating an electron from other molecules (Cheeseman and Slater, 1993). Superoxide anion radical, oxygen singlet and

peroxynitrite radical has more oxygen containing free radicals in diseases conditions. These compounds caused damaging of carbohydrate, lipids and proteins; they are highly reactive species. Enzymatic reactions are involved in phagocytosis and respiratory chain, resulting into formation of free radicals (Liu and Roberts, 1999). Initiation of ionizing oxygen reactions with organic compounds are examples of non-enzymatic source of free radicals. Free radicals can be externally or internally produced (Ebadi, 2001)

2.8   Human diseases in relation to oxidative stress

Different conditions such as heart attack have been linked with roles of oxidative stress. Oxidation of lipids and proteins is triggered by excess of oxidative stress, resulting to changes in proteins fatty acids structure and functions (Stefanis *et al.*, 1997).

2.9   Lipid peroxidation product hazards

Thiobarbituric acid reactive substances assay is the most frequently used method of malondialdehyde quantification. Malondialdehyde is the major end products of lipid peroxidation. When thiobarbituric acid reacts with malondialdehyde, a fluorescent product is formed. When malondialdehyde is not removed immediately after its generation, it causes cell membrane damage especially lipids thus may become carcinogenic and mutagenic in the system (Marnett, 1999).

2.10   Antioxidants

Any factor that eliminates or inhibits oxidative damage to aimed molecule is called antioxidants. Superoxide dismutase, glutathione-S transferases, catalase and Glutathione peroxidase are the most widely studied antioxidants enzymes. Peroxiredoxins and sulfiredoxin are also important but are not extensively studied (Gulcin and Beydemir, 2013).

2.10.1 Mechanism of action

Rice-Evans and Diplock (1993) suggested two foremost ways of antioxidants mechanism of action. One is through transfer of electron to the free radical in the body. Two is the stopping of chain reaction initiating catalyst by clearing reactive oxygen

species. Other methods include gene expression control and chelation of metal ion (Gulcin and Beydemir, 2013).

2.10.2 Antioxidant action

The antioxidants action is involve at different levels. Firstly, antioxidants can avert creation of free radicals. The free radical formed by chelation of metal ion *in vivo* has been reported and the reaction is reduced by antioxidants to alcohol and H_2O_2 without free radicals release (Gulcin and Beydemir, 2013). Antioxidants such as catalase, peroxidase and phospholipid hydroperoxide and glutathione peroxidase decompose lipid hydroperoxides to corresponding alcohols (Reczek and Chandel, 2015).

Secondly, antioxidants scavenge the free radicals to stop chain initiation reactions. Several endogenous radical scavenging antioxidants are in two forms: water lovers (hydrophilic e.g albumin, bilirubin, thiols and vitamin C) and lipid lovers (lipophilic e.g ubiquinol and vitamin E) with vitamin E been the most potent (Turkoglu *et al.*, 2014).

De novo antioxidants include proteases, proteinases and peptidases. This proteolytic enzymes repair cellular damage by identifying, destroying and removing oxidatively modified proteins (Pajares *et al*., 2015). Enzymes like nucleases and glycosylases repair deoxyribonucleic acid system by preventing oxidative damage (Husain and Kumar, 2012).

CHAPTER THREE
STUDY 1: CARDIOPROTECTIVE POTENTIAL OF *ANDROGRAPHIS PANICULATA* LEAF EXTRACT ON ISOPROTERENOL-INDUCED MYOCARDIAL INFARCTION.

3.1 Introduction

Myocardial infarction occurred when there is imbalance between heart metabolic demand and supply of nutrients and oxygen through the coronary circulation to the myocardium causing cell damage (Boudina *et al*., 2002) and is a known cause of heart disease death. The annual account of death from heart disease is about 20% of world mortality; hence heart disease will become the main and the most prevalent threats to life (Evran *et al*., 2014). Most frequent presentation of heart disease is myocardial infarction and is an important source of mortality in developed and developing world. Though rapid advancements have been done in the management of cardiovascular ailments, myocardial infarction is still a major pathological issue in global world (Boudina *et al*., 2002). There is damage to the heart muscle is due to lack of enough oxygen (Abel, 2004). However, in an ischemic heart as a result of reduced oxygen, glucose becomes the main source of energy; therefore glycolysis shifts from aerobic to anaerobic conditions. There is therefore a resultant shifting of metabolic utilization of substrates toward glucose from fatty acids. Under normal physiological condition heart utilizes fatty acids as source of energy (Nagoshi *et al.*, 2011).

Isoproterenol is β-adrenergic agonists, in large doses it can be used to replicate an animal model of myocardial infarction (Kawabata *et al*., 2010).

Enalapril is an example of ACE inhibitor. Enalapril and other drugs in this group were basically produced for the management of elevated blood pressure. It was later discovered that this class of drug is effective in the management of other heart and kidney diseases (Jackson, 2006). The reference drug used in this study was enalapril.

Andrographis paniculata is a dietary component, used in traditional medicine with extensive kind of therapeutic effects e.g immunosuppressant, antithrombotic, antineoplastic, anti-viral, anti-bacterial, anti-diabetic, anti-inflammatory, anti-oxidative stress, antipyretic, anti-oedematogenic, and anti-nociceptive activities (Jarukamjorn and Nemoto, 2008).

In this study, Isoproterenol was used to induce acute myocardial infarction and cardioprotective potential of enalapril and *Andrographis paniculata* leaf extract were evaluated in rats

3.2   Materials and methods

3.2.1   Drugs and Chemicals

Sample bottles, Ethanol, enalapril, normal saline, potassium iodide, sodium potasium tartarate, thiobarbituric acid, Griss reagent, o-dianisidine, hydrogen peroxide, phosphate buffer, Ellman's reagent, epinephrine. All other reagents and chemicals used were of standard.

3.2.2   Plant collection and processing

Andrographis paniculata leaves were collected from botanical garden, University of Ibadan, Ibadan North Local Government Area. Then identified and valid with voucher number: UIH-2846, Botany department, University of Ibadan, Ibadan, Oyo State, Nigeria. Air dried for six weeks and ground to coarse powder using an electric blender.

3.2.3   Extract preparation

The dried leaves of *Andrographis paniculata* were extracted using Panovska *et al.* (2005) method. Cold maceration was used for the extraction of the coarse powder with 100 % ethanol for 72 h, with constant shaking. Each batch of harvested solvent were stored in glass containers and refrigerated at 4 ^{0}C.

3.2.4   Separation of the extract

The harvested solvent was filtered with filter paper and the filtrate was reduced to dryness using rotatory evaporator to obtain semi-solid crude extract at 40 °C. Small volumes of the semi-solid crude extract was placed in porcelain dishes in the oven set at low temperature of 4 °C to remove the remaining ethanol. The extract obtained from

the leaves came as semi-solid greenish-black paste. Small portions of the extract were weighed and dissolved in normal saline for use in this study.

3.2.5 Experimental animals

Forty nine wistar rats of the male sex (100-160 body weight) were purchased from the experimental animal unit of the Faculty of Veterinary Medicine, University of Ibadan for the experiment. Fed with rat pellets and water *ad libitum* with a 12 hour light duration and $25 \pm 2\ ^0C$ temperatures and acclimatized for two weeks. Experiments and protocols described in the study follow the OECD approved Standard Operation Procedures (SOPs). Also, it was accepted by University of Ibadan Animal Care and Use Research Ethics Committee, University of Ibadan (UI-ACUREC/App/2016/030).

3.2.6 Animal grouping

The animals were randomly grouped into groups A to G and each group had seven animals. Animals in the control (group A) were administered normal saline, group B; isoproterenol at 85mg/k,g, while group C, D, E and F were pretreated with enalapril 10mg/kg, EEAP 100, 200 and 400mg/kg respectively for 7 days and thereafter administered ISO (85mg/kg) day 8 and 9. Group G was administered isoproterenol on day 1 and 2, thereafter was given 200 mg/kg of EEAP

3.2.7 Myocardial infarction induction in experimental rats

ISO was dissolved in normal saline and 85 mg/kg dose was given subcutaneously for 2 days, 24 hours apart on 1st and 2nd day for post treated group and 8th and 9th day for pretreated groups respectively to induce animal model myocardial infarction (Prince *et al.*, 2011)

3.2.8 Non-invasive blood pressure determination (indirect method)

Blood pressure was done on day 10. Blood pressure parameters were taken by tail plethysmography using an electrosphygnomanometer (CODA, Kent Scientific, USA).

3.2.9 Electrocardiogram

Electrocardiograph was also recorded on day 10. Electrocardiogram was done using 5-lead electrocardiography equipment (EDAN VE-1010, Shanghai, China). Parameters

recorded include heart rate, QT-interval, Bazett's correction of the QT interval and QT segment

3.2.10 Blood collection

On day 11, up to 5 ml of blood was drawn into two separate blood sample tubes for haematology and serum chemistry tests. The one in heparinized bottles were used for haematology. The bloods in plain bottles were allowed to clot and centrifuged at 4000 revolutions per minute for 10 minutes. Supernatant was pipetted into another clean tube and was used for the assay of serum alanine trasnsferase (ALT), serum aspartate transferase (AST), myeloperoxidase (MPO) and total protein.

3.2.11 Preparation of tissue homogenate

The heart of each rat was carefully removed, perfused immediately with normal saline and blotted with filter paper. After which it was homogenized in cold 0.1 M potassium PBS (pH 7.4) using a homogenizer (Teflon). The homogenate obtained was centrifuged at 10000 revolutions per minute for 10 minutes using a cold centrifuge at 4 ^{0}C. The supernatant was used as a sample for the biochemical assay for the estimation of total protein, generation and malondialdehyde (MDA), reduced glutathione (GSH), glutathione-S- transferase (GST), superoxide dismutase (SOD), total thiol, non-protein thiol, hydrogen peroxide (H_2O_2) and glutathione peroxidase (GPx).

3.2.12 Haematology

Jain (1986) method was used for the haematology analysis.

3.2.12.1   Erythrocyte count

Red blood cell diluting pipette marked 101 above the bulb was filled with blood sample to exactly 0.5 mark and put in erythrocyte diluting fluid (Grower's solution). Then filled with the fluid to 101 mark above the bulb. After which it was gently rotated for 3 minutes by a simple wrist movement with pipette held horizontally between the thumb and the middle finger. The diluted blood in the pipette was used to fill the haemocytometer (allowed to settle for few minutes). High power microscope lens was used to count the RBC in 5 of the 25 small squares in the central area of the counting chamber. The erythrocyte was calculated from the addition of all cells in five squares X 10,000 per cubic millimetre.

3.2.12.2 Estimation of Haemoglobin

Cyanmethaemoglobin method which measures total haemoglobin including carboxyhaemoglobin was used. 4 ml of Drabkin's diluents liquid was put in a tube into which 0.02 ml of blood sample measured with a pipette was added, rinsing the pipette three times and stand for ten mins. After thorough mixing, optical density was recorded using spectrophotometer at 540 nanometer wavelength.

Hb = Photometer reading of unknown x gm% Hb of standard x Dilution factor

Photometer reading of standard

Dilution factor = Volume of whole blood used + Volume of diluents

Volume of whole blood used

3.2.12.3 Determination of Packed Cell Volume

The heparinised capillary tubes were filled with blood samples to about two-third of the tube and the vacant end of each tube was sealed with plasticine. The tubes were then placed in the haematocrit-centrifuge (Perkin Elmer, USA) for 5 min at 3000 rpm, the capillary tubes were then removed and read in the graphic reader.

3.2.12.4 Total Leucocyte Count

The white blood cell diluting pipette marked 11 above the bulb was filled with blood sample to the mark 0.5 and then leucocyte diluting solution was added to the mark 11. This was gently rotated for three minutes to mix well. 2-3 drops were discarded and the remaining one was used to fill the counting chamber of the haemocytometer (allowed to settle for a minute). The number of the WBC in the four large corner squares of the counting chamber was counted under low power microscope lens. The sum of the cells in the four corner squares X 50 per cubic millimeter.

3.2.13 Biochemical Assays

3.2.13.1 Nitric oxide level determination

Griess reagent was used to evaluate production of nitric oxide by measuring the level of nitrite (Olaleye *et al.* 2007). Griess reagent which was first described in 1879 and because of it easy, it has been used widely in analysis of several biological samples.

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Phosphate acid solution (5 ml of phosphoric acid plus 95 ml of distilled water). 1% Sulphanilamide (0.5 g of sulfanilic acid was put in 50mls of Phosphate acid solution to make Solution A). 0.1% N-(naphthyl) ethyl-enediamine dihydrochloride (0.05g of NED was added to 50 mls of Phosphate acid solution to make Solution B). Both were added together to make 100mls of Griess reagent. Then 250µl of test sample was added to 250 µl of Griess reagent in test tubes and kept at 25^0C for 20 minites, read at 540nm.

y = 3.6755x - 0.0256
R² = 0.9887
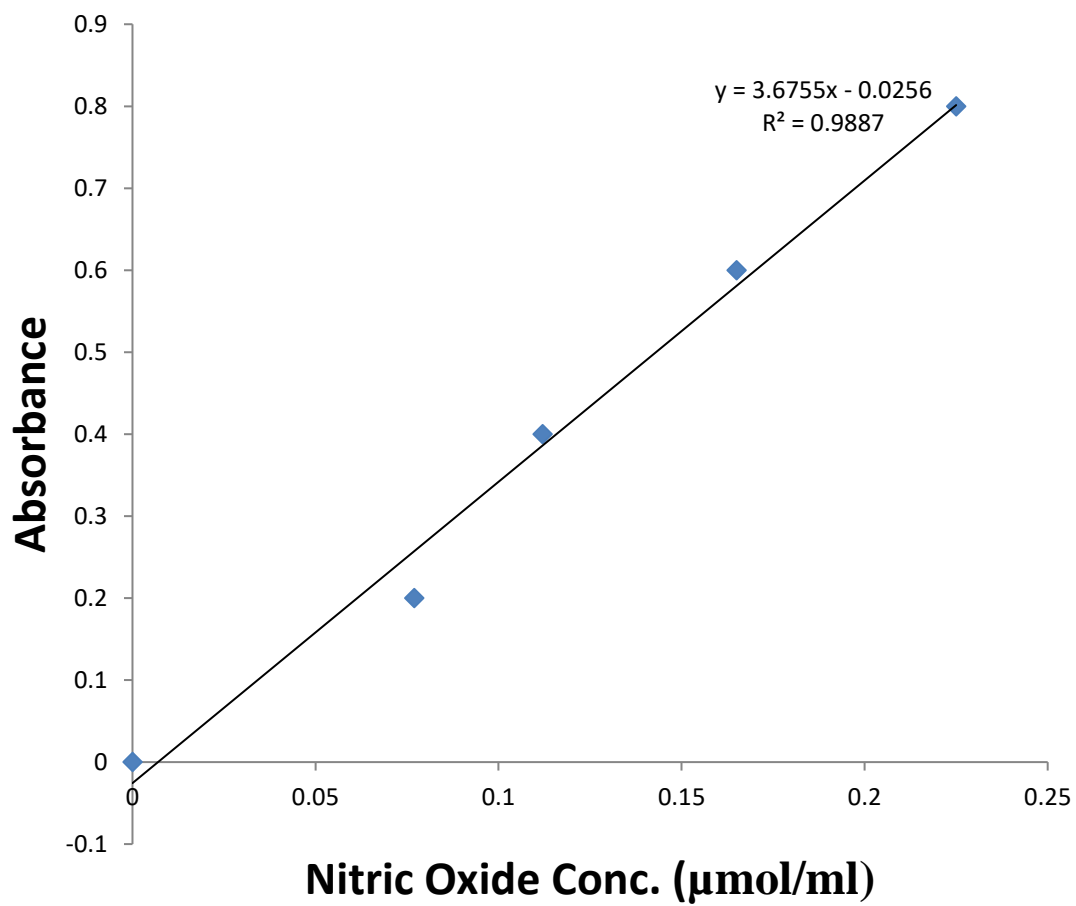
Figure 3.1: Nitric Oxide Standard Curve

3.2.13.2 Myeloperoxidase activity

Xia and Zweier (1997) method was used. Reagents used include 16.7 mg of O-dianisidine dihydrochloride (O-dianisidine), 0.05 M of Potassium Phosphate buffer (pH 6.0) prepared by using 3.40 g of KH_2PO_4 and 4.3545 g of K_2HPO_4 were put in 450 ml of distilled H_20 (pH 6.0) and make up to 500 ml. Diluted H_2O_2 was prepare by adding 2 µL of 59% $H202$ to 48µL of distilled water. 70 µL of homogenate was added to 2 ml O-dianisidine mixture in curvette. Read at 450 nm and recorded every 30 secs for 1 minute. Myeloperoxidase activity was calculated using this formula; change in activity divided by 1.13×10^{-2}

T1 = 30 -0 and T2 = 60-30 seconds (µmole/L).

3.2.13.3 Aspartate Aminotransferase level

Reitman and Frankel (1957) described method using RANDOX kit was used. The manufacturer protocol was duly followed.

3.2.13.4 Alanine aminotransferase (ALT) activity

Reitman and Frankel (1957) method was used to determine Alanine aminotransferase activity in serum sample. This was done using RANDOX kit and manufacturer protocol was duly followed

3.2.13.5 Determination of Reduced Glutathione

This was done using Beutler *et al*., (1963) method. 40 mg GSH in 0.1M PBS (pH 7.4) made up to 100ml. Ellman Reagent (0.04 g of Ellman reagent in 0.1M PBS made up to 100 ml). Precipitating Solution was prepared using 4% of sulphosalicyclic acid prepared by adding 4.8g of sulphosalicyclic acid to 120ml of DW.

The GSH level was estimated using 500 µl of sample and 500 µl of the precipitating solution, centrifuge at 4000 revolutions per minute for 5 minutes. 20 µl of the supernatant and 180 µl of Ellman's reagent pipette into micro plate and read at 412nm against blank (DW). Blank was prepared with 2ml of the 0.1M PBS and 3ml of diluted precipitating solution. 1ml of the above mixture was added to 4.5ml Ellman's reagent and absorbance was measured at 412nm.
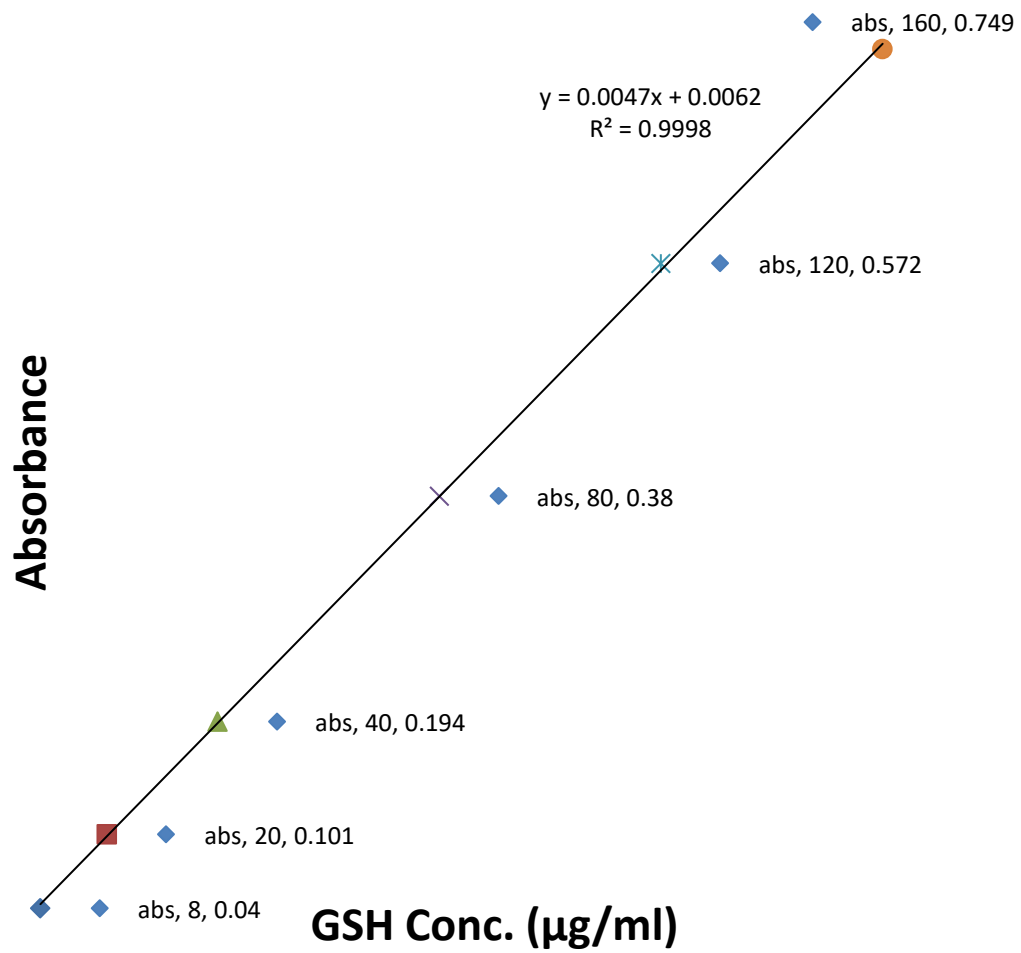
Figure 3.2: Reduced GSH standard curve

The figure shows a plot with y-axis labeled "Absorbance" and x-axis labeled "GSH Conc. (µg/ml)". Data points labeled: abs, 160, 0.749; abs, 120, 0.572; abs, 80, 0.38; abs, 40, 0.194; abs, 20, 0.101; abs, 8, 0.04. The trendline equation is $y = 0.0047x + 0.0062$ with $R^2 = 0.9998$.

3.2.13.6   Superoxide dismutase Activity

This was done using method of Misra and Fridovich (1972) with slight modification. 50 milligram of adrenaline was put in 50 ml DW and acidified with 0.25 ml concentrated HCl. 110 microliter carbonate buffer (pH 10.2) was added to thirty microliter of sample.   70 microliter of adrenaline was added and read at 480 nm every 30 s for 150 s. Increase in absorbance per minute is equal to A_f minus A_i divided by 100.

$$\% \text{ inhibition} \quad = \quad \frac{\text{increase in Abs for substrate X} \quad 100}{\text{increase in Abs of blank}}$$

A_i = absorbance after 30 seconds A_f = absorbance after 150 seconds

3.2.13.7   Determination of Glutathione Peroxidase Effect

Using Rotruck *et al*., (1973) method in which hydrogen peroxide was used as substrate to oxidize reduced glutathione to oxidized glutathione (GSSG).

Table 3.1: Glutathione peroxidase Assay protocol

Reagent	Volume
Phosphate buffer	100
NaN_3	20
GSH	40
H_2O_2	20
Sample	100
Distilled water	120

The compound was kept at 37˚C for 5 minutes. 100 µl of Trichloroacetic acid was added and after which it was centrifuged at 3000 rpm for 5 minutes. 100 µl of DTNB and 200 µl of K_2HPO_4 were added to 100 µl of each of the supernatants and read at 412 nm against a blank. The remaining GSH was calculated from the curve. GSH consumed is equal to 245.34 minus remaining GSH. GPx activity equal to GSH consumed divide by milligram protein

3.2.13.8   Estimation of Glutathione S-transferase Activity

Habig *et al*., (1974) method was used. This is based on glutathione-S-transferase exhibit a relatively great activity with CDNB as the second substrate. Reagents used include: 20mM CDNB (33.7mg CDNB was put in10ml of ethanol). Reduced Glutathione {61.46 mg of GSH was put in 2ml of 0.1M PBS (pH 6.5)}.   30 microliter of sample, 2.79 ml of PBS, 150 microliter CDNB and 30 microliter of GSH was added together and read against the blank at 340nm with extinction coefficient of CDNB = $9.6mm^{-1}$ Cm^{-1}. Glutathione-S-Transferase level in µmole/min/mg protein was then calculated using this formula:

$$\text{GST activity} = \frac{\text{optical density per min}}{9.6} \times \frac{1}{0.03ml \ * \ \text{miligram protein}}$$

3.2.13.9   Hydrogen Peroxide Generation

The Hydrogen peroxide was estimated using Wolff's method (1994). Reagents used include 250mMol/L ammonium ferrous sulfate (MW = 392.14) prepared by dissolving 0.01g of ammonium ferrous sulfate in 100 mls of distilled water, 100 µmol/L Xylenol orange (760.6) prepared by dissolving 0.00375g of xylenol orange in 50mls of distilled water. 250 mMol/L H_2SO4 by adding 1ml of 1M H_2SO_4 to 40 mls of distilled water. 100Mm/L sorbitol (MW = 182.2) prepared by adding 0.91g of sorbitol in 50mls of distilled water. All added to 50µl of sample vortexed and kept at 25^0C for 30 minutes. After which it was read at 560nm.

Table 3.2: Hydrogen peroxide generation

Reagents	Volume
Sorbitol	20 µl
XO	20 µl
AFS	50 µl
H_2SO_4	10 µl
Buffer	100 µl
Sample	10 µl

3.2.13.10   Lipid peroxidation estimation

Using method described by Varshney and Kale (1990). This principle is based on the reaction between MDA and 2-thiobarbituric acid (TBA). The pink chromophore can be extracted into organic solvents such as butanol. The maximum fluorescence at 553 nm and absorbance at 532 nm.
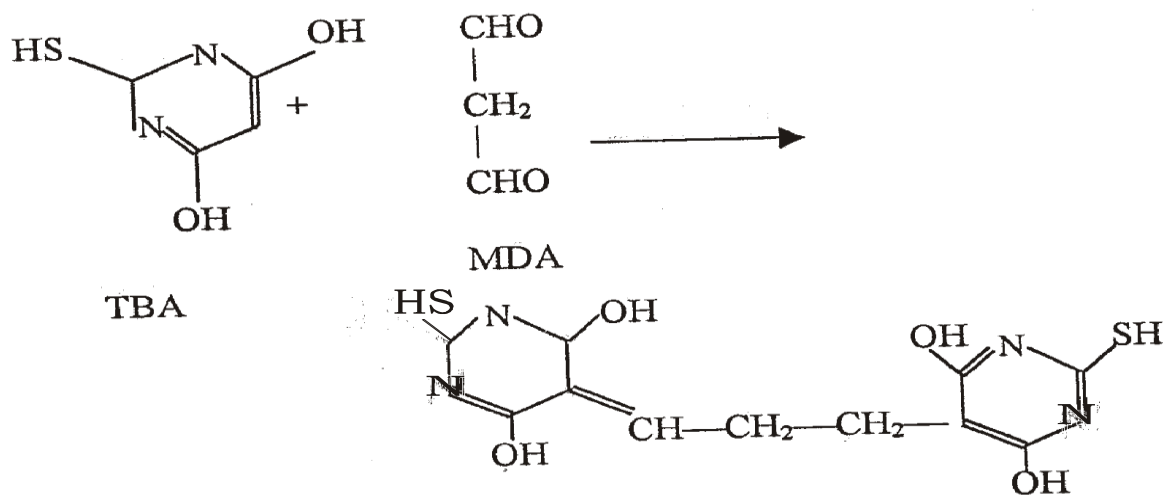
Figure 3.3: Reaction of TBA with MDA gives pink colouration

0.2ml of the test sample was mixed with 0.8 ml of Tris-KCl buffer which 0.2ml of 30% TCA and 0.25ml of 0.75% TBA was added. The mixture was put in a water bath for 45 minutes at 80°C and cooled in ice, centrifuged at 3000 rpm. 0.2 ml of the supernatant was plated in a micro plate and read using micro plate reader at 532nm. MDA level was calculated in units per mg protein using molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{Cm}^{-1}$ to compute it

MDA in units per milligram protein = $\dfrac{\text{Absorbance x volume of mixture}}{E_{532nm} \text{ x volume of sample x mg protein}}$

3.2.13.11   Protein Thiol Level

Protein thiol level was estimated according to Ellman (1959). 0.008 g of DTNB was dissolved into 20 ml of phosphate buffer solution. 196 µL of Phosphate Buffered solution and 6 µl of DTNB were added to 10 µL of sample, incubated in a dark chamber (30 minutes) and read at 412 nm.

3.2.13.12   Non Protein Thiol Level

Non protein thiol level was estimated according to Ellman (1959).). 500 µL of sample was added to 500 µL of TCA, spin at 4000 revolutions per minute for 5 minutes, 20 µL of supernatant was plate on the micropipette plate, 170 µL of phosphate Buffer Saline and 10 µL of DTNB was added. The plate was read with spectrophotometer at wavelength of 405 nm.

3.2 .13.13   Protein Determination

Gornal *et al.*, (1949) method was used. 150 microliter of biuret reagent was added to 50 microliter of serum sample and incubated at room temperature for 30 minutes. Read at 540 nm with micro plate reader and the value was extrapolated from total protein standard curve.

3.2.14 Histopathological Studies

Histopathology was done using Drury *et al.*, (1976) method. Harvested hearts from each group were rinsed in ice cold saline, and then tissues were put in a sample bottle containing 10% buffered formalin solution preservation. The tissues were processed

and then fixed in paraffin wax and cut to serial transverse sections. Haematoxylin and eosin was used to stain and viewed under light microscope.

3.2.15 Immunohistochemistry of CRP, IL-10 and Cardiac troponin in the heart

This was done using Todorich *et al.,* (2011) method. Slides were labeled with Hb pencil. Samples were dewaxed using xylene. Rehydrated with ethanol in decreasing concentration of 100-80 %. 3% H_2O_2 per methanol (endogenous peroxidase) was used for peroxidase quenching. 0.01 M citrate buffer (pH 6.0) was used to retrieve antigen by boiling in microwave and allowed to cool. Normal goat serum was used to block sections and probed with CRP, IL-10 and Cardiac troponin antibodies overnight at 25^0C for 16 h (Abclonal®). Biotinylated (goat anti-rabbit, 2.0 µg/ml) as secondary antibody and after which, Horseradish Peroxidase- streptavidin was used (HistoMark®, KPL, Gaithersburg MD, USA). Enriched with diaminobenzidine (DAB Amresco®, USA) for 3-5 minutes. High definition haematoxylin (Enzo®, NY – USA) was used to stain and dehydrated using 80-100% ethanol. Covered slides were viewed with photo microscope attached to digital camera at X400 magnification.

3.3   Data analysis

Data were presented as mean ± S.D and level of significance is α <0 .05, One- way analysis of variance (ANOVA) with Tukey's post –hoc test was performed using Graph Pad Prism version 4.00.

3.4   Results

3.4.1   Haemodynamics

Table 3.3: There is a significant decrease ($\alpha<0.05$) of haemodynamics in ISO (Group B) compared to control (Group A).   All treated groups (C, D, E & F) show a significant increase ($\alpha<0.05$) compare to control (Group A).

Table 3.3: Effect of EEAP on haemodynamics in isoproterenol induced myocardial infarction.

				Extracts			
	Control	**ISO only**	**ISO + Enalapril**	**Pre-treated groups**			**Post–treated group**
Systolic BP mmHg	130±2.3	102.33±1.0^a	128.7±1.8^b	129.6±2.0^b	133.00±1.0^{bc}	131.50±2.1^{bc}	136.00±1.3^{bc}
Diastolic BP mmHg	105.75±1.4	82.67±1.8^a	103.7±1.2 ^{b}	104.60±1.4^b	103.25±2.4^b	101.75±1.3^{ab}	101.00±2.1^{ab}
Mean AP mmHg	114.50±1.4	89.00±2.0^a	111.8±0.9 ^{b}	115.00±1.3^{bc}	113.00±1.1^b	114.00±1.5^b	113.00±1.2^b

Superscripts a indicates when related with control, whereas superscripts b indicates when related with ISO treated only, superscript c indicates when related with ENA.

3.4.2   Electrocardiogram

Figure 3.4: show that there is an increase in heart rate in ISO (Group B) compared to control (Group A). There is an increase in heart rate in 200mg + ISO & 400mg + ISO (Group E & F) compared with ISO (Group B). 100 mg + ISO reduced the heart rate compared to control (Group A) & ISO (Group B). 200 mg/kg + ISO post treated show no alteration in compared with the control (Group A).

Figure 3.5: show that there is a significant different in QT interval in ISO (Group B) compared to control (Group A). There is different in all treated groups except 200 mg + ISO post treated (Group G) compared to control (Group A). There is a marked decrease in QT interval in 200 mg + ISO post treated (Group G) compared to ISO (Group B).

Figure 3.6: Show that there is significant different in QTc Bazzett in ISO (Group B) compared to control (Group A). There is a significant different in all treated groups groups except 200 mg + ISO post treated (Group G) compared to control (Group A).). There is a decrease in QTc Bazzett in 200 mg + ISO post treated (Group G) compared to ISO (Group B).
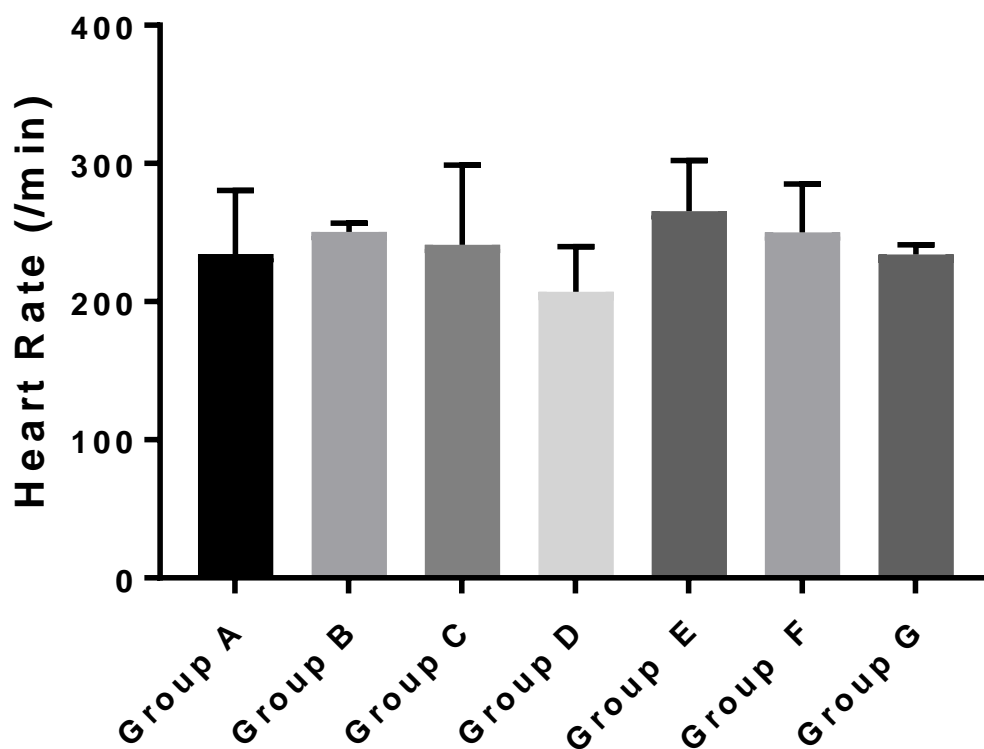
Figure 3.4: Effect of EEAP on heart rate in isoproterenol induced myocardial infarction.

Grp A (Control), Grp B (ISO treated only), Grp C (ENA+ 85 mg/kg ISO), Grp D (100 mg/kg AP + ISO), Grp E (200 mg/kg AP + ISO), Grp F (400 mg/kg AP + ISO) & Grp G (ISO +200 mg/kg Ap Post treated).
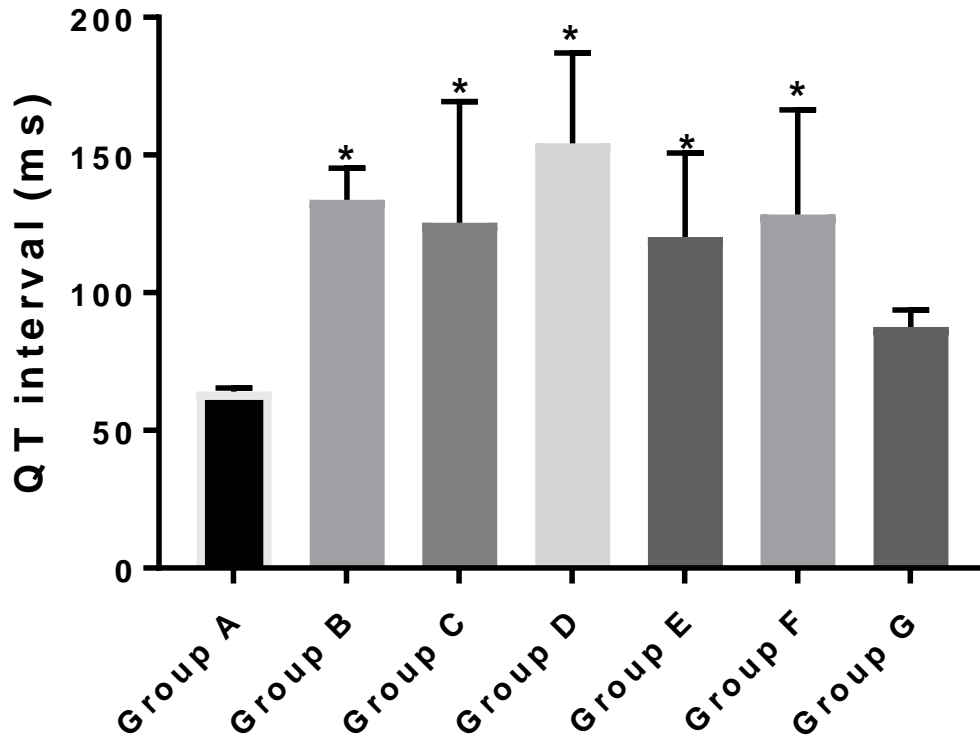
Figure 3.5: Effect of EEAP on QT interval in isoproterenol induced myocardial infarction.

Superscripts (*) indicates when compared with control (Grp A), Grp A (Control), Grp B (ISO treated only), Grp C (ENA+ 85 mg/kg ISO), Grp D (100 mg/kg AP + ISO), Grp E (200 mg/kg AP + ISO), Grp F (400 mg/kg AP + ISO) & Grp G (ISO +200 mg/kg Ap Post treated).
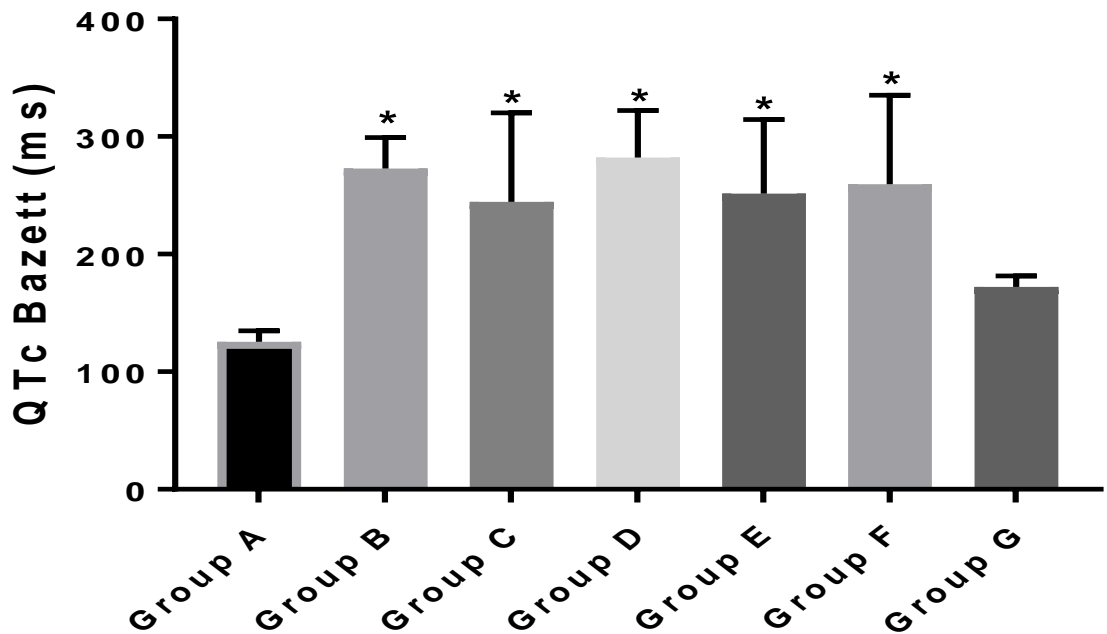
Figure 3.6: Effect of EEAP on QTc Bazzett in isoproterenol induced myocardial infarction.

Superscripts (*) when compared with control (Grp A). Grp A (Control), Grp B (ISO treated only), Grp C (ENA+ 85 mg/kg ISO), Grp D (100 mg/kg AP + ISO), Grp E (200 mg/kg AP + ISO), Grp F (400 mg/kg AP + ISO) & Grp G (200 mg/kg Ap + ISO Post treated).

3.4.3 Haematology

Table 3.4: show that there is significant ($\alpha < 0.05$) increase in WBC in ISO group related with the control. The extract treated groups also show significant ($\alpha < 0.05$) increase in WBC compared with the ISO group while enalapril show significant decrease compared with ISO group.

Table 3.4: EEAP effects on haemogram in isoproterenol induced myocardial infarction

				Extracts			
				Pre- treated groups			**Post-treated group**
	Control	**ISO**	**Enalapril**	**100 mg/kg**	**200 mg/kg**	**400 mg/kg**	**200 mg/kg**
RBC($\times 10^{12}$/L)	4.75±0.90	4.96±0.43	5.03±0.69	5.51±1.05	5.86±0.47[abc]	5.08±0.59	6.71±0.34[bc]
WBC (10^3/μL)	5.47±0.38	6.71±1.13[a]	4.68±1.68[b]	5.95±1.64	7.50±1.64[ac]	6.70±1.29[ac]	7.73±1.09[ac]
HB (g/dl)	13.33±1.40	15.15±1.84	14.95±1.62	15.25±1.71	15.24±1.83	13.64±1.61	14.95±1.37
PCV (%)	45.75±4.65	54.25±4.25[a]	50.25±3.10	52.75±1.89[a]	49.25±3.86[b]	47.00±2.58[b]	49.67±0.58[a]
MCV (fl)	96.32±9.03	109.37±30.12	99.90±22.76	95.73±14.08	84.04±16.31	92.52±9.73	74.02±6.83[b]
MCH (pg)	28.06±3.48	30.54±8.08	29.72±2.25	27.68±5.30	26.01±5.24	26.85±2.56	22.28±3.17
MCHC (g/dl)	29.14±2.05	27.93±2.38	29.75±2.37	28.91±4.46	30.94±2.81	29.02±1.32	30.09±1.68

n =5, a=compared with control, b = compared with ISO, c =compared with enalapril.

3.4.4 Antioxidant defense system

Figure 3.7: Show that there is decrease in SOD of ISO treated rats as compared with control (grp A). Extract treated (grp D, E & F) increase SOD compared with ISO treated. 200 mg/kg AP Post treated (grp G) almost increase the SOD to normal level compare with the control (grp A).

Figure 3.8: Compared with control (grp A) there is increase in GPX activity in all treated group. There is increase in GPX compared with ISO (grp B) except 200mg+ISO (grp E).

Figure 3.9: ISO (grp B) show decrease in GST activity compared with control. There is increase in GST activity in all treated group compared with the control. There is in 100mg+ISO and 200mg+ISO compared with ISO (grp B).

Figure 3.10: ISO (grp B) treated show decline in GSH activity compared with the control (grp A). There is increase in all treated group compared with ISO (grp B)
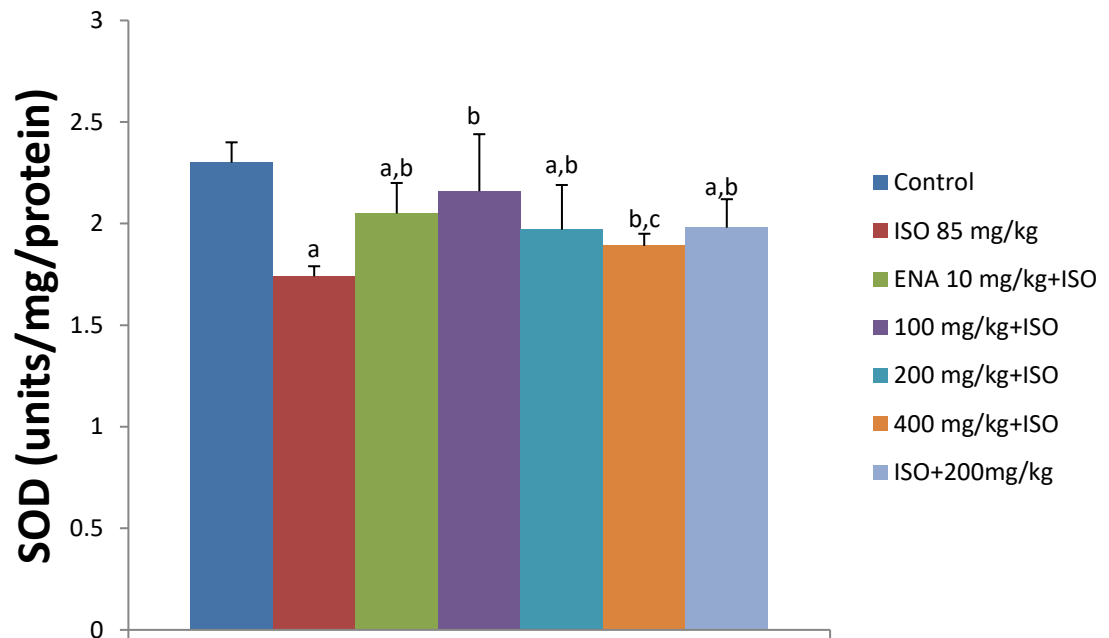
Figure 3.7: EEAP effects on SOD enzyme in isoproterenol-induced myocardial infarction

Superscripts a indicates when related with control, whereas superscripts b indicates when related with ISO treated only, superscript c indicates when related with ENA. n=7
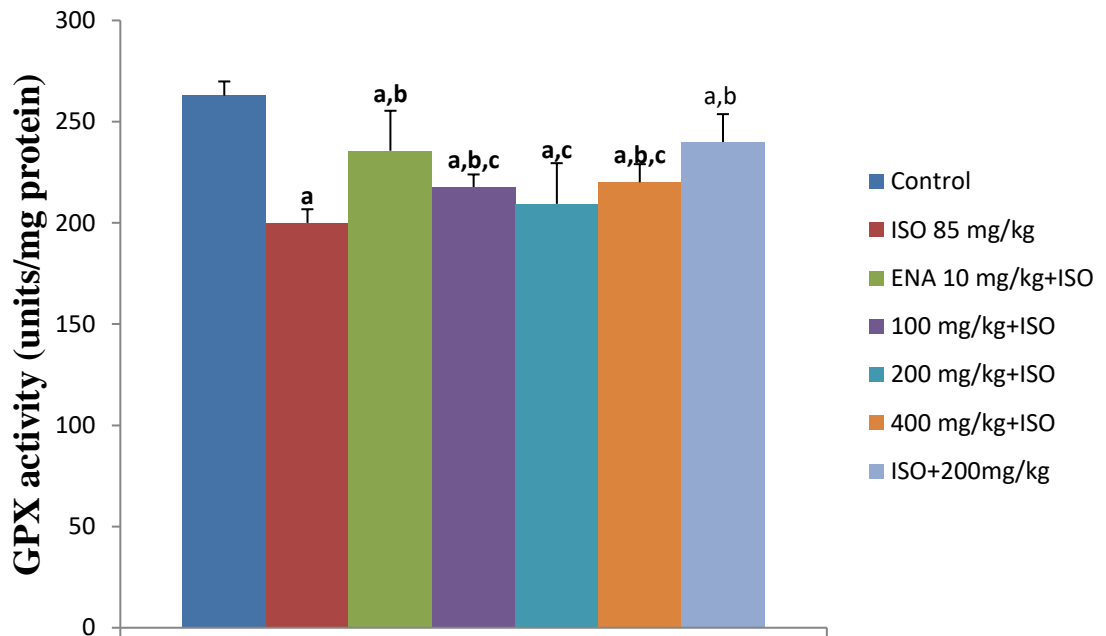
Figure 3.8: EEAP effects on GPx enzyme in isoproterenol-induced myocardial infarction

Superscripts a indicates when related with control, whereas superscripts b indicates when related with ISO treated only, superscript c indicates when related with ENA. n=7
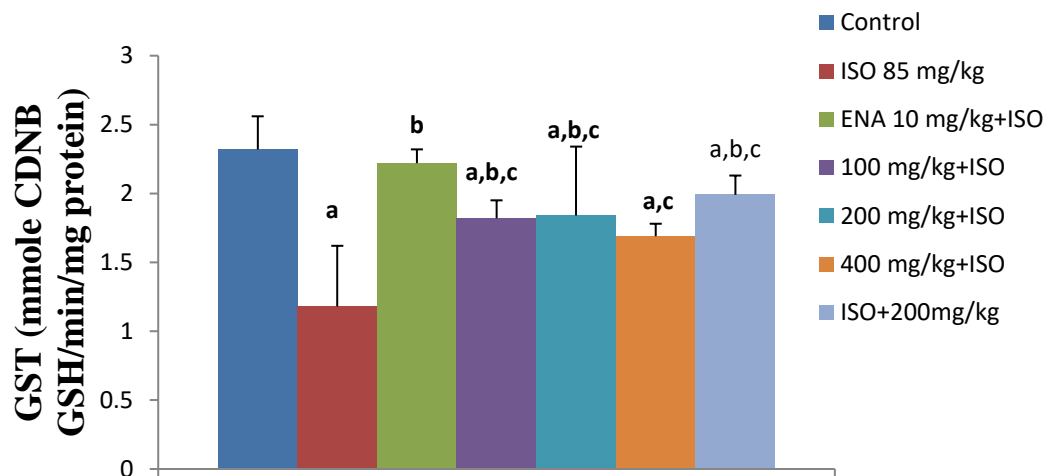
Figure 3.9: EEAP effects on GST enzyme in isoproterenol induced myocardial infarction

Superscripts a indicates when related with control, whereas superscripts b indicates when related with ISO treated only, superscript c indicates when related with ENA. n=7
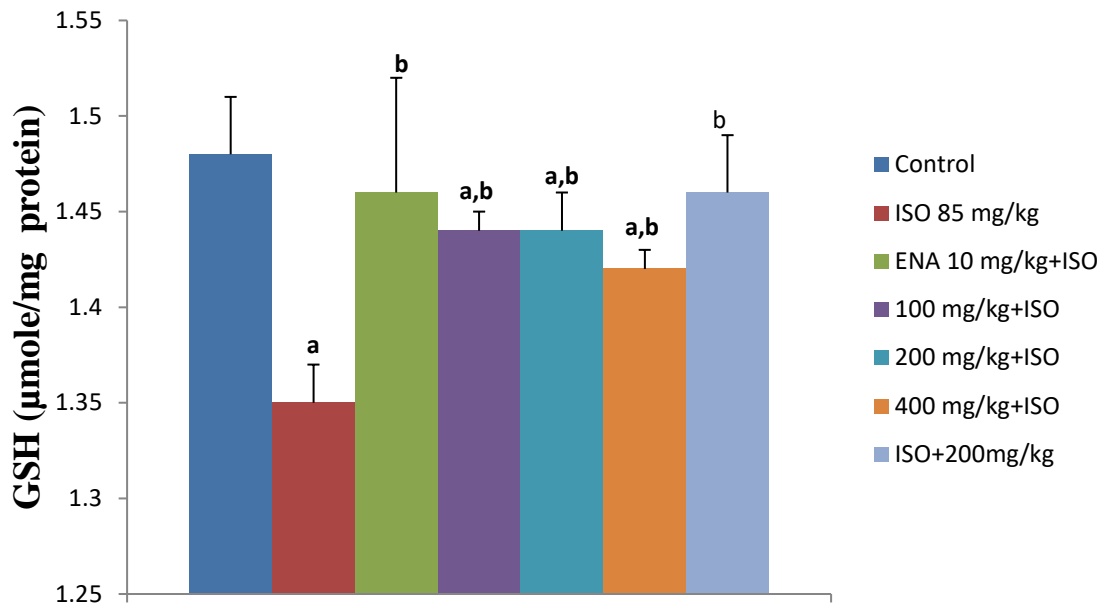
Figure 3.10: EEAP effects on GSH in isoproterenol induced myocardial infarction

Superscripts a indicates when related with control, whereas superscripts b indicates when related with ISO treated only, superscript c indicates when related with ENA. n=7

3.4.5 Markers of Oxidative Stress

Figure 3.11: There is an increase in MDA activity in ISO (grp B) treated compared with control (grp A). MDA generation was reduced in all treated groups.

Figure 3.12: There is an increase in H_2O_2 generation in ISO (grp B) compared with the control (grp A). Treated groups show decrease in H_2O_2 generation compared with ISO (grp B).

Figure 3.13: There is an increase in MPO in ISO (grp B) compared with control (grp A). 100mg + ISO, 200 + ISO and 400mg + ISO have decrease compared with ISO (grp B) treated.

Figure 3.14: There is a decrease in PT in ISO (grp B) compared with control (grp A). 100mg + ISO and 400mg + ISO have increase compared with ISO (grp B) treated

Figure 3.15: ISO (grp B) treated show decline in NPT compared with control (grp A). All treated groups show increase compared with ISO (grp B). 200 mg/kg post treated group increased the NPT to normal control level.
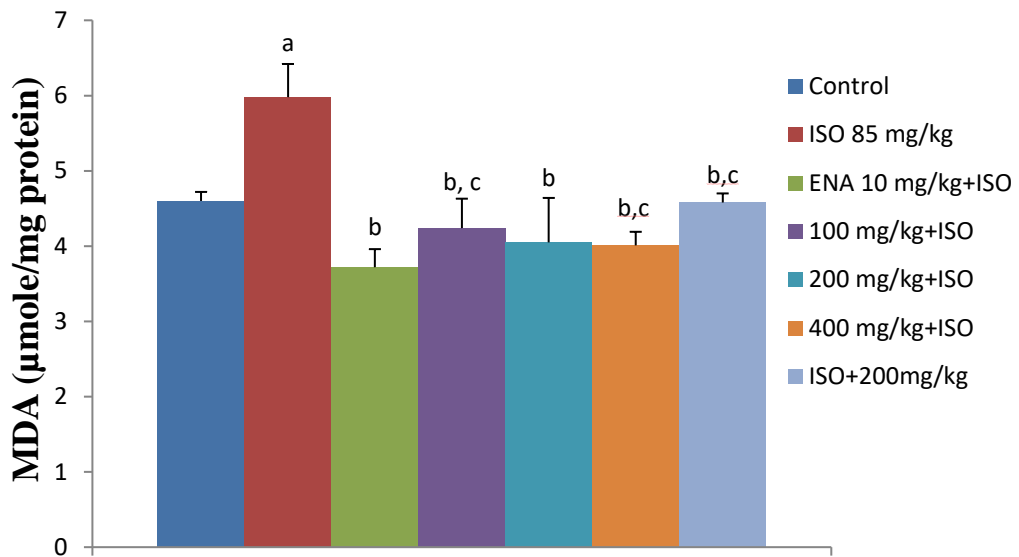
Figure 3.11: EEAP effects on MDA in isoproterenol induced myocardial infarction

Superscripts a indicates when related with control, whereas superscripts b indicates when related with ISO treated only, superscript c indicates when related with ENA. n=7
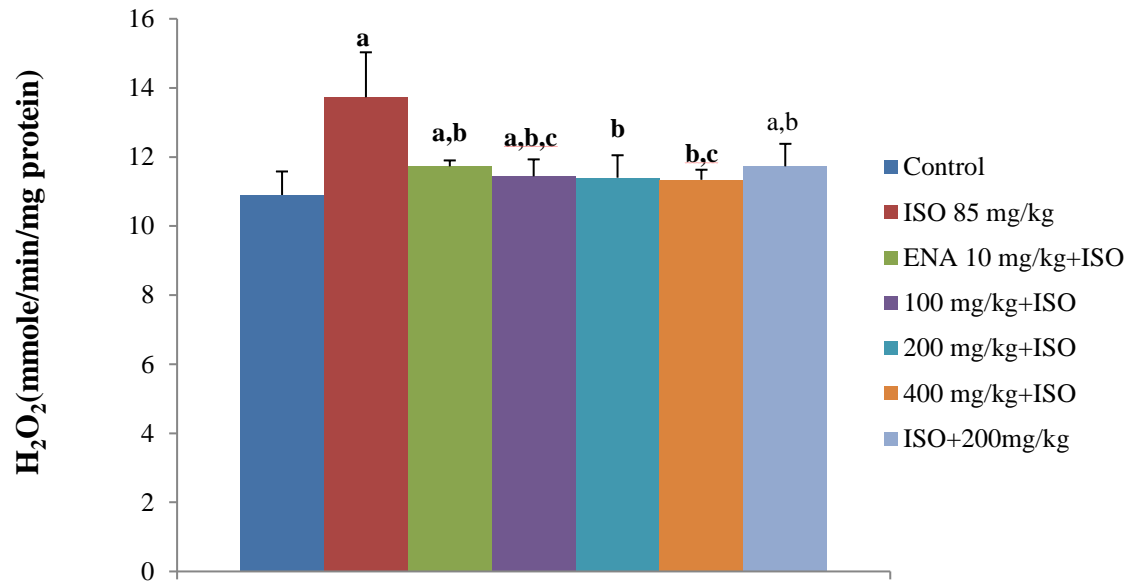
Figure 3.12: EEAP effects on hydrogen peroxide in isoproterenol induced myocardial infarction

Superscripts a indicates when related with control, whereas superscripts b indicates when related with ISO treated only, superscript c indicates when related with ENA. n=7
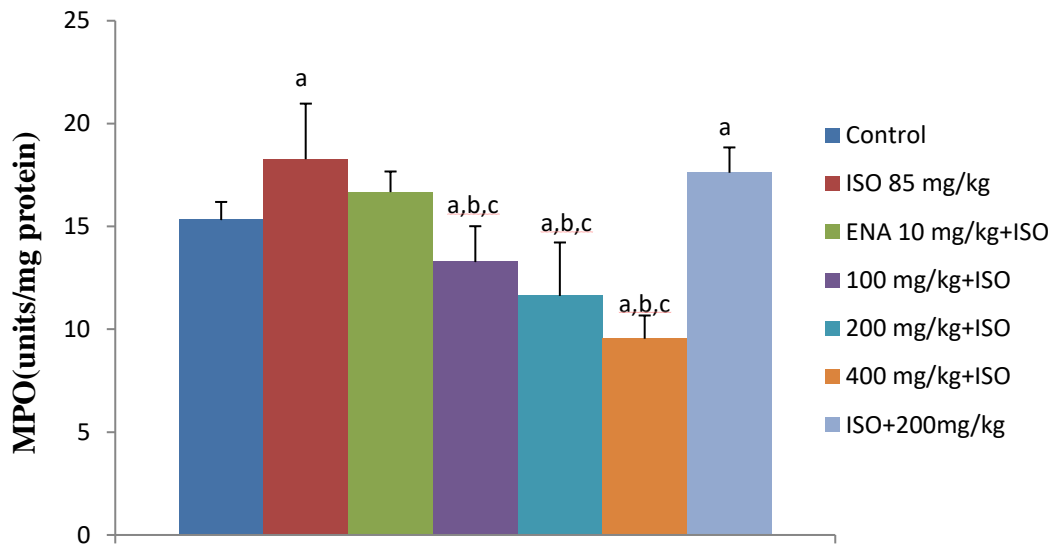
Figure 3.13: EEAP effects on myeloperoxidase in isoproterenol induced myocardial infarction

Superscripts a indicates when related with control, whereas superscripts b indicates when related with ISO treated only, superscript c indicates when related with ENA. n=7
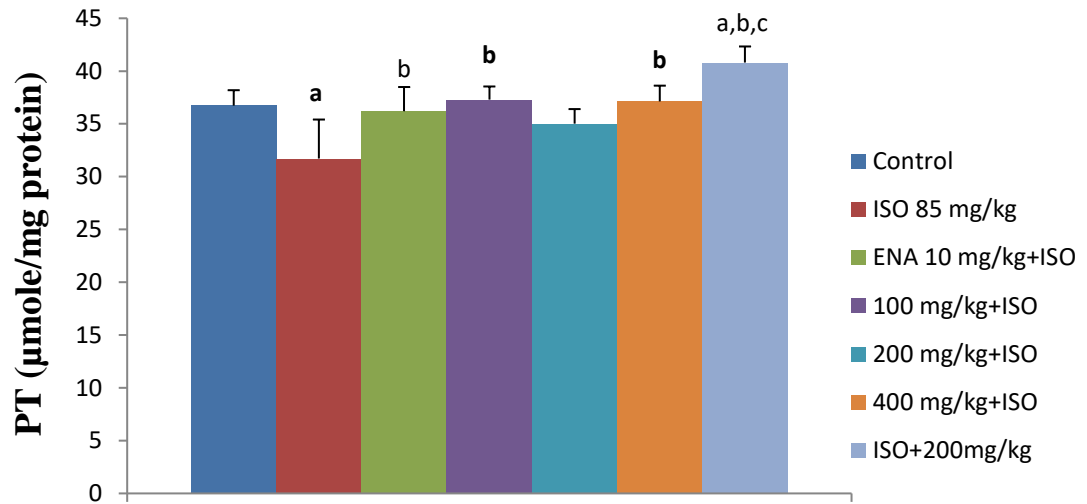
Figure 3.14: EEAP effects on protein thiol in isoproterenol induced myocardial infarction

Superscripts a indicates when related with control, whereas superscripts b indicates when related with ISO treated only, superscript c indicates when related with ENA. n=7
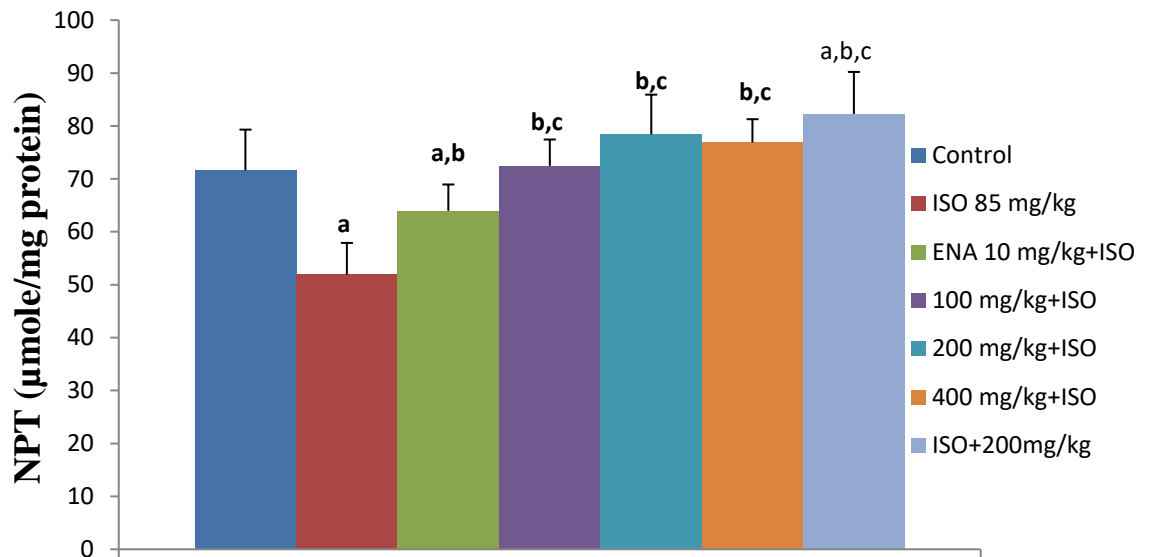
Figure 3.15: EEAP effects on protein thiol in isoproterenol induced myocardial infarction

Superscripts a indicates when related with control, whereas superscripts b indicates when related with ISO treated only, superscript c indicates when related with ENA. n=7

3.4.6   Serum chemistry

Table 3.5: There is a significant increase ($\alpha<0.05$) in ALT and AST in ISO (grp B) compared with control (grp A). The enalpril and the extract treated groups have a significant decrease ($\alpha<0.05$) compared with ISO (grp B) treated group.

Table 3.5: EEAP effects on serum chemistry in isoproterenol induced myocardial infarction

				Extracts			
				Pre- treated groups			Post-treated group
	Control	ISO	Enalapril	100 mg/kg	200 mg/kg	400 mg/kg	200 mg/kg
ALT (U/L)	14.51±0.02	14.67±0.05[a]	14.41±0.05[ab]	14.45±0.04[ab]	14.48±0.04[b]	14.45±0.01[b]	14.40±0.01[ab]
AST (U/L)	19.91±0.01	19.97±0.02[a]	19.87±0.02[ab]	19.88±0.02[b]	19.88±0.01[ab]	19.88±0.02[ab]	19.87±0.02[ab]

 n =5, Superscripts a indicates when related with control, whereas superscripts b indicates when related with ISO treated only, superscript c indicates when related with ENA.

3.4.7 Histopathology

Group A (Control) shows no visible lesion. Group B (ISO): shows severe infiltration of inflammatory cells. Group C (enalapril) has no visible lesion. Group D (100 mg/kg+ISO) and group E (200 mg/kg + ISO) show slight inflammatory cells infiltration. Group F (400 mg/kg +ISO) have no lesion. G (ISO+200 mg/kg) have mild inflammatory cells infiltration.
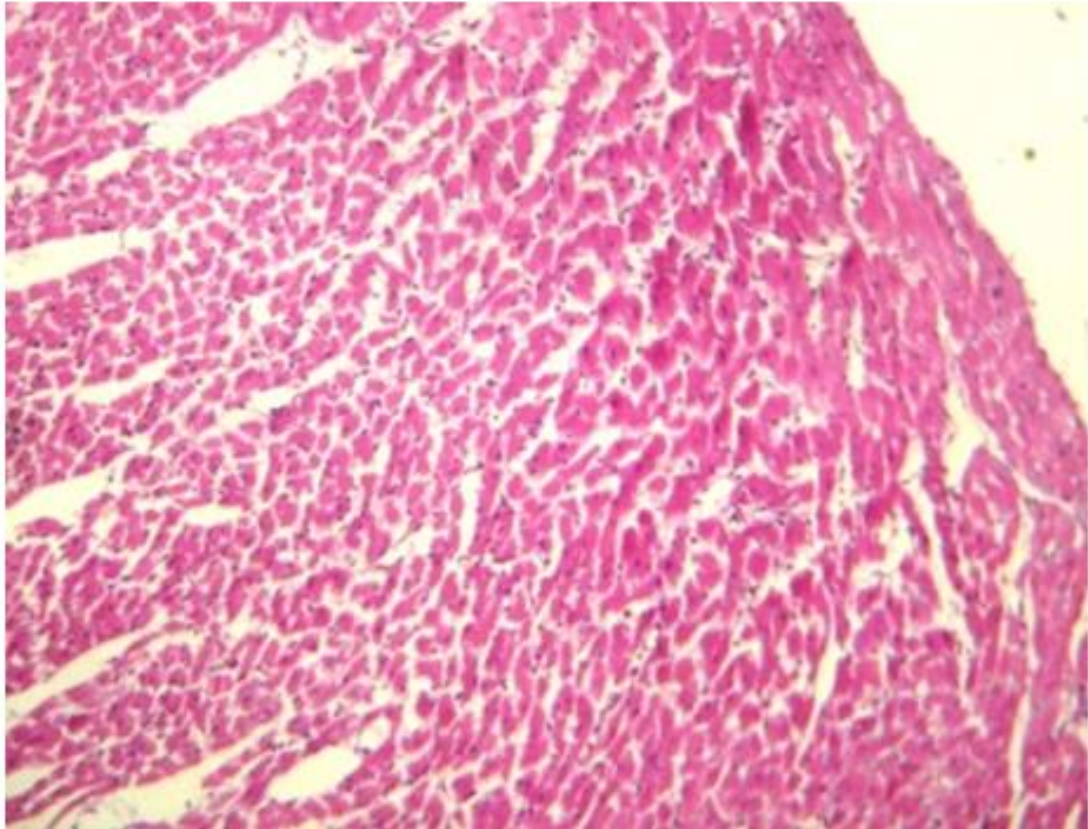
Figure 3.16a: The heart section of rat in the control group (group A)

showing no visible lesion (mag x 100).
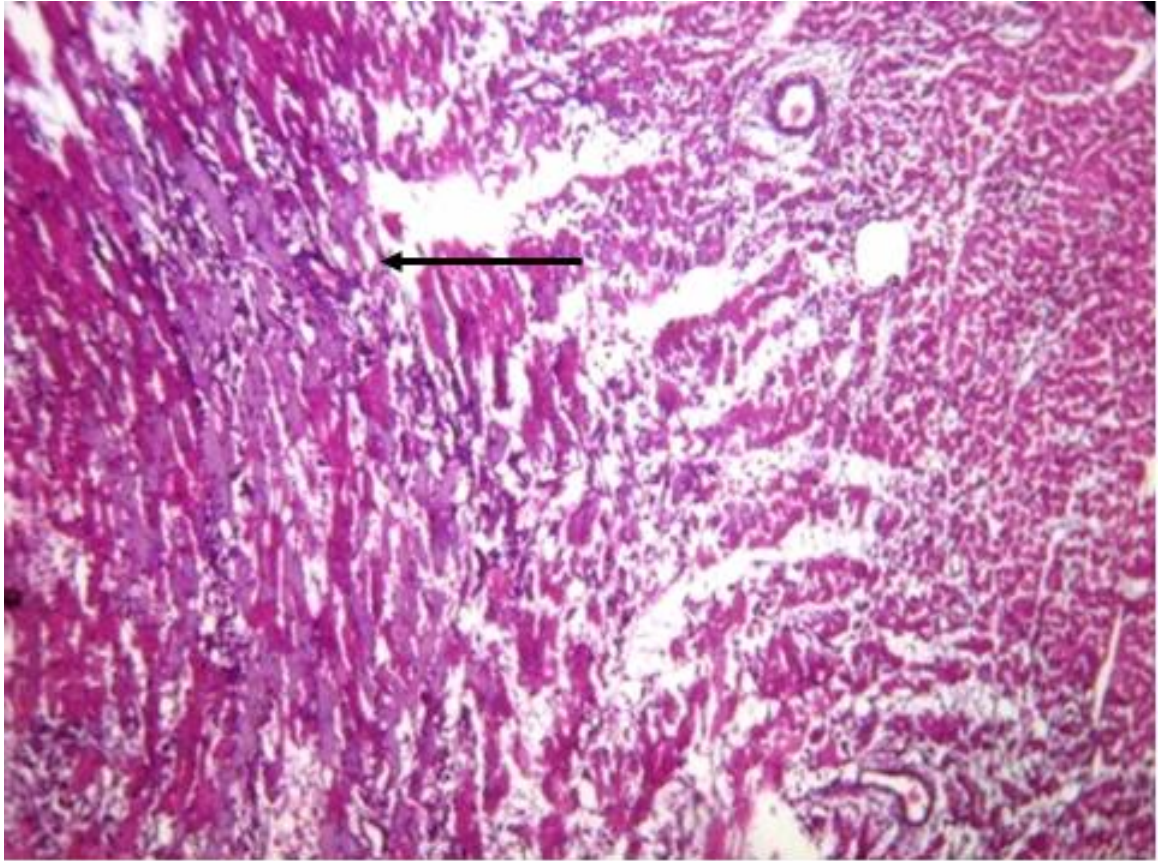
Figure 3.16b: The heart section of rat administered with isoproterenol only (group B)

showing severe infiltration of inflammatory cells (mag x 100).
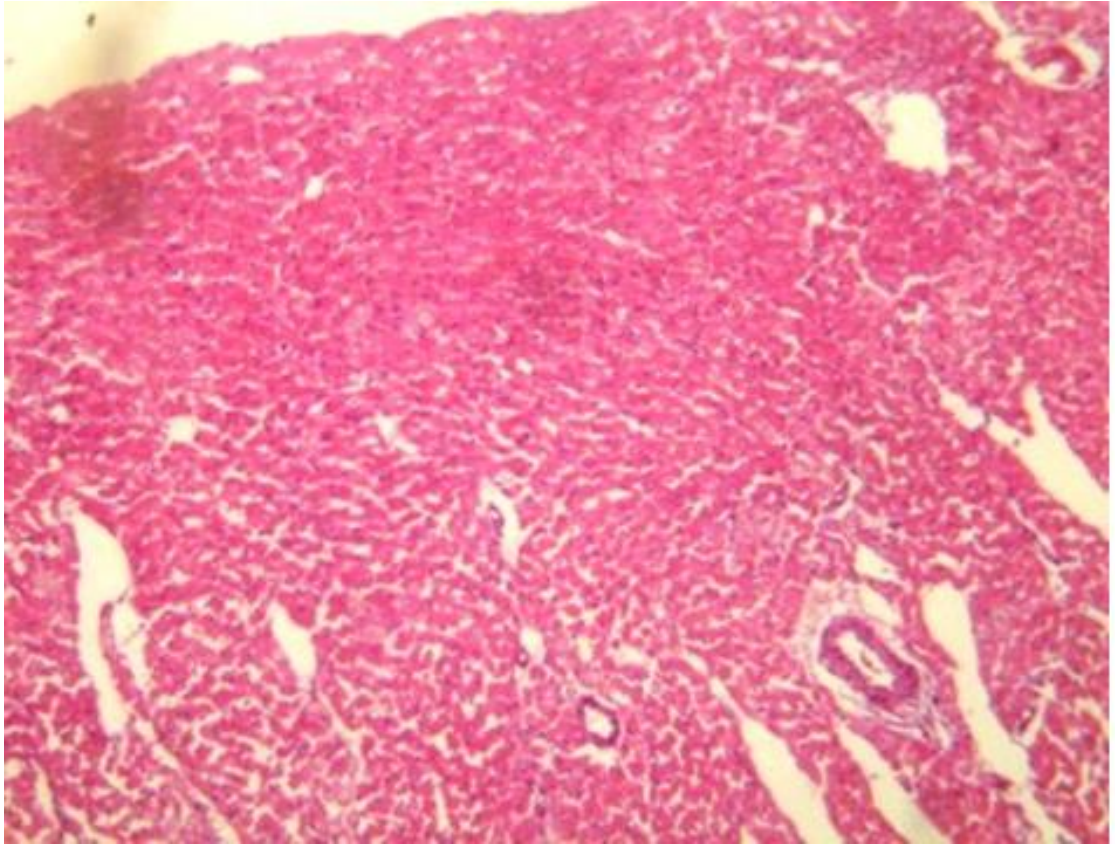
Figure 3.16c: The heart section of rat pretreated with 10 mg/kg (group C) of enalapril

showing no visible lesion. (mag x 100).
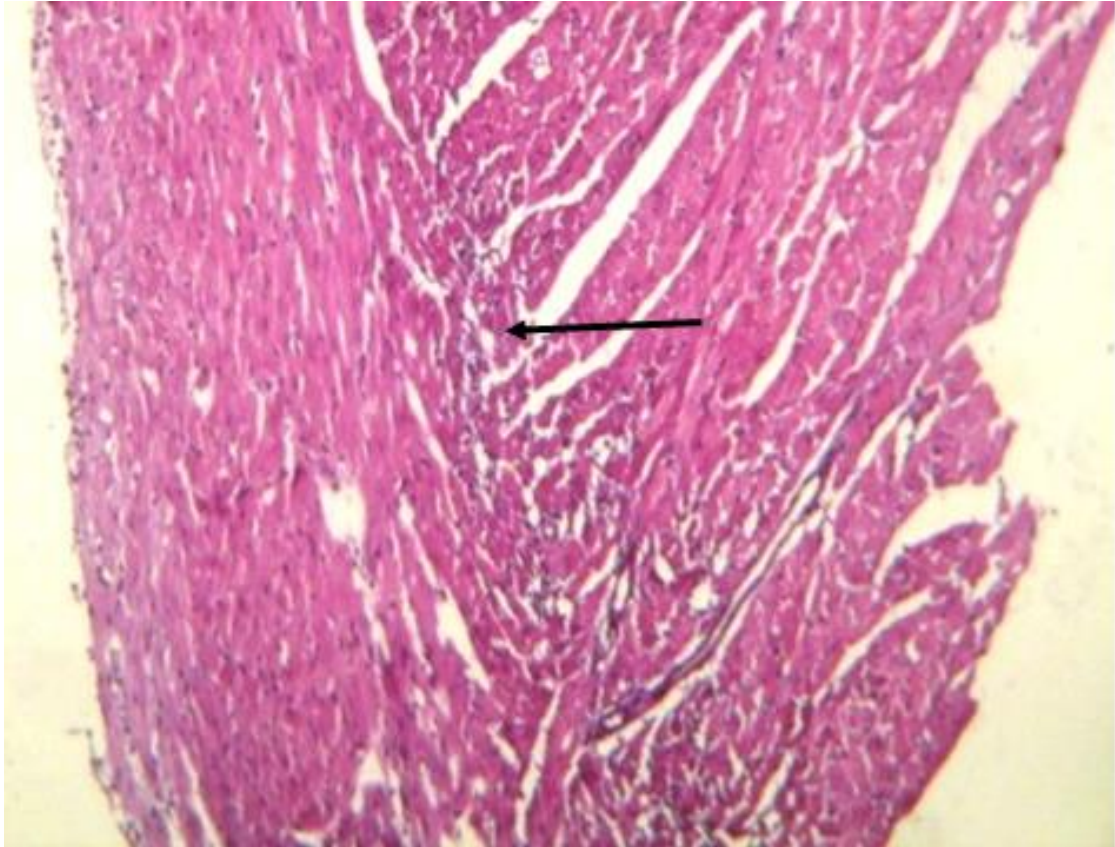
Figure 3.16d: The heart section of rat pretreated with 100 mg/kg (group D) of EEAP

showing mild infiltration of inflammatory cells. (mag x 100).
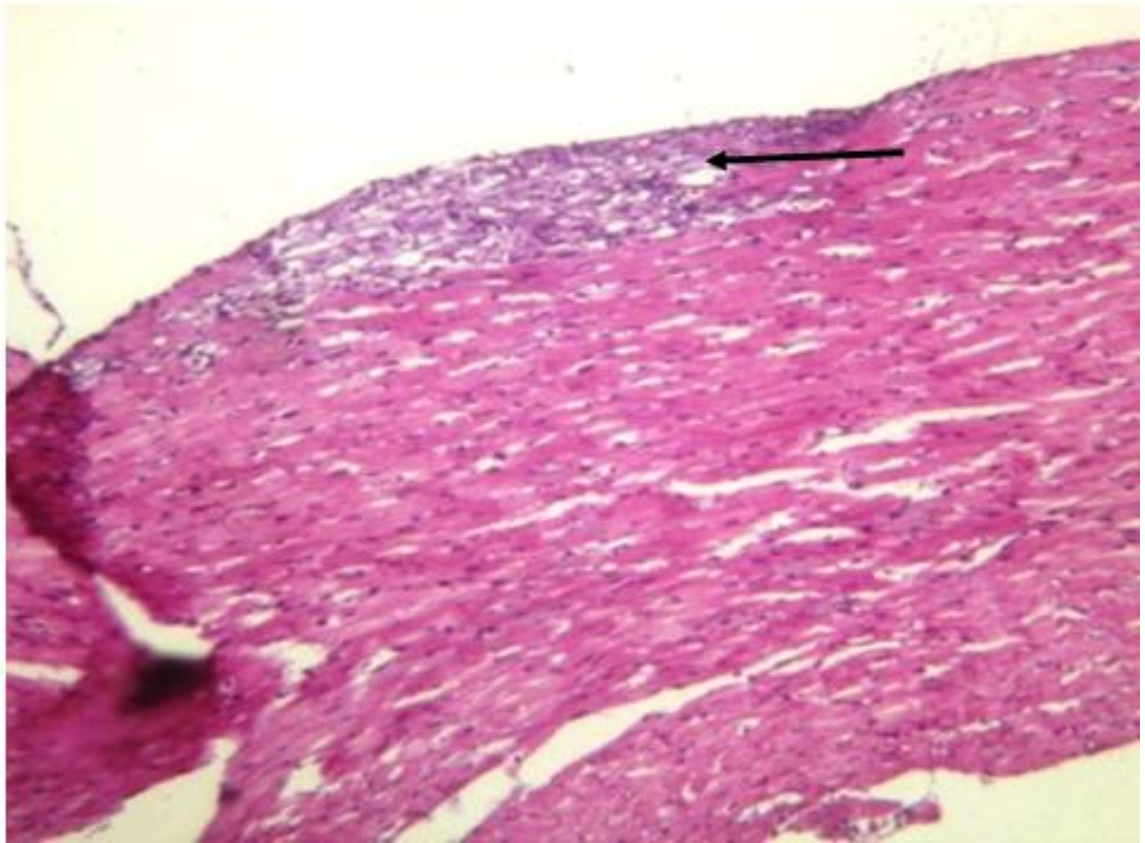
Figure 3.16e: The heart section of rat pretreated with 200 mg/kg (group D) of EEAP

showing mild infiltration of inflammatory cells. (mag x 100).
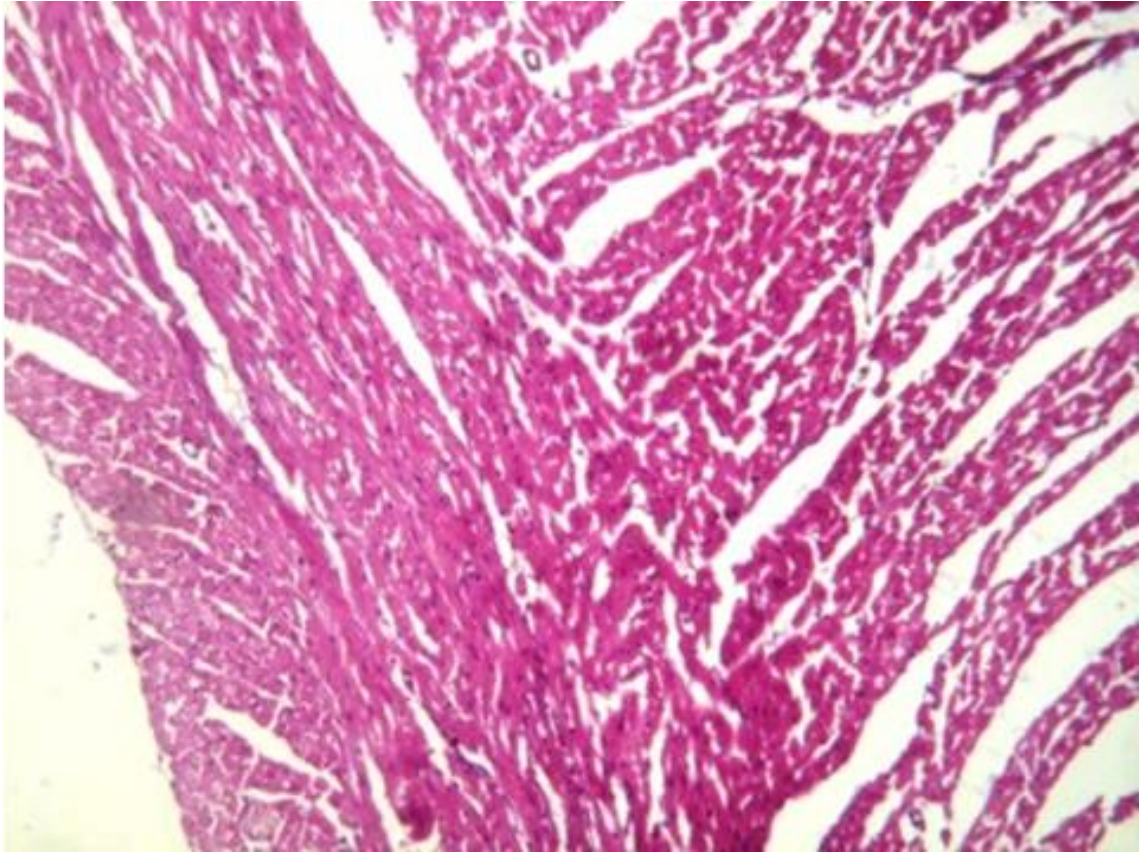
Figure 3.16f: The heart section of rat pretreated with 400 mg/kg (group F) of EEAP

showing no visible lesion (mag x 100).
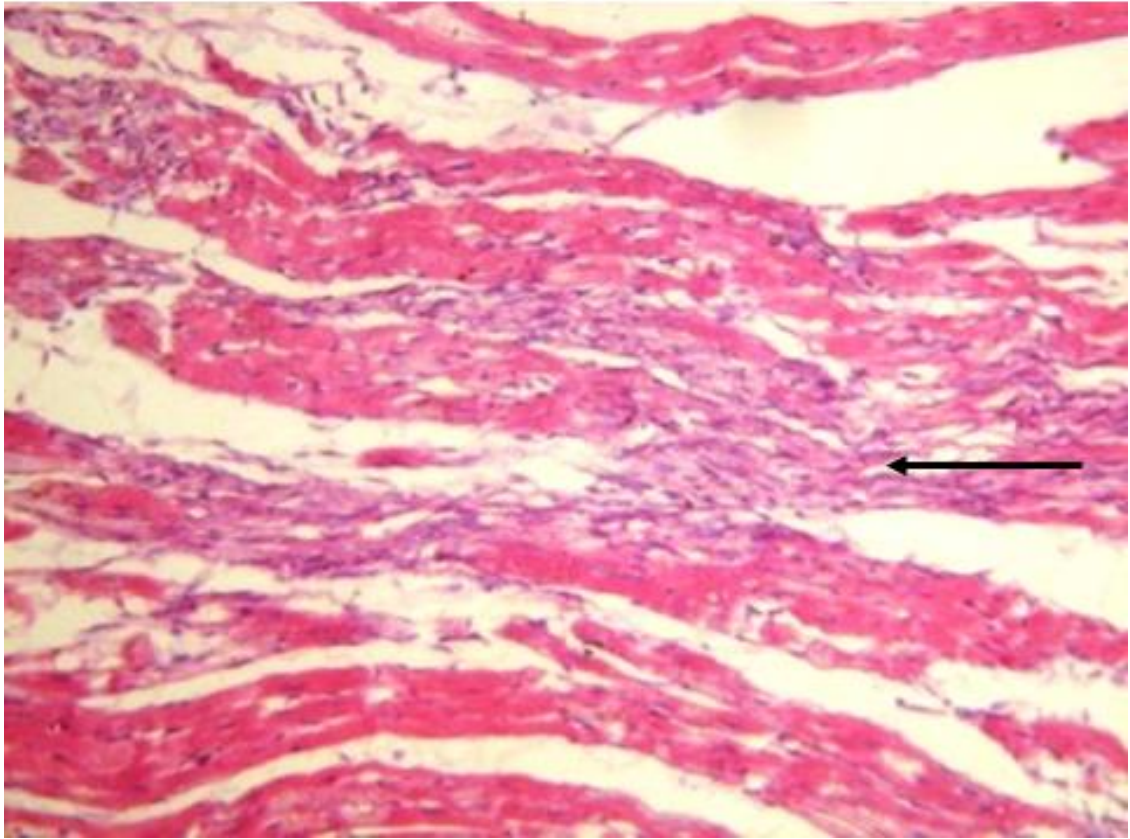
Figure 3.16g: The heart section of rat posttreated with 200 mg/kg (group G) of EEAP

showing mild infiltration of inflammatory cells (mag x 100).

3.4.8 Immunohistochemistry of cardiac troponin in heart tissue of myocardial infarction caused by isoproterenol.

Group A (Control): show positive and low expression of cTn, group B (ISO): shows more expression of cTn than control, group C (enalapril,), group D (100 mg/kg+ISO), group E (200 mg/kg + ISO), group F (400 mg/kg +ISO) and group G (ISO+200 mg/kg) exhibited lower expression of cTn than group B (ISO). (Figure 3.17 a-g)
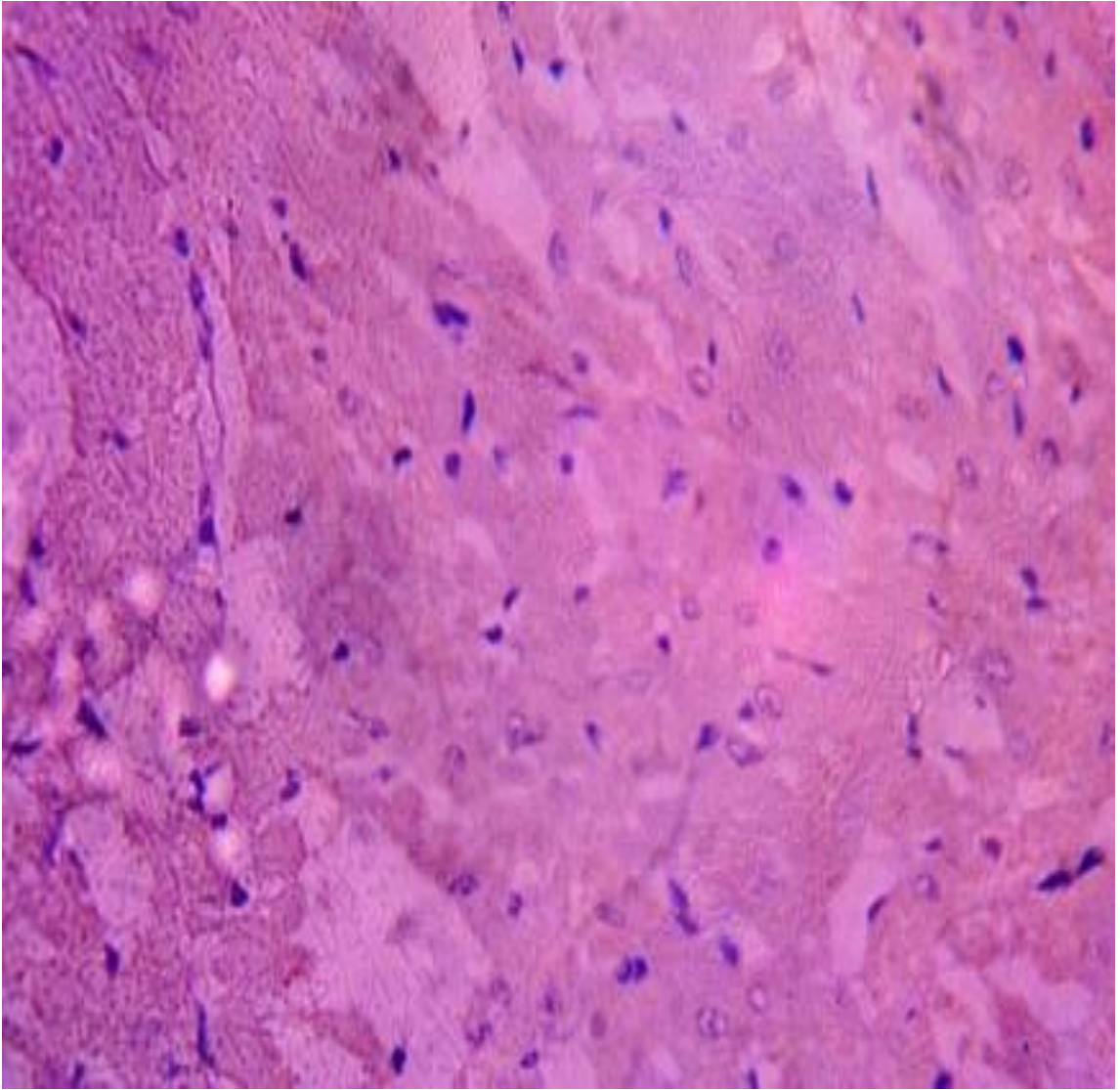
Figure 3.17a: Immunohistochemistry of cardiac troponin in heart tissue

Control (group A) shows low expression of cTn
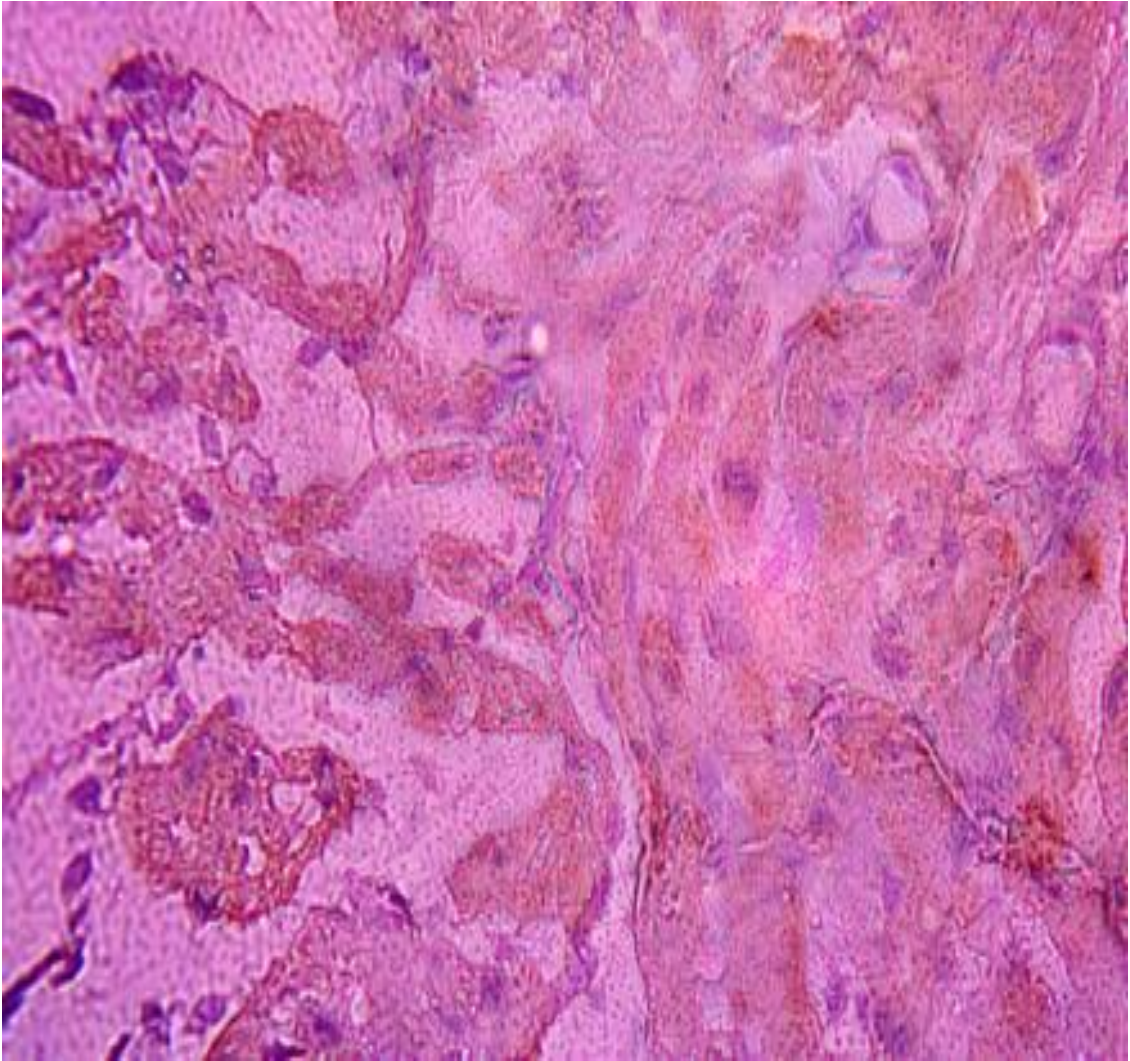
Figure 3.17b: Immunohistochemistry of cardiac troponin in heart tissue

ISO only (group B) shows higher expression of cTn than control.
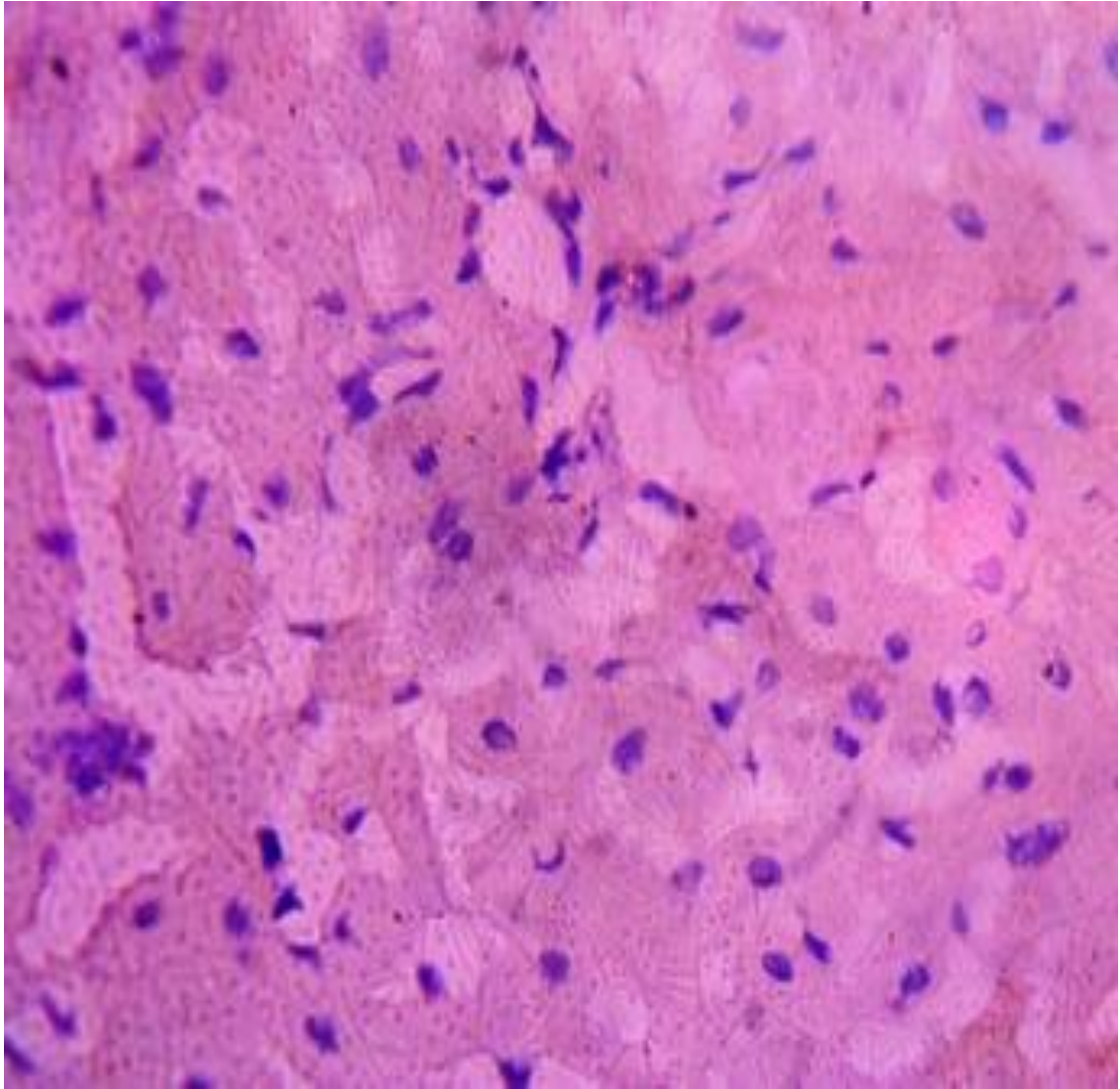
Figure 3.17c: Immunohistochemistry of cardiac troponin in heart tissue

Enalapril + ISO (group C) shows lower expression of cTn
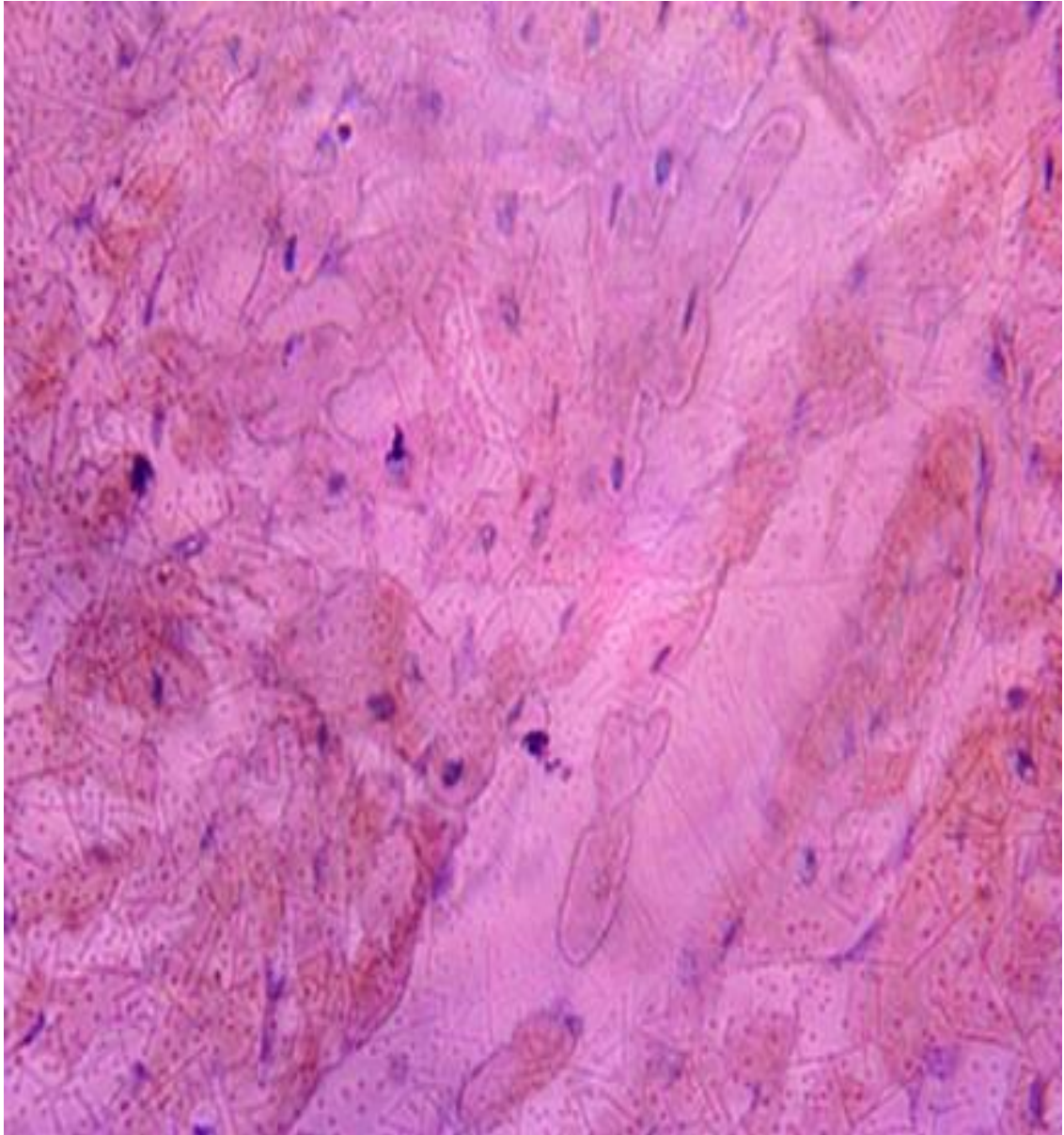
Figure 3.17d: Immunohistochemistry of cardiac troponin in heart tissue

100 mg/kg EEAP + ISO (group D) shows lower expression of cTn.
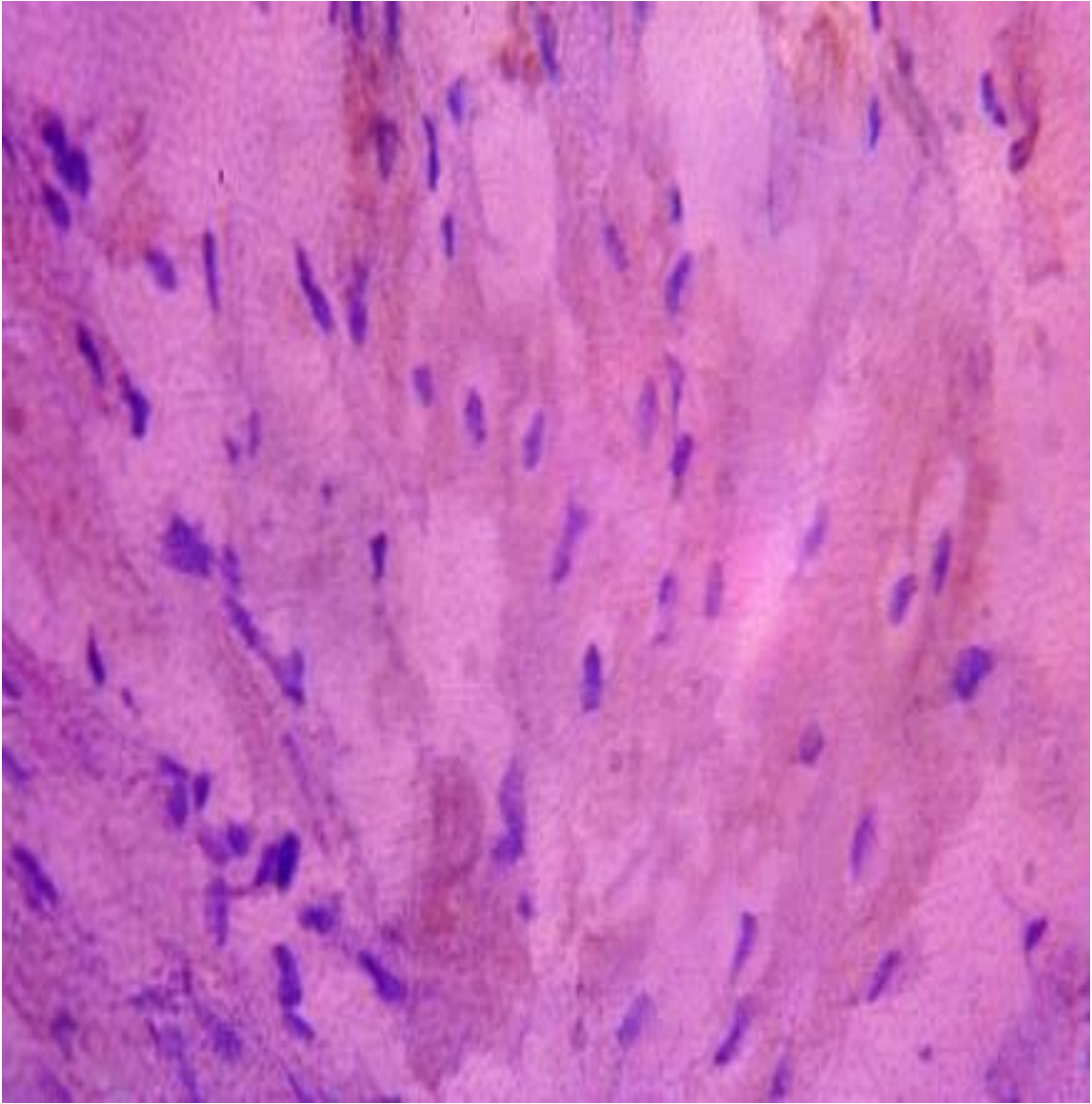
Figure 3.17e: Immunohistochemistry of cardiac troponin in heart tissue

200 mg/kg EEAP + ISO (group E) show lower expression of cTn.
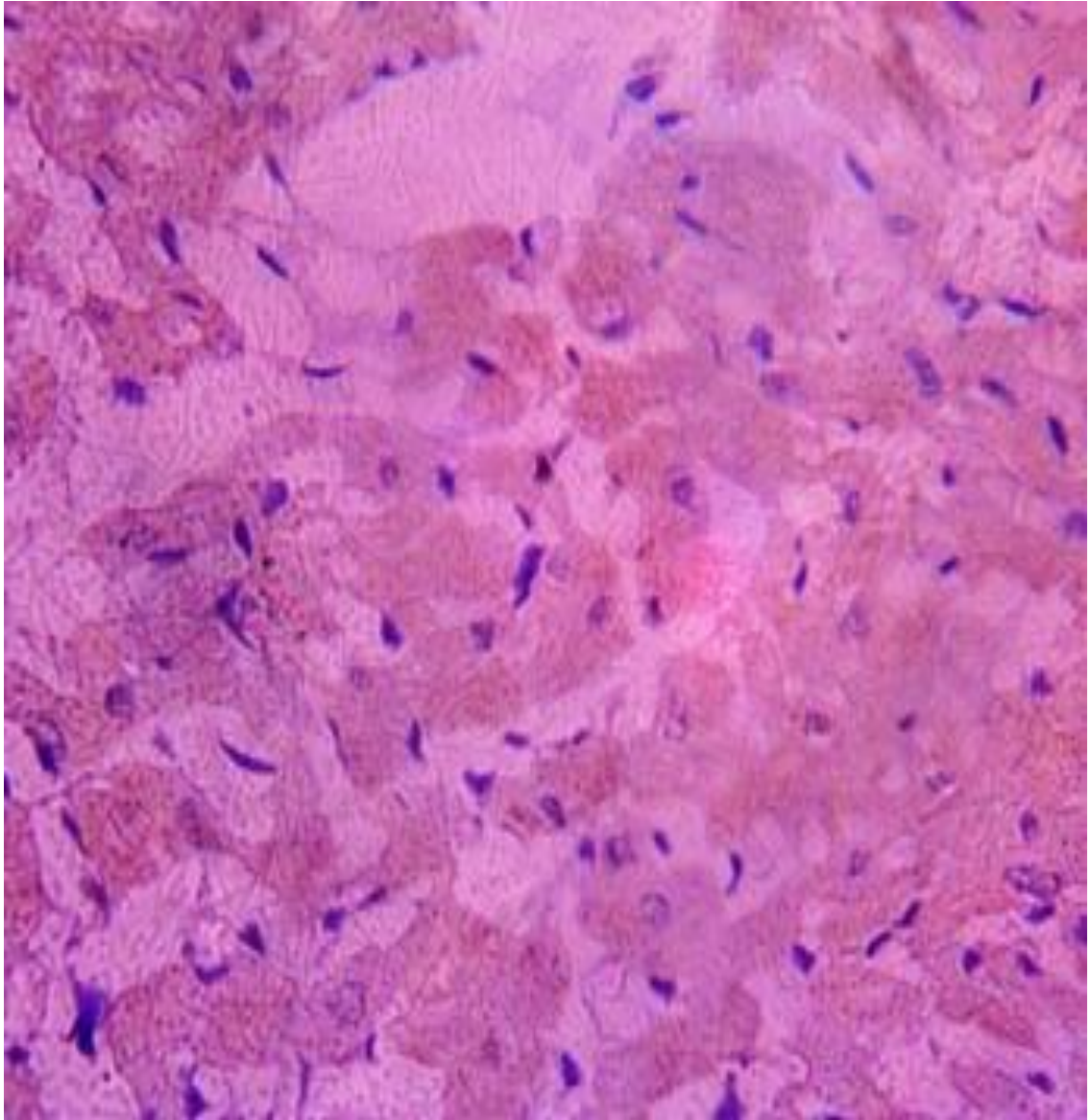
Figure 3.17f: Immunohistochemistry of cardiac troponin in heart tissue

400 mg/kg EEAP +ISO (group F) shows lesser expression of cTn.
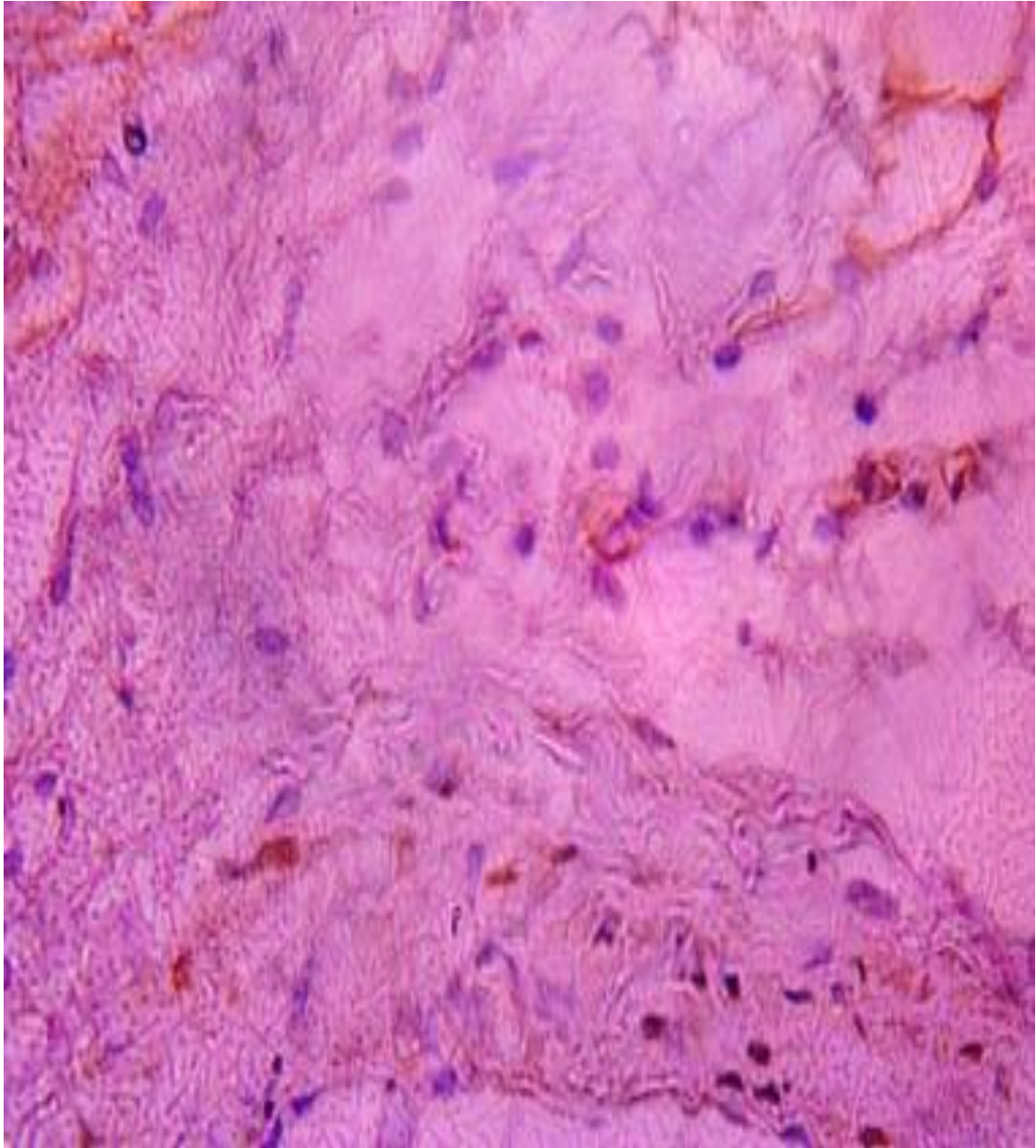
Figure 3.17g: Immunohistochemistry of cardiac troponin in heart tissue

ISO+200 mg/kg EEAP (group G) shows lesser expression of cTn.

3.4.9 Immunohistochemistry of C-reactive protein in heart tissue of myocardial infarction caused by isoproterenol.

Group A (control) show low expression of CRP, group B (ISO): Higher expression of CRP than group A, group C (enalapril,), group D (100 mg/kg+ISO), group E (200 mg/kg + ISO), group F (400 mg/kg +ISO) and group G (ISO+200 mg/kg) exhibited lower expression of CRP than group B (ISO) (Figure 3.18 a-g)
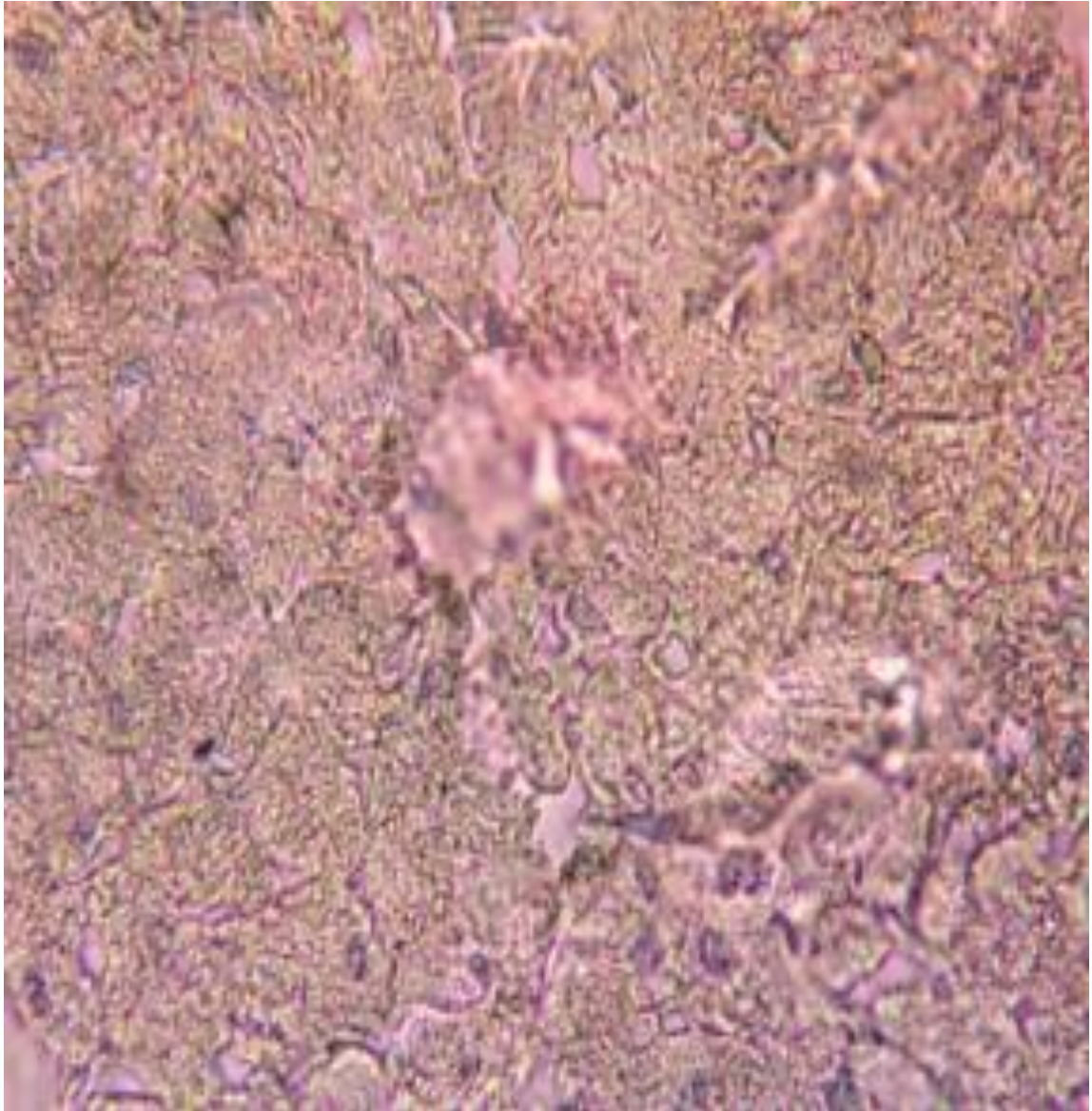
Figure 3.18a: Immunohistochemistry of C- reactive protein in heart tissue

Control (group A) shows Positive and low expression of CRP
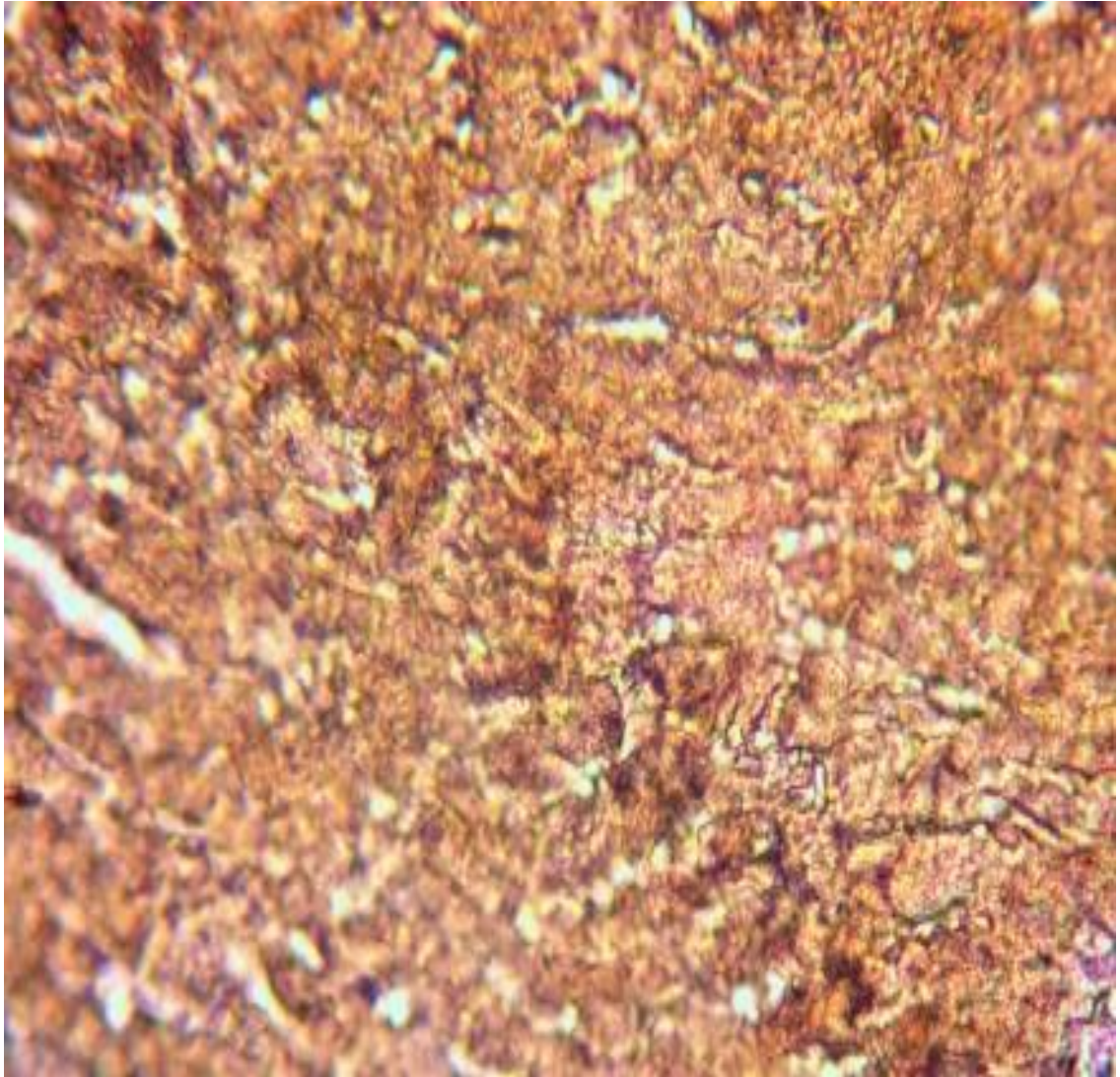
Figure 3.18b: Immunohistochemistry of C- reactive protein in heart tissue

ISO only (group B) shows Higher expression of CRP
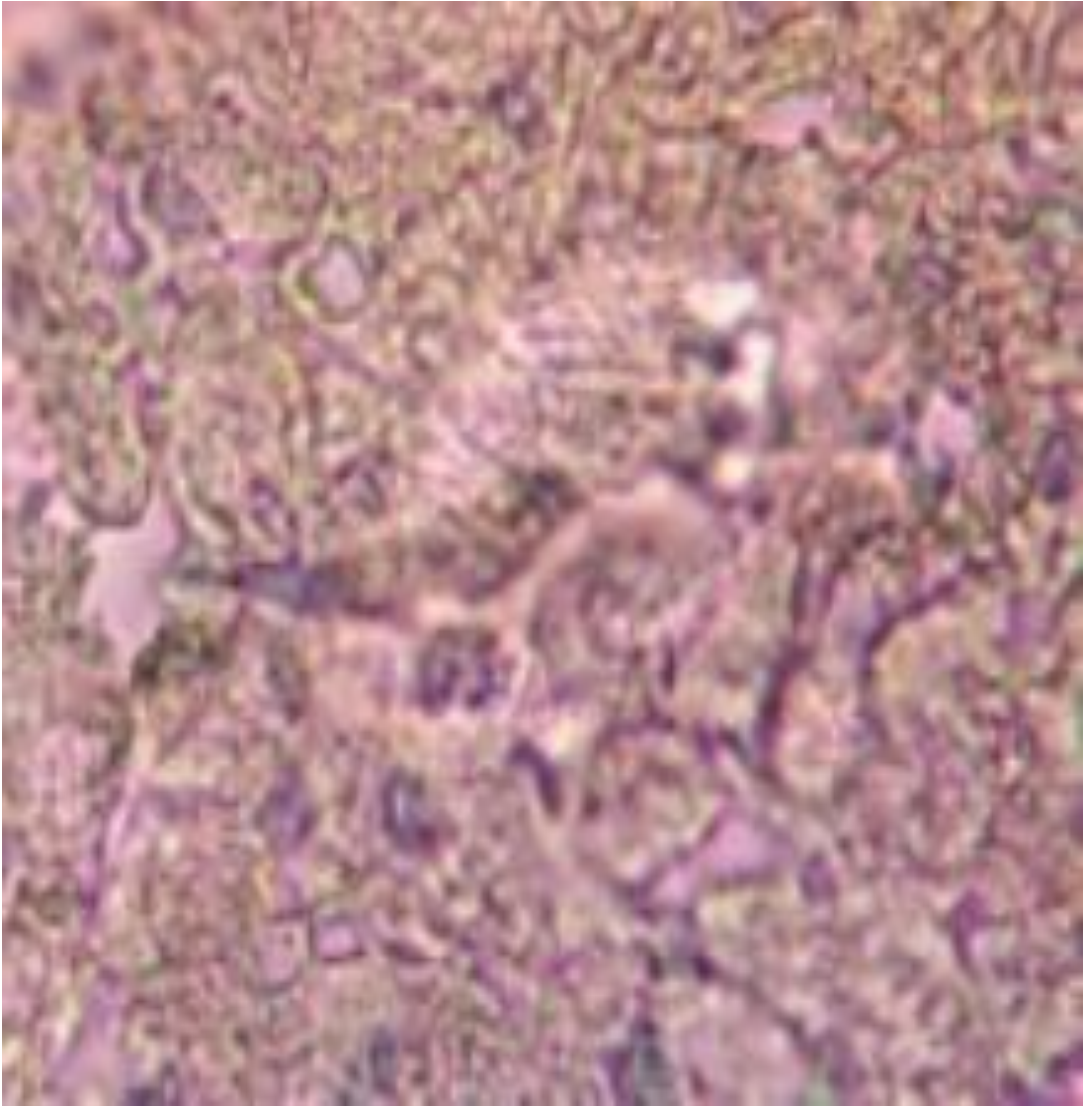
Figure 3.18c: Immunohistochemistry of C- reactive protein in heart tissue

10 mg/kg ENA+ ISO (group C) shows lower expression of CRP.
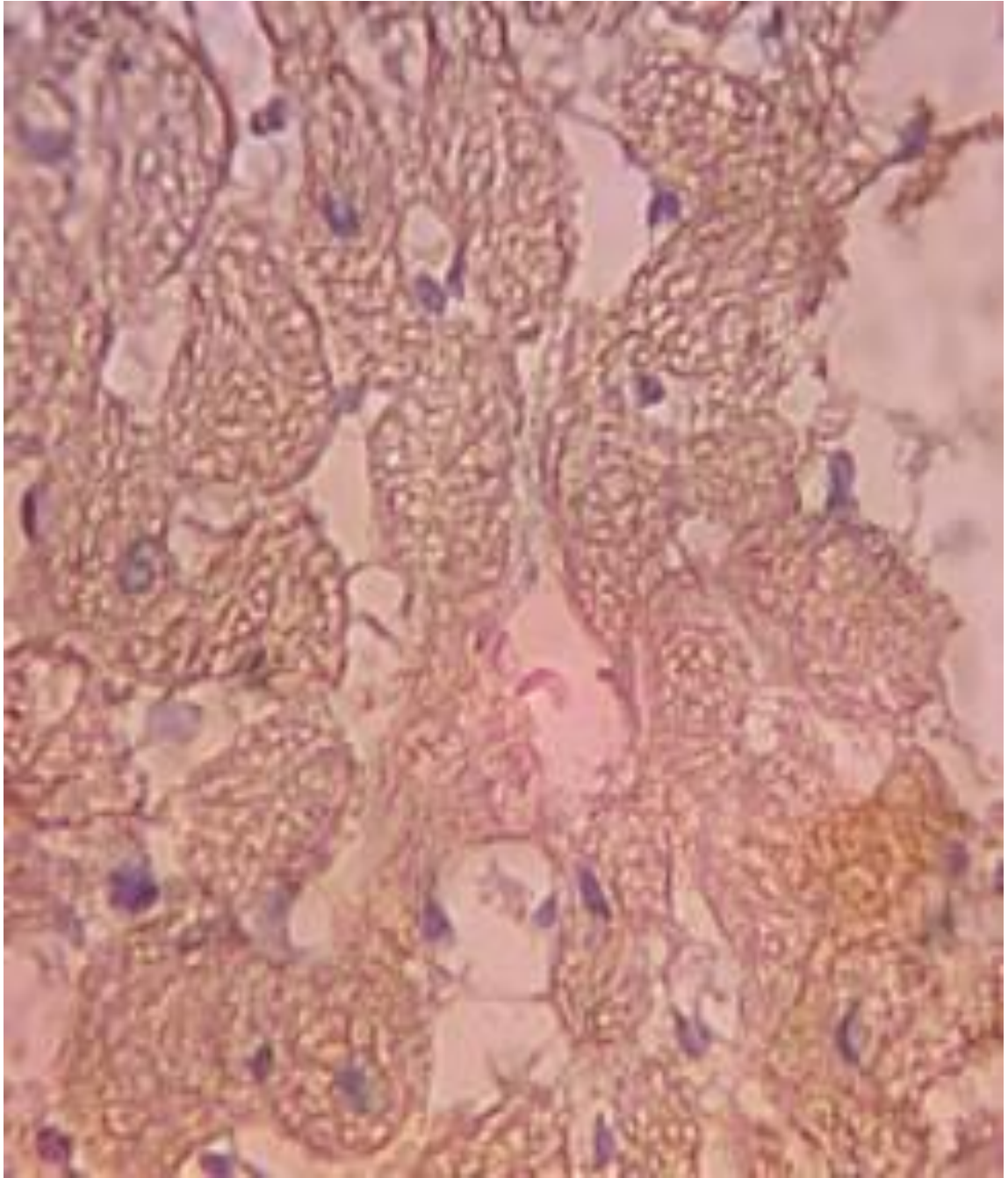
Figure 3.18d: Immunohistochemistry of C- reactive protein in heart tissue

100 mg/kg EEAP+ISO shows lower expression of CRP.
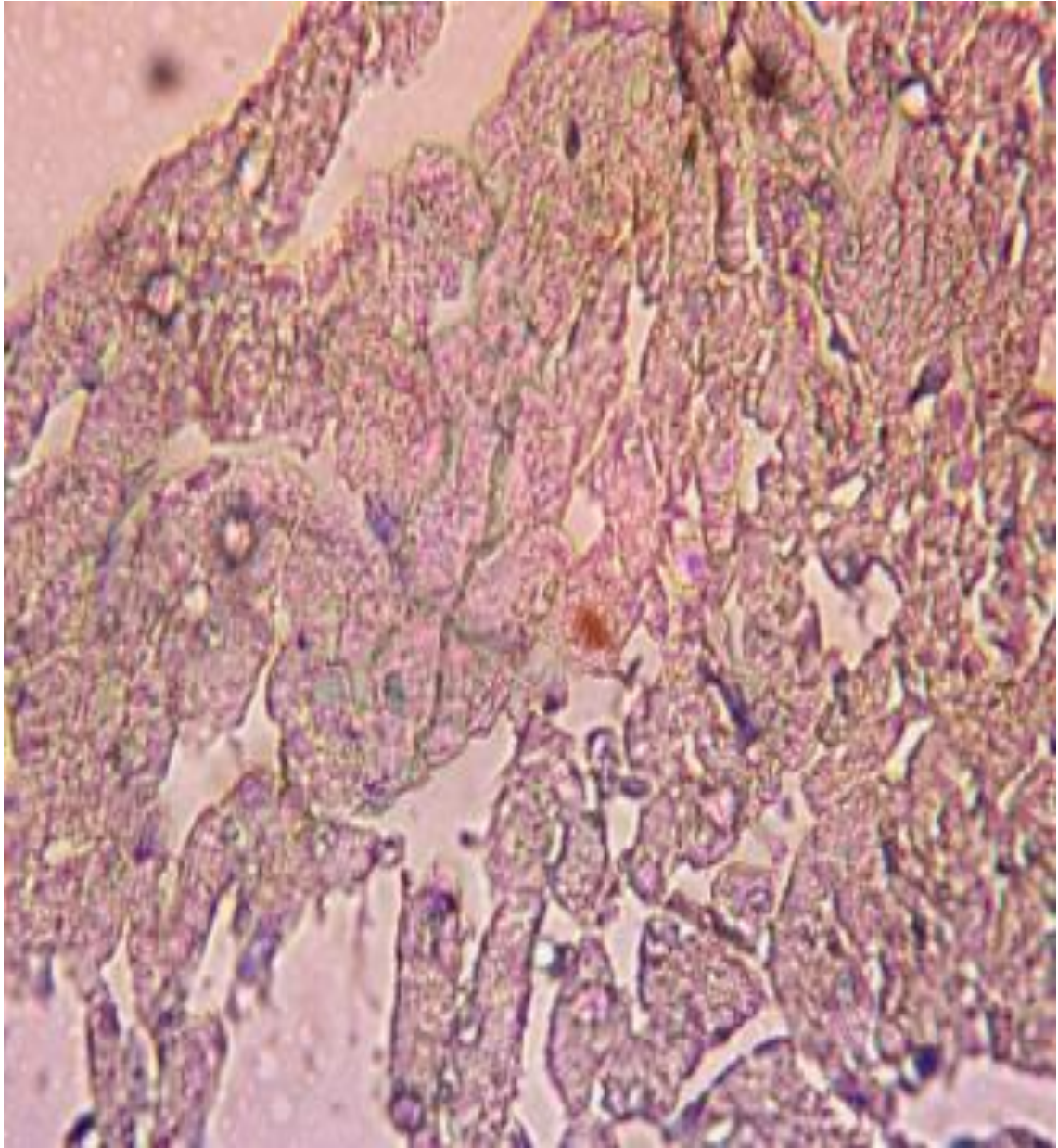
Figure 3.18e: Immunohistochemistry of C- reactive protein in heart tissue.

200 mg/kg EEAP + ISO shows lower expression of CRP.
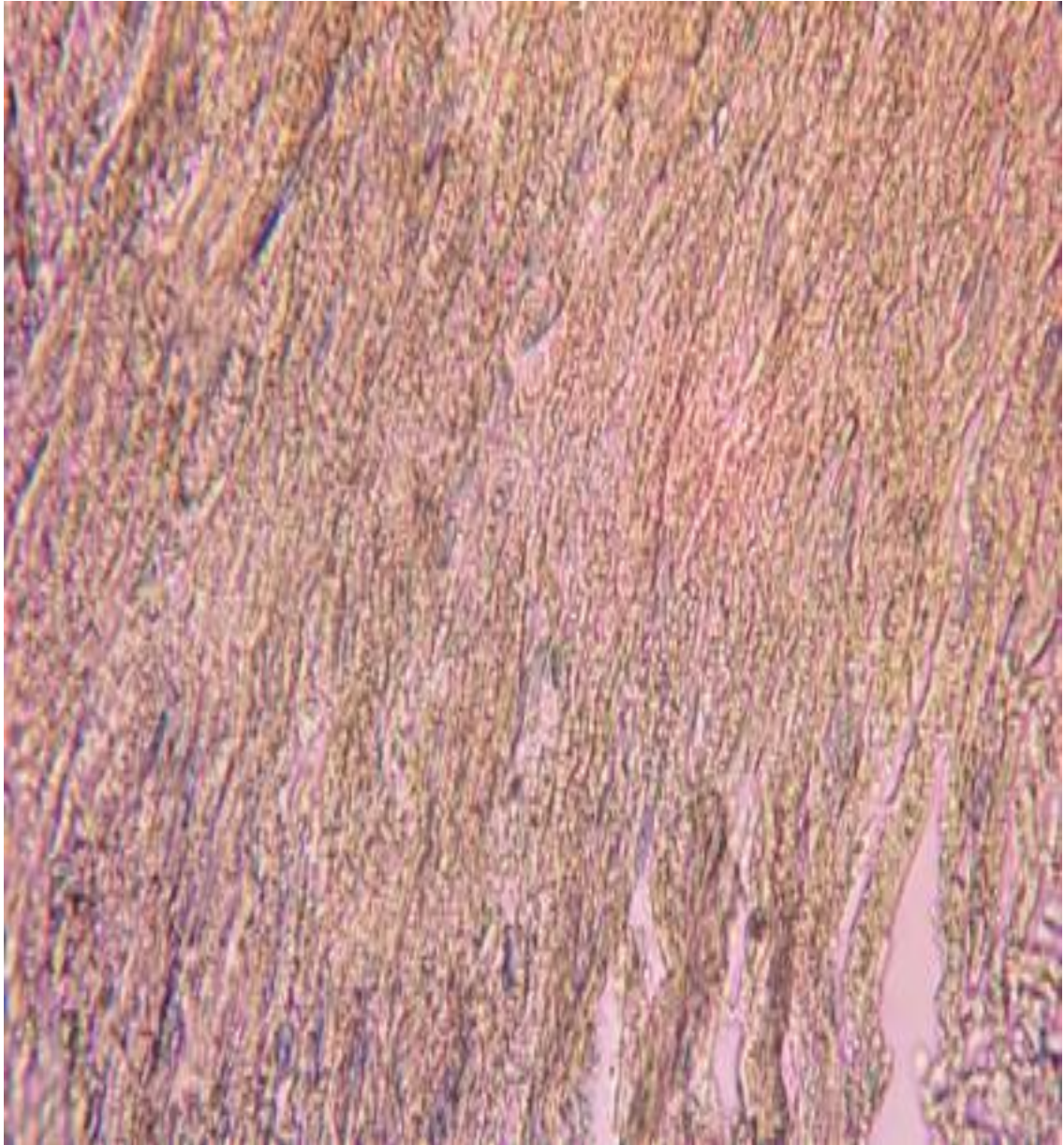
Figure 3.18f: Immunohistochemistry of C- reactive protein in heart tissue.

400 mg/kg EEAP +ISO shows lower expression of CRP.
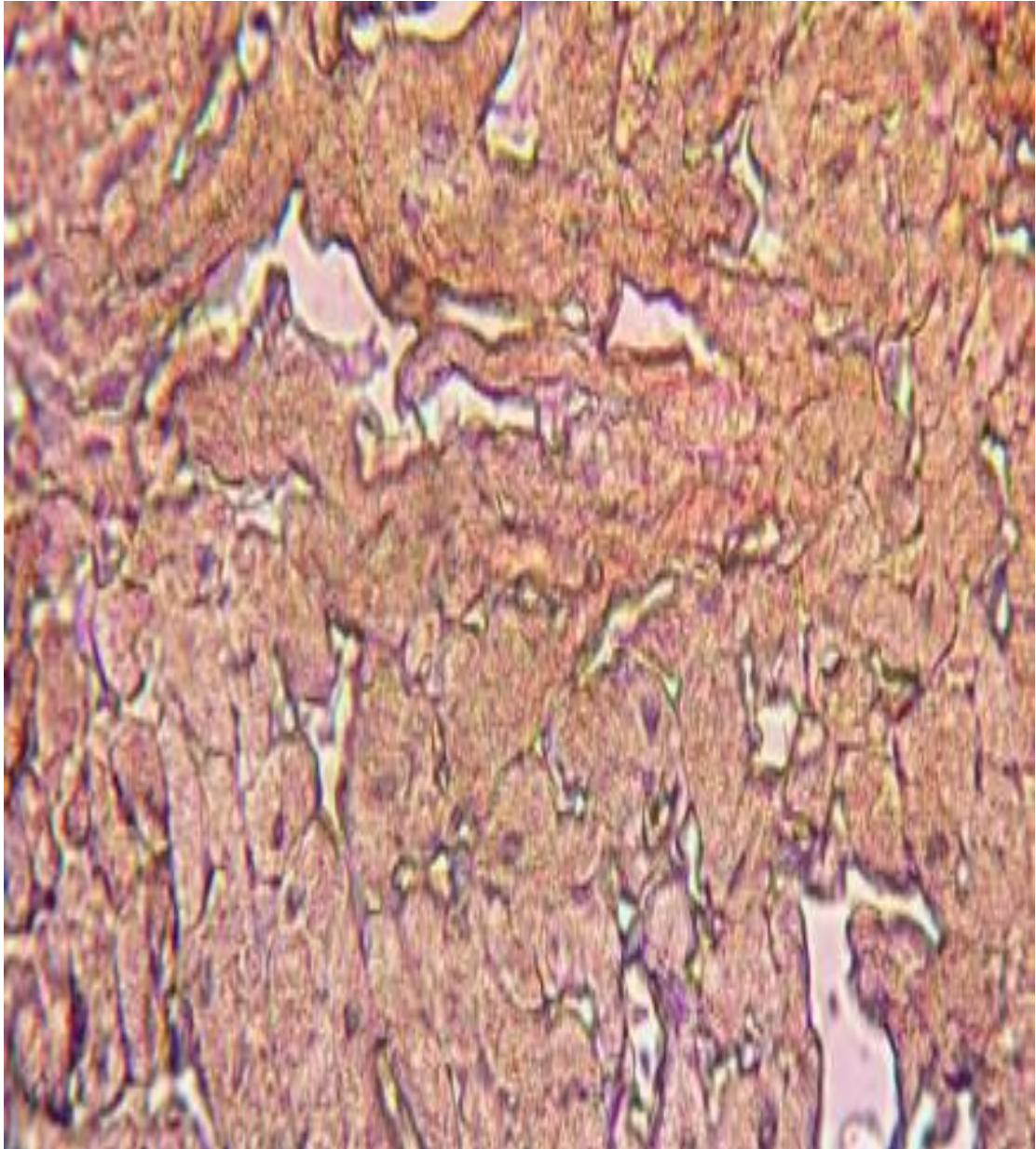
Figure 3.18g: Immunohistochemistry of C- reactive protein in heart tissue

ISO+200 mg/kg EEAP shows lower expression of CRP

3.4.10 Immunohistochemistry of interleukin-10 in heart tissue of myocardial infarction caused by isoproterenol.

Group A (control) show more expression of IL-10, group B (ISO): lower expression of IL-10 than group A, group C (enalapril,), group D (100 mg/kg+ISO), group E (200 mg/kg + ISO), group F (400 mg/kg +ISO) and group G (ISO+200 mg/kg) exhibited higher expression of IL-10 than group B (ISO) (Figure 3.19 a-g).
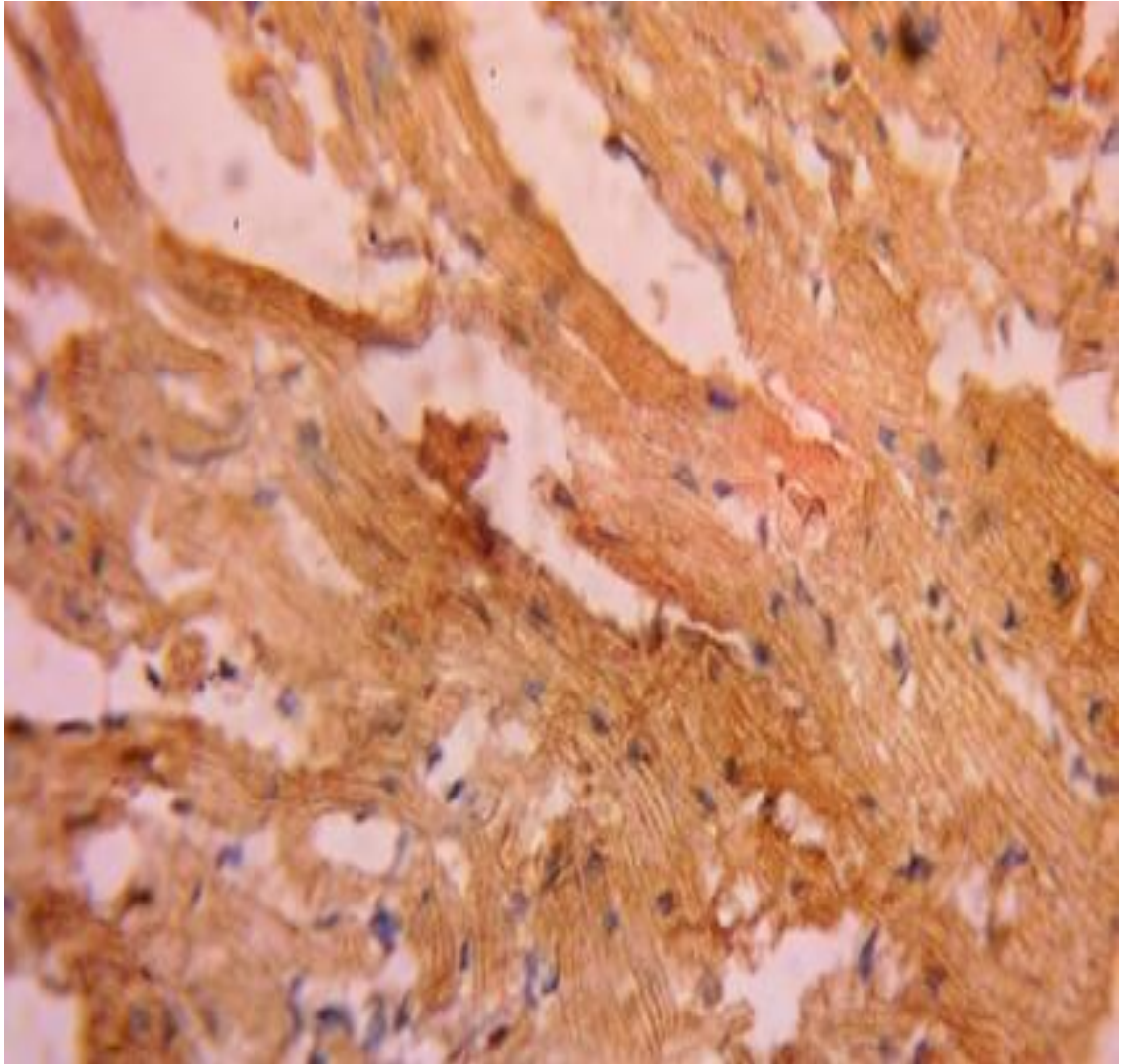
Figure 3.19a: Immunohistochemistry of interleukin-10 in heart of isoproterenol induced myocardial infarction rat

Control (group A) shows positive and higher expression of IL-10.
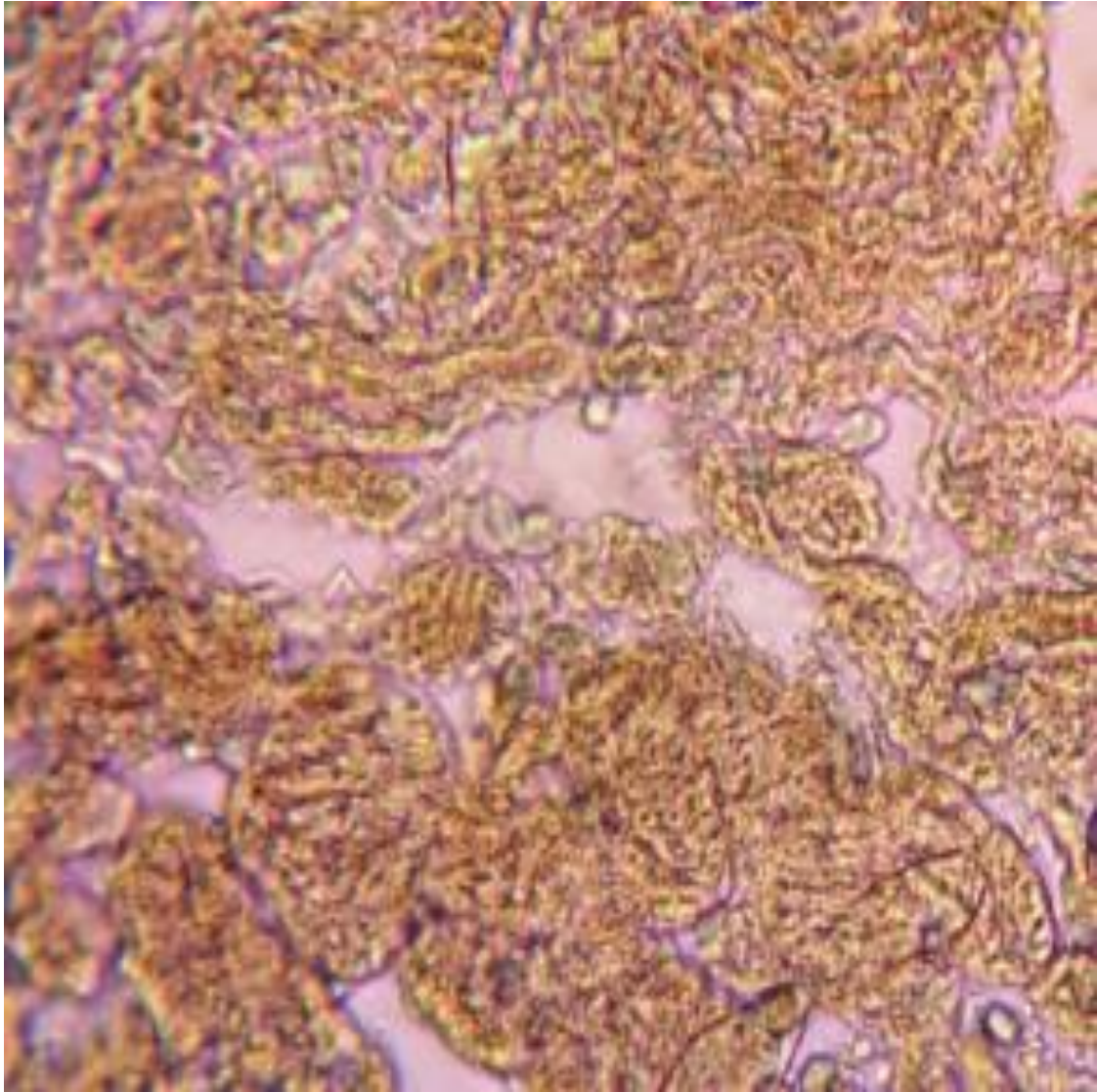
Figure 3.19b: Immunohistochemistry of interleukin-10 in heart of isoproterenol induced myocardial infarction rat.

ISO only (group B) shows lower expression of IL-10.
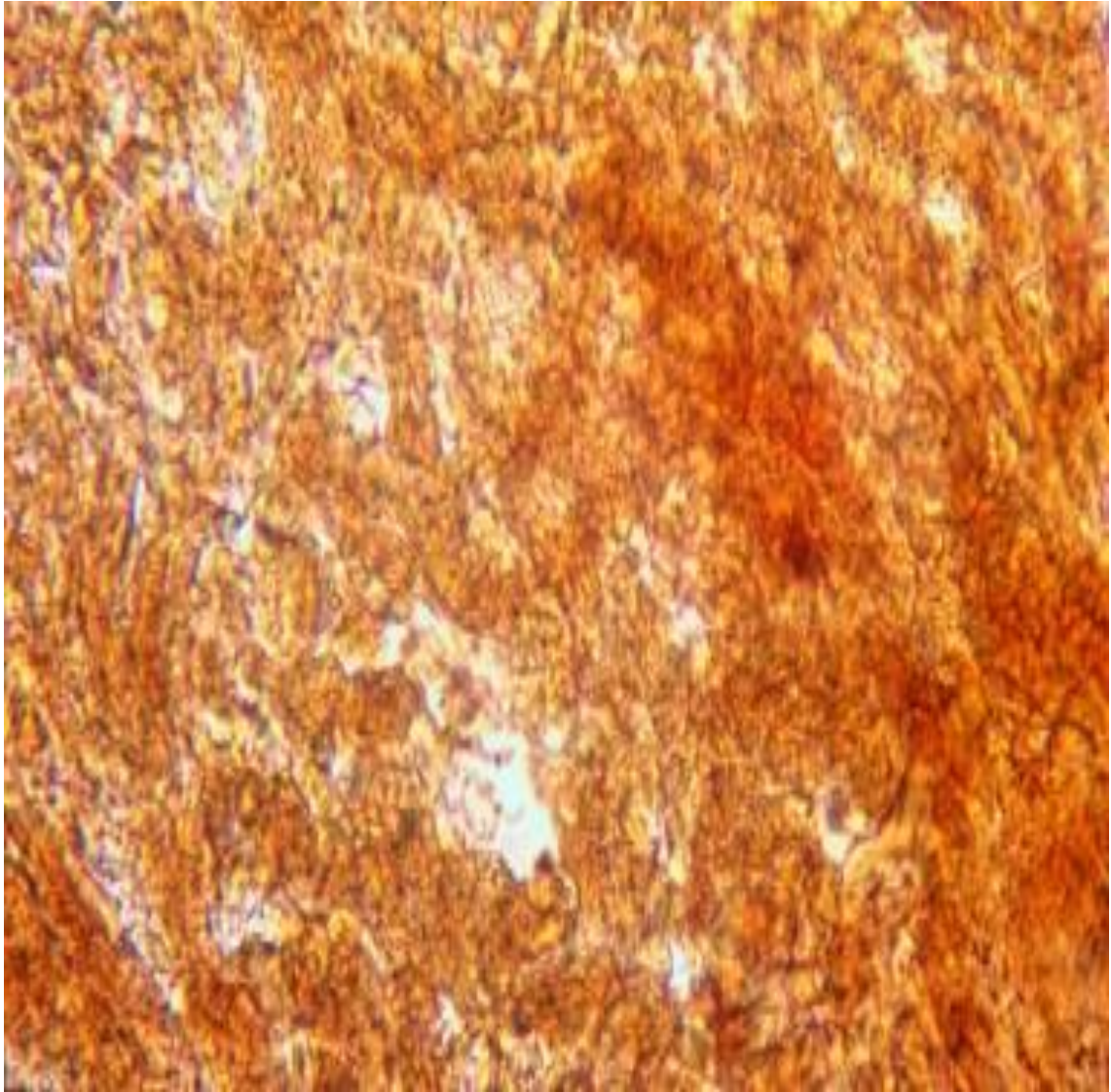
Figure 3.19c: Immunohistochemistry of interleukin-10 in heart of isoproterenol induced myocardial infarction rat.

10 mg/kg ENA + ISO shows higher expression of IL-10.
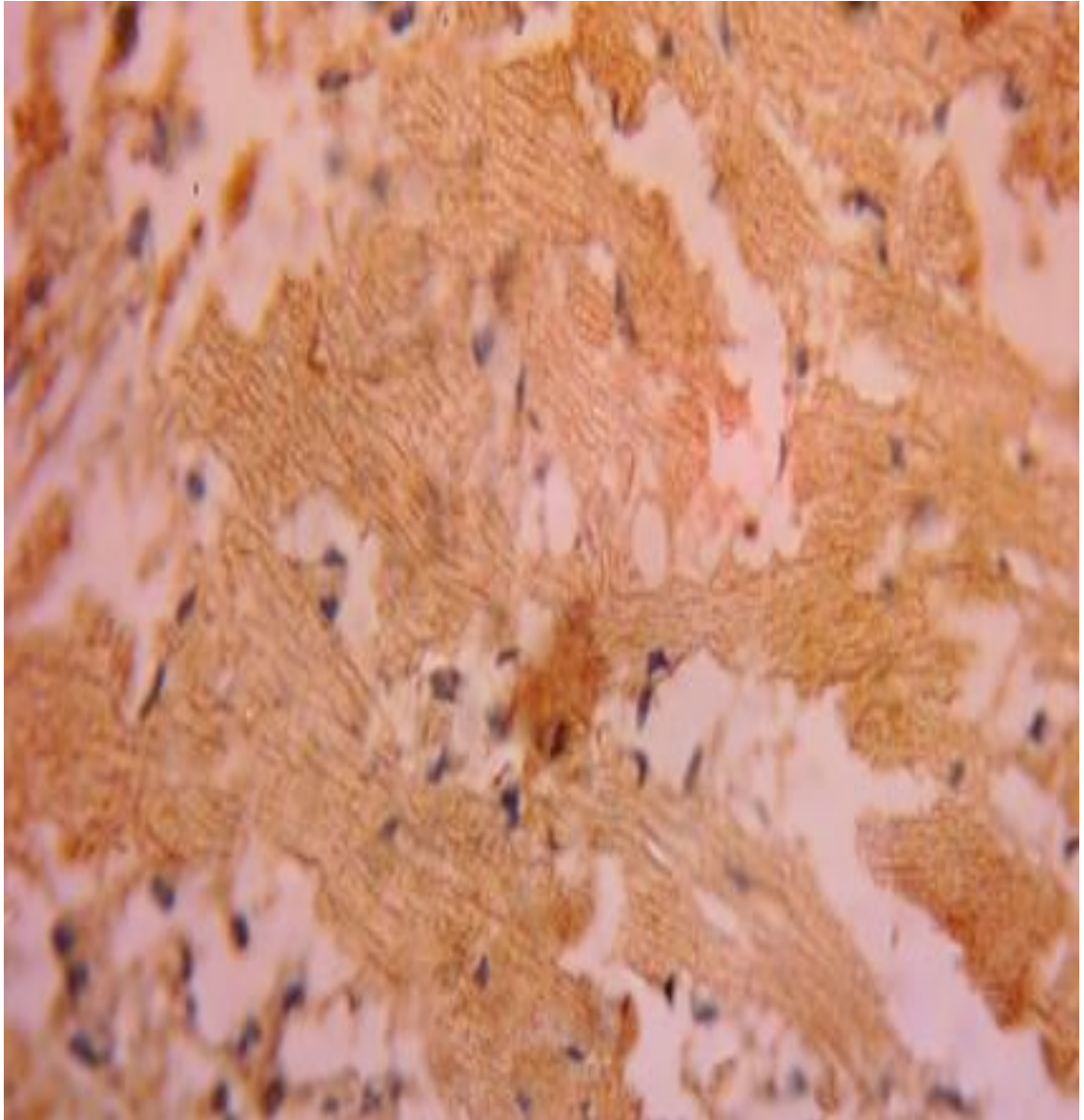
Figure 3.19d: Immunohistochemistry of interleukin-10 in heart of isoproterenol induced myocardial infarction rat.

100 mg/kg EEAP + ISO shows higher expression of IL-10.
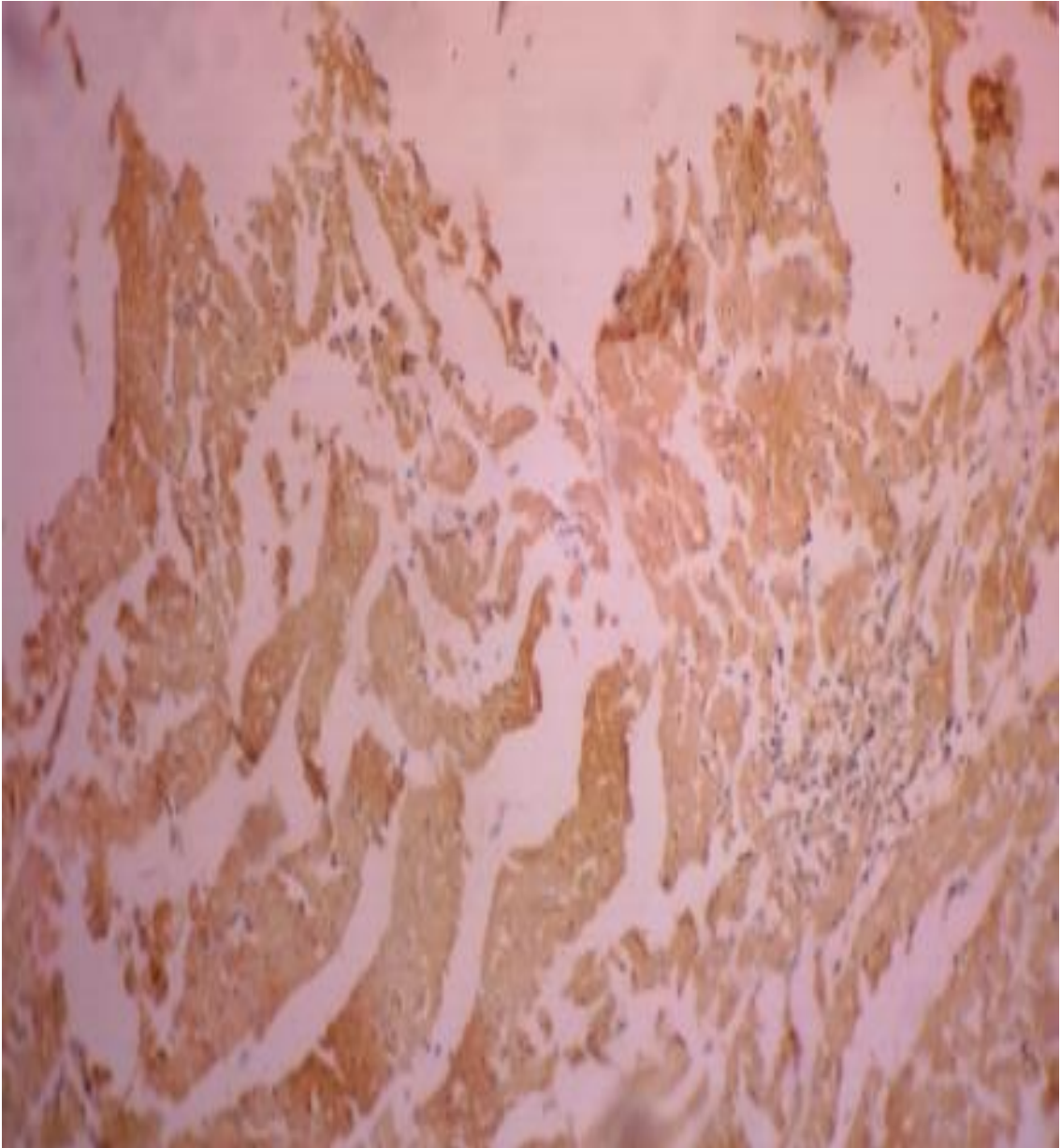
Figure 3.19e: Immunohistochemistry of interleukin-10 in heart of isoproterenol induced myocardial infarction rat

200 mg/kg EEAP + ISO shows higher expression of IL-10.
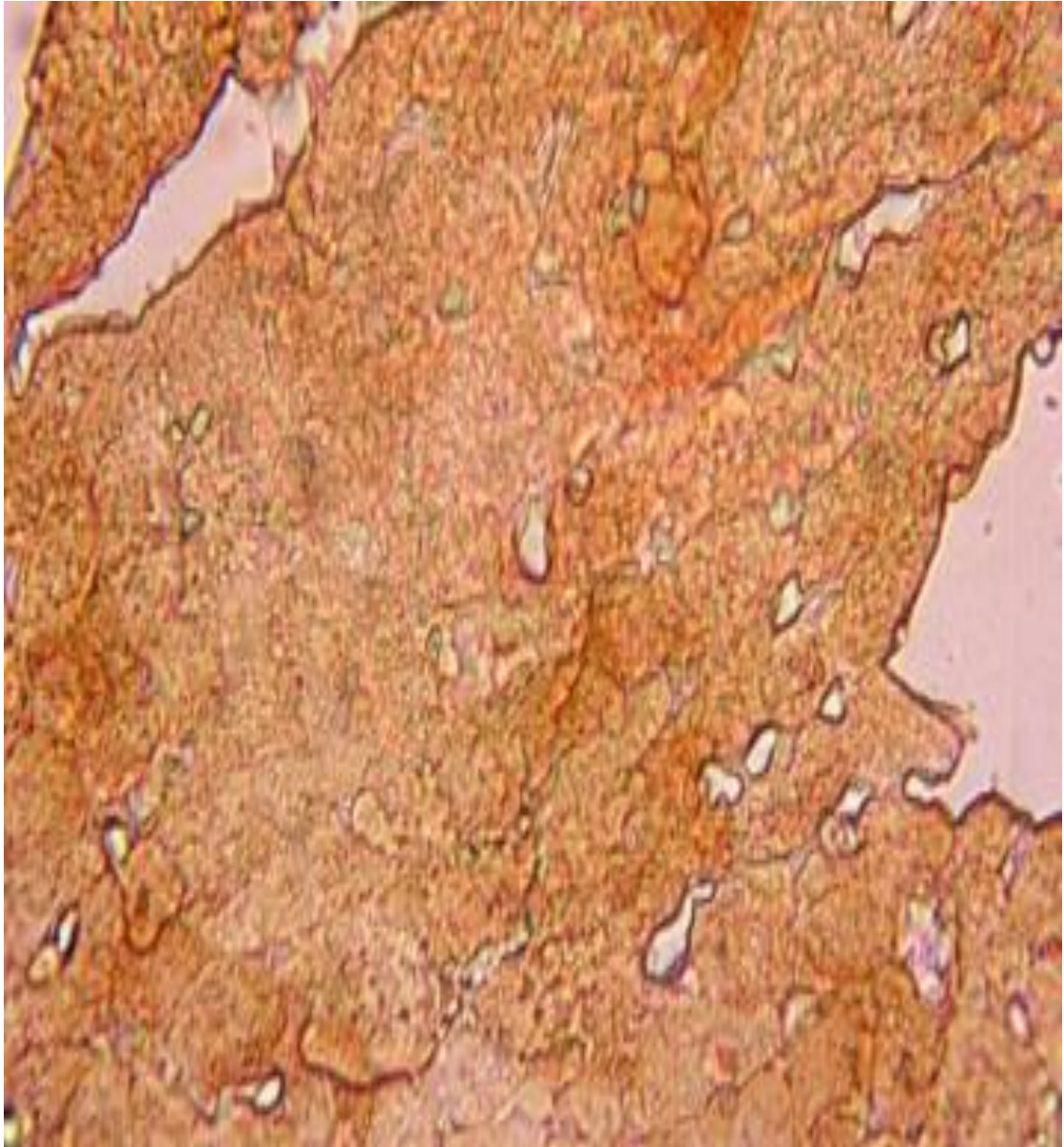
Figure 3.19f: Immunohistochemistry of interleukin-10 in heart of isoproterenol induced myocardial infarction rat.

400 mg/kg EEAP + ISO shows higher expression of IL-10.
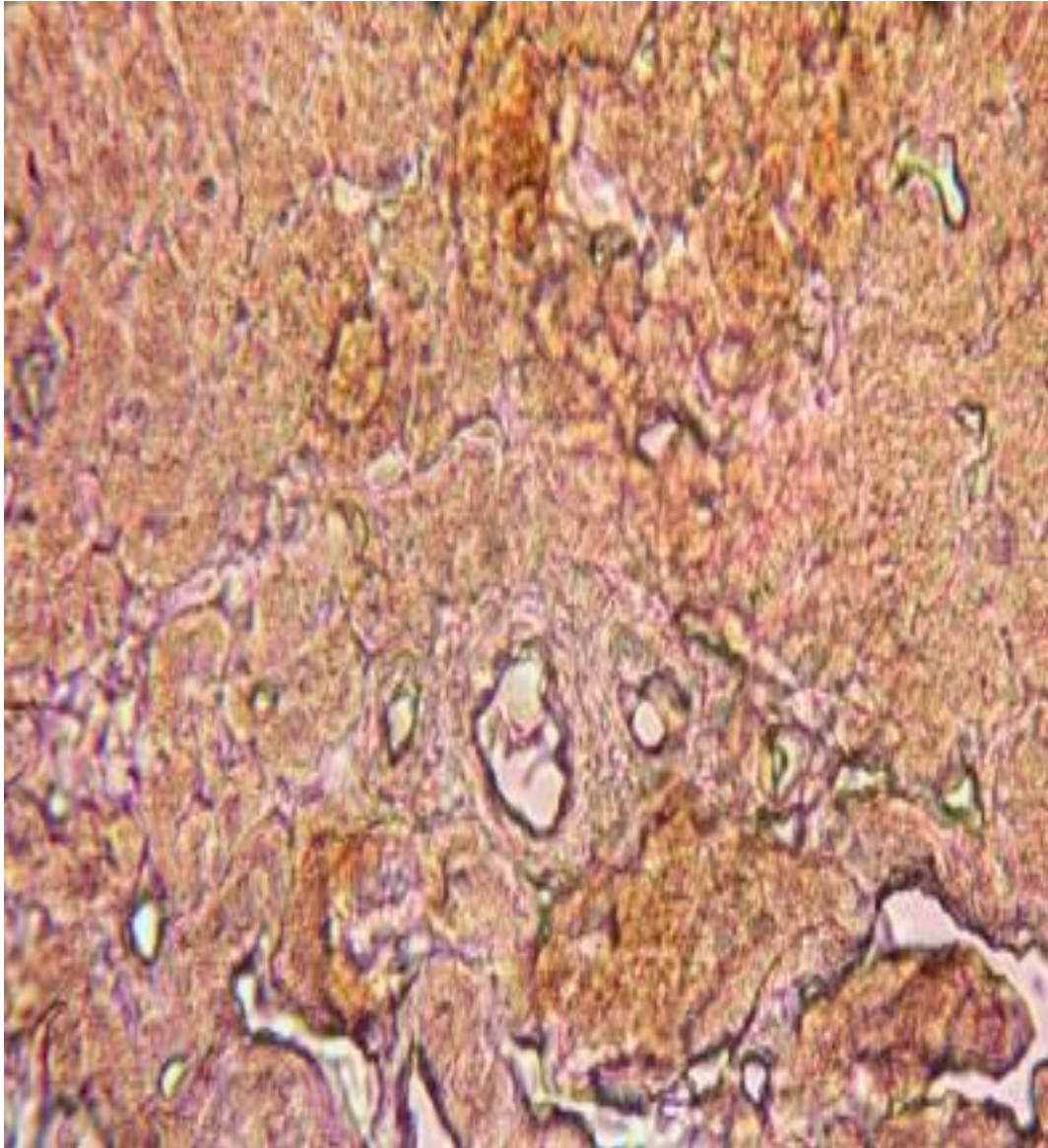
Figure 3.19g: Immunohistochemistry of interleukin-10 in heart of isoproterenol induced myocardial infarction rat.

ISO +200 mg/kg EEAP (group G) shows higher expression of IL-10.

3.5   Discussion

Andrographolide, the functioning constituent of *Andrographis paniculata* medicinal properties have been reported (Tan *et al.*, 2016). Enalapril (ENA) possesses antioxidant properties and the potential to remove free radicals (Taskin *et al*., 2016). Enalapril was used as a reference drug in this study due to its use in the treatment of cardiovascular ailments. Enalapril might have proffered its ability by down regulating free radical production.

Haemodynamic parameters were determined in this experiment to know the protective effects of EEAP on cardiac condition. It is believed that supramaximal dosages of isoproterenol caused ventricular dysfunction and impediment of diastolic and systolic functions, comparable to those pathophysiological changes in myocardial infarction patients (Gupta *et al.*, 2004). Isoproterenol also produced an aggressive myocardial necrosis with a profound reduction in blood pressure parameters. The reduction in the levels of systolic diastolic and mean arterial pressure caused by isoproterenol could lead to coronary hypotension as recorded in this study. In a study by Owens and O'Brien (1999), it was established that patients affected by cardiovascular disease and hypotension have symptomatic and ischaemia occurred in a temporally causal relation with hypotension, especially for diastolic pressures. It thus suggests that low blood pressure seen in patients with coronary disease may be due to ischaemic events. This experiment revealed that enalapril and EEAP meaningfully blocked isoproterenol-induced cardiac abnormalities.

In this study, prolong QT interval was noted in isoproterenol induced rat. Enalapril and treatment with EEAP inhibited isoproterenol induced QT interval elongation, indicative of its cell membrane defensive properties. In this present experiment, no noteworthy variance in heart rate was observed among all experimented rat groups.

The results of haematological analysis also showed that isoproterenol resulted in significant increase levels of WBC and PCV while enalapril caused significant decrease in WBC and no different in other parameters compare to isoproterenol treated only. The increase in the level of WBC could be explained in terms of necrosis caused by this agent leading to white blood cell mobilization (Khalil *et al*., 2015). The reduction in the level of these parameters by enalapril could also be due to its ability to negate the toxic effect of isoproterenol. EEAP caused a significant increase in WBC

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and this could be due to immunostimulant effect of the extract. Puri *et al.* (1993) was of the view that increase WBC level may be link to the effect of andrographolide that possess immunostimulatory properties.

Oxidative stress that results in necrotic damage in the myocardium of rats can be induced by using supramaximal dose of isoproterenol (Rona, 1985). There is decrease in SOD, GST and GPx in isoproterenol only treated group in agreement to earlier study by Dianita *et al.,* (2015). GPx, GST and SOD, evaluated in this study were increased in enalapril treated rats. This view is clearly supported by Chandran *et al.* (2014), where it was established that enalapril has anti-oxidative property and this may be responsible for its cardioprotective property. As seen in rats treated with EEAP, there is increased activity of these enzymes, which strongly suggested that EEAP has the capability to check the deleterious properties of free radicals in isoproterenol-induced rats in agreement Sivakumar and Rajeshkumar (2015) who reported that AP enhanced antioxidant enzymes.

Reduce glutathione, protein thiol and non-protein thiol are important non-enzymatic antioxidant, for maintaining cellular integrity because of its reducing properties (Hadi *et al*., 2016). GSH and thiols function to serve as the reductant of toxic free radicals and it also helps in keeping the enzymes in functional state, by preventing the turning of sulfhydryl group to disulfide group (Halliwell and Gutteridge, 1989). When there is deficiency or depletion of this enzyme, it causes damage to the macromolecules or membranes. The deficiency of GSH produced by isoproterenol shows its interaction with biomembrane and subsequent peroxidizing action. The inhibitory action on depletion of GSH levels by the enalapril (10 mg/kg), pretreatment and post treatment with EEAP may be as a result of the antioxidant property of EEAP and enalapril. This was earlier reported by Hassan *et al.* (2017).

In the pathogenic progression of myocardial necrosis, lipid peroxidation is very essential. Increased level of MDA, one of the lipid peroxidation end products, reflects the intensity of damage of cardiac constituents (Khalil *et al*., 2015). In this present study, there is increase in MDA level in isoproterenol treated group and Zhou *et al*., (2006) reported that isoproterenol mediated myocardial infarction might be through stimulation of free radical-mediated fatty acid peroxidation. The results in this study

showed that enalapril and EEAP could drop isoproterenol-induced MDA level elevation.

After the onset of injury to myocytes as a result of deficient oxygen or energy supply and oxidative stress initiated by isoproterenol, the heart membranes become leaky or rupture and caused escape of enzymes (Selaraj *et al*., 2012). This study showed that isoproterenol caused high MPO activity, indicative of necrosis caused inflammation of cardiac tissue. Use of enalapril and EEAP was established to meaningfully reduce ISO-induced elevation in the activity of MPO. This present study is in agreement with the study by Adedapo *et al*., (2015) who reported anti-inflammatory properties of AP. Also, De Cavanagh *et al.,* (2001) reported that enalapril inhibits free radical formation and attenuates oxidative stress and also averts injury to the liver and kidney

In heart failure, the heart has a decreased ability to supply blood to the body. Many explanation may be offered to this, for instance a heart attack can damage part of the muscular wall and because of that, the delivery of blood to the organs like liver and kidney reduces. In an attempt to raise the blood pressure the kidneys may react by conserving fluid and electrolytes and thus puts more strain on the heart, worsening the state. The fluids in the body accumulate even more. The liver can become dysfunctional, and liver enzymes can be liberated into the blood. The liver enzymes can be released for a number of problems. First, the liver may be receiving an enough supply of blood, thus injuring the cells and secondly, due to heart not functionally pumping blood, blood and fluids can back up into the liver, further injuring the cells (Macfarlane *et al.*, 2000). It thus means that the increased noted for the liver enzymes in this study implied that isoproterenol could impair liver functions. In this study, serum ALT and AST of isoproterenol-induced rats was increased while Enalapril and EEAP had protective potential.

Furthermore, to elucidate the possible mechanism of EEAP on isoproterenol-induced myocardial infarction; serum NO was evaluated. In hypertrophy and cardiac damage NO is known to play an essential part (Zhang *et al.*, 2016). Decrease production of NO may also add to and exacerbate the proliferation of renal diseases by both hemodynamic and renal growth-promoting actions. These study demonstrated that there is increased level of NO in serum of animals treated with enalapril and EEAP

treated rats. Previous report on eNOS-overexpressed mouse model had proved that released NO can reduced isoproterenol-induced hypertrophy (Ozaki *et al.*, 2002).

There is increase in exhibition of cTn in cardiac tissue of isoproterenol-induced rats. This increase in troponin expression predicts the risk of cardiac damage following infarction. Earlier report by Othman *et al.* (2017) supported this. Enalapril and EEAP treatment decreased expression of cTn. Down regulation of cardiac troponin by enalapril and EEAP showed their potential to protect against myocardial injury in rats. This could be as result of due to the lower level of injury in the myocardium by enalapril and EEAP.

C-reactive protein level increased in myocardial necrosis caused an inflammatory reaction (Balbay *et al.*, 2001). Previous study by researchers established that the level of C-reactive protein increased in myocardial infarction. C-reactive protein has been implicated with greater risk of myocardial infarction and potentially vital device in basic prevention of cardiovascular diseases thus it is as a marker of systemic inflammation and tissue damage (Oh *et al.*, 2017). Acute phase protein that was first discovered was C-reactive protein to be described (Rosselli *et al.*, 1998). In this study, there is up-regulation of C-reactive protein in isoproterenol treated group and this established the hypothesis that myocardial damage occurred as result of pro-inflammatory cytokines. It is a recognised fact that tissue necrosis can potentially stimulate acute-phase. This response in myocardial infarction is indicating the extent of necrosis as a major response (De Beer *et al.*, 1982). In acute myocardial infarcts, C-reactive protein is co-deposited with activated complement. Research evidence has shown that the C-reactive protein response indicate tissue damage and complicate myocardial injury (Griselliet *et al.*, 1999). In the pathogenesis of cardiovascular disease, C-reactive protein role has been established and it as been used as a predictor and marker of cardiovascular disease. In a reviewed by Prasad (2006) it was concluded that therapeutic agent such as antioxidants (vitamin E); platelet aggregation inhibitors (e.g clopidogrel, abciximab); lipid lowering agents (e.g fenofibrate, statins, ezetimibe, niacin; beta-adrenoreceptor antagonists; cyclooxygenase inhibitors (e.g aspirin); and angiotensin converting enzyme inhibitors (e.g aptopril, fosinopril), reduce serum levels of C-reactive protein in health and disease but trandolapril and enalapril effects is yet to be known. The lowering of the level of C-reactive protein in this study by enalapril and EEAP is a pointer to its potent to halt cardiovascular disease. Many

immunological cell types like macrophages or monocytes produced IL-10 cytokine (Akdis and Blaser, 2001). Blockage of cytokine production (e.g., IL-6 TNF-α) and their natural function on aim cells is initiated by IL-10 to produce immunosuppressive effects. An acute lipid infusion is usually accompanied by muscle insulin resistance but simultaneous use of IL-10 prevented such occurrence (Pestka *et al.*, 2004). It has been reported that inflammation, angiotensin II-mediated vascular dysfunction and oxidative stress were decreased by IL-10, elucidating its protective effects in cardiovascular diseases (Kim *et al.*, 2004). During diabetes and atherosclerosis, endothelial and vascular superoxide increases and this blocked by IL-10. In a study, patients having arterial hypertension treated with enalapril have increase plasma concentration of IL-10 (Schieffer *et al.*, 2004). It thus confirmed that cardioprotective ability of enalapril could be linked to its anti-inflammatory property as shown by the up regulation of IL-10. In this study there is increase expression of IL-10 in cardiac tissues of rats treated with EEAP showing its cardioprotective effect.

Isoproterenol-induced myocardial infarction is also manifested by modified histopathological features including marked necrosis, severe infiltration of inflammatory cells, and disorganization of myocardium of myofibrillar loss. This increase in the inflammatory cells may have been responsible for the increase in the levels of WBC noted in this study. In the study on the isoproterenol-induced myocardial damage, it was recorded that the pathologic lesions seen on the heart varied with doses and duration of treatments and that many macrophages were observed in the necrotic areas. Also, it was recorded myocardial infarction is associated with inflammatory infiltration, myocardial basement damage, myocardial degeneration, and interstitial oedema which was interpreted as reversible symptoms of myocardial infarction lesions. These changes are usually not important in the pathogenesis of cell death. But with increase in duration and doses, complicated with program cell death, necrosis with bursting of cell membrane and fibroblast proliferation was categorise irreversible cell injury (Zhang *et al.*, 2008). The Normal control rats showed normal cardiac tissue. The tissue from enalapril and EEAP treated rats showed a near normal myocardium.

3.6 Conclusion

The results indicated a protective potential of EEAP doses similar to that seen in the enalapril (10 mg/kg) group. Amongst the groups treated with isoproterenol (85 mg/kg) and EEAP, the best results were achieved in the EEAP (ISO+200 mg/kg) group ie the post treated group.

Thus, these results showed that EEAP has cardioprotective effect against myocardial infarction in experimental rats. The mechanism of prevention might have restored fatty acid peroxidation levels, potentiate myocardial endogenous antioxidants, decreases cardiac biomarker enzymes, decrease C-reactive protein, Cardiac Troponin and increase IL-10. In addition, EEAP also protected the cardiac tissue as established in histopathology.

CHAPTER FOUR
STUDY 2: NEPHROPROTECTIVE POTENTIAL OF *ANDROGRAPHIS PANICULATA* LEAF EXTRACT ON CISPLATIN-INDUCED RENAL INJURY

4.1 Introduction

Cisplatin (CP) is the first of the platinum coordination complex with alkylating activity introduced into clinical medicine. Cisplatin is an active medication for the management of different types of cancer (Wang and Lippard, 2005). As effective as cisplatin is in management of cancers, its toxicity can result in acute renal failure (Pabla and Dong, 2008). Direct tubular toxicity, inflammation, vascular factors, and oxidative stress are the resultant outcome of cisplatin-induced nephrotoxicity (Ramesh and Brian, 2002). High dose of CP result to severe renal dysfunction (Hoek *et al*., 2016). Cytotoxic lesion seen in tumours and other dividing cells after administration of cisplatin is caused by cisplatin-DNA cross links and toxicity in non-proliferating cells is minimal. CP selectively injured proximal tubule cells of the kidney leading to high accumulation of it in the kidney than in other organs (Atessahin *et al*., 2003). In the presence of CP, Xanthine-xanthine oxidase system, NADPH oxidase and mitochondria in cells produce ROS; thereby connected to renal damage. Also, CP induces hexokinase and glucose-6-phosphate dehydrogenase activity that result to increase free radical production and reduce level of antioxidant defence system.

Andrographis paniculata has a broad therapeutic action such as anti-inflammatory, anti-oxidative stress, immunosuppressant, antithrombotic, antineoplastic, anti-viral, anti-bacterial, anti-diabetic, antipyretic and analgesic activities (Akbar 2011; Kabir *et al*., 2014 and Greco *et al.,* 2016)

This experiment was designed to evaluate the potential of *Andrographis paniculata* leaf extract on cisplatin-induced renal damage.

4.2 Materials and Methods

4.2.1 Chemicals and reagents

All chemicals, drugs and reagents used were of standard grade.

4.2.2. Collection and processing of plant

Andrographis paniculata leaves were collected, identified and authenticated as described in study one (section 3.2.2).

4.2.3 Extract preparation

Andrographis paniculata leaves extract was prepared according to the method of Panovska *et al*. (2005) as described in section 3.2.3 of study 1

4.2.4 Experimental animals

Forty nine male wistar rats with body weight of 100-160g were purchased from the Faculty of Veterinary Medicine experimental animal unit, University of Ibadan for the experiment. They were fed with pellets and fresh water *ad libitum* with 12 hour light duration and $25 \pm 2\ ^0C$ temperature and acclimatised for two weeks. All experiments and protocols described in present study follow the OECD accepted by Standard Operation Procedures (SOPs). Also, it was approved by the Animal Care and Use Research Ethics Committee, University of Ibadan (UI-ACUREC/App/2016/030).

4.2.5 Induction of experimental nephrotoxicity

Cisplatin (CP) was dissolved in physiological saline and 10 mg/kg single dose (intraperitoneally) on 1st day for post treated group and 8th day for pretreated group to induce nephrotoxicity.

4.2.6 Animal grouping

The animals were group into groups A-G. Each group has seven animals.

Group A: normal control were administered normal saline orally,

Group B: received normal saline orally for 7 days, then a single dose of CP 10 mg/kg i.p on day 8.

Group C: received 200 mg/kg EEAP orally for 7 days, then a single dose of CP 10 mg/kg i.p on day 8.

Group D received 400 mg/kg EEAP orally for 7 days, then a single dose of CP 10 mg/kg i.p on day 8.

Group E: received a single dose of CP 10 mg /kg on day 1 i.p, then 3 days after the CP injection; they were administered normal saline for the7 days.

Group F: received a single dose of CP 10 mg /kg on day 1 i.p, then 3 days after the CP injection; they were administered 200 mg/kg EEAP for the 7 days.

Group G: received a single dose of CP 10 mg /kg on day 1 i.p, then 3 days after the CP injection; they were administered 400 mg/kg EEAP for 7 days.

4.2.7   Urine sample collection

Urine sample for protein analysis was collected on day 10 using metabolic cages.

4.2.8   Collection of blood sample

Up to 5 ml of blood was drawn from the retro orbital venous plexus using haematocrit tubes into two separate blood sample bottles (plain and heparinized) for haematology and serum chemistry tests. Blood collected in plain bottles were left for 30 minutes to clot and centrifuged at 4000 rpm for 10 minutes. The serum obtained was stored at -4^0 C and used for analysis.

4.2.9   Preparation of tissue homogenate

The kidney of each rat was carefully removed and Processed as described in the first study. The supernatant was used as a sample for the biochemical assay for the estimation of GSH, GPx, GST, SOD, protein thiol, non-protein thiol, advanced oxidative protein product (AOPP), hydrogen peroxide (H_2 O_2) generation, malondialdehyde (MDA) and protein carbonyl.

4.2.10 Haematology

Hb concentration, RBC count, PCV and WBC count were done following methods described in Study 1 section 3.2.11.

4.2.11 Determination of serum creatinine

Randox kit was used for estimation for serum creatinine. 25 ml of reagent R1a which contains picric acid and equal volume of reagent R1b containing NaOH are mixed to form the working reagent and 1ml of working reagent and 100 µL of standard solution then 100 µL of sample are added into the cuvette, this sample is mixed and read the absorbance after 30 seconds as A1 and at exactly 2 minutes A2 absorbance readings is taken at 492 nm

Contents	Initial concentration of Solution
CAL. Standard	
RIa. Picric Acid	35mmol/1
Rib. Sodium Hydroxide	0.32mol/1

Procedure	
Wavelength	492 (490-510nm)
Cuvette	1cm light path
Temperature	$25/30/37^0$C
Measurement	Against air

Pipette in cuvette

Working reagent	Standard	Sample
Standard Solution	1.0ml	1.0ml
Sample	0.1ml	-
	-	0.1ml

The concentration was the calculated using

A2-A1=ΔA sample or ΔA standard.

Concentration of creatinine in serum

$\dfrac{\Delta A\ sample}{\Delta A\ standard}$ * Standard concentration (mg/dl) = mg/dl

4.2.12 Serum urea concentration

The estimation of serum urea was carried out using Randox kit. In the presence of the enzyme urease urea serum was hydrolyzed to ammonia. The level ammonia was measured against blank with spectrophotometer at 546 nm

4.2.13 Myeloperoxidase activity

Xia and Zweier (1997) method as described in first study was used to determine myeloperoxidase activity.

4.2.14 Determination of Nitric Oxide Levels

Nitric oxide concentration determination was determine using the method of Olaleye *et al*., (2007) as described in first study

4.2.15 Determination of xanthine oxidase (XO) activity

Akaike *et al.,* (1990) method was used to determine activity of xanthine oxidase. Reagents used include PBS (50 mM), pH 7.4 (1.79 g Na_2 $HPO4.12H_2O$ and 0.60 g $NaH_2PO4.2H_2O$ in 500 ml of diluted H_2O), 10 g of Trichloroacetic acid (TCA) was added to 100 ml distilled H_2O and 0.013 g xanthine (dissolved in NaOH) was made up in 500 ml of the PBS. 2, 950 µl of the xanthine-buffer was put in 50 µl of the test sample making a total reaction mixture of 3,000 µl. The reaction mixture was prepared in duplicates and kept at 25^0C for 40 minutes. First duplicates was terminated after 40 minutes of incubation and the other after 20 minutes extra with the addition of 100 µL TCA. It was then centrifuge at 4, 000 rpm for 20 minutes the resulting supernatant was read at 293 nm wavelengths in units/mg protein or µmole/L.

The activity of xanthine oxidase was calculated as using Difference in change in absorbance at O minute and 20 minutes divided by mg-protein Δ AT2- Δ AT1/mg-protein

4.2.16 Determination of protein thiol level

Ellman (1959) method as described in first study was used to determine the level of protein thiol.

4.2.17 Determination of non-protein thiol level

Ellman (1959) method as described in first study was used to determine the level of non-protein thiol.

4.2.18 Determination of Reduced Glutathione (GSH) Levels

Using the method of Beutler *et al*. (1963) as described in first study

4.2.19 Determination of Glutathione Peroxidase Activity

The method of Rotruck *et al*., (1973) in which hydrogen peroxide was used as substrate to oxidize reduced glutathione to oxidized glutathione (GSSG) as described in first study was used.

4.2.20 Glutathione S-transferase Activity
Method of Habig *et al*., (1974) as described in first study was used

4.2.21 Determination of Superoxide dismutase Activity

Misra and Fridovich (1972) method as described in first study was used

4.2.22 Malondialdehyde level

MDA concentration was measured using Varshney and Kale (1990) method as described in first study

4.2.23 Hydrogen Peroxide Generation

The Hydrogen Peroxide Generation of the samples was determined by means of Wolff's method (1994) as described in first study

4.2.24 Determination of advanced oxidation protein products (AOPP)

AOPP level was measured using Kayali *et al.,* (2006) method. 800 μL of phosphate buffer and 400 μL of sample were added in test tubes after 2mins 100 μL of KI and 200 μL acetic acid was added. The absorbance was read at 340 nm against distilled water as blank.
Acetic acid, 17.418 g of K_2HPO_4 and 13.601g of KH_2PO_4 was dissolved in 900ml of distilled water (PH to 7.4), 1.16 M potassium iodide: Dissolve 192.56 g of potassium iodide in 1litre of distilled water.

The calculation was done using the concentration of AOPP for each sample using the extinction coefficient of 261 cm^1 mM^1 and the results were expressed as nmoles/mg protein.

4.2.25 Determination of protein carbonyl content

Protein carbonyl level was determined according to Reznick and Packer (1994)

i   Reagent:

1. 10 mM DNPH in 2 M HCl:
    Dissolve 0.099 g of DnPH in 50 ml 2 M HCl (made by taking 9.8 ml of Conc. HCl into 40.18ml of distilled water).
2. 20% TCA: 20 g of TCA added to 100 ml of DW
3. Absolute Ethanol
4. Absolute Ethyl acetate
5. 6M Guanidine HCl:
    Dissolve 573 g of Guanidine HCl in 1litre distilled water.

ii   Procedure:

1. Add 500 μL of DnPH solution into all the test tubes
2. Add 100 μL of Sample
3. Incubate for 1 hr at room temperature, Vortexing the sample every 15 mins
4. Add 500μL TCA
5. Place the tubes on ice for 5 mins
6. Centrifuge at 4,000 rpm for 10mins to collect protein precipitate
7. Wash pellet two times with 1 ml Ethanol-ethyl acetate (V/V)
8. Dissolve final precipitate in 600μl Guanidine HCl Solution
9. Incubate for 15 mins at 37°C
10. Read the absorbance at 370 nm using distilled water as blank

iii   Caculation:

. The carbonyl content was calculated based on the molar extinction coefficient of DNPH (e ¼ 2.2 10^4 cm^1 M^1) and expressed as nmoles/mg protein.

4.2.26 Histopathological studies

Kidneys were dissected out, washed in ice cold saline. Processed as described in study one.

4.2.27 Immunohistochemistry

Immunohistochemistry of kidney tissue embedded in paraffin was used for the immunohistochemistry. It was process as described in study one with the use of Kim-1and Nrf$_2$ antibodies (Abclonal).

4.2.28 Data analysis

Data were presented as mean ± S.D and level of significance is α <0 .05, One- way analysis of variance (ANOVA) with Tukey's post –hoc test was performed using Graph Pad Prism version 4.00.

4.3 Results

4.3.1   Haematology

The results of haematology in this study is as shown in Table 4.1

Table 4.1: *Androgryphis paniculata* leaf extract effects on haemogram in cisplatin-induced renal damage

	Pre- treated groups				Post-treated groups		
			Extract			**Extract**	
	Control	**CP 1**	**200 mg/kg**	**400 mg/kg**	**CP 2**	**200 mg/kg**	**400 mg/kg**
RBC $(\times 10^{12}/L)$	4.41±0.87	6.48±0.72^{a}	4.38±0.19^{a*}	4.48±0.17*	5.69±0.83^{a}	4.92±0.47$^{a£}$	4.83±0.43$^{a£}$
WBC $(10^{3}/\mu L)$	4.68±0.48	2.23±0.79^{a}	2.88±0.63^{a}	2.73±0.31^{a}	3.26±0.36^{a}	4.63±0.47$^{£}$	4.33±0.99$^{£}$
HB (g/dl)	10.53±0.76	12.75±0.99^{a}	9.03±0.53^{a*}	10.18±0.53*	11.07±1.52	10.25±1.19	10.82±1.75
PCV (%)	32.33±2.31	39.00±3.65^{a}	28.00±1.41^{a*}	31.75±1.5^{a*}	36.33±2.89^{a}	33.33±0.58$^{£}$	34.5±3.32$^{£}$
MCV (fl)	73.31±2.78	60.18±2.99^{a}	63.93±1.30^{a}	70.87±3.05*	63.85±1.21^{a}	67.74±2.77^{a}	71.13±3.78$^{£}$
MCH (pg)	23.88±0.61	19.68±0.89^{a}	20.62±0.67^{a}	22.72±1.24*	19.46±0.58^{a}	20.83±0.94^{a}	22.31±0.99$^{£}$
MCHC (g/dl)	32.57±0.34	32.69±0.76	32.25±1.24	32.06±1.18^{a}	30.47±0.52	30.75±0.56	31.36±1.35$^{£}$

n =5, a= compared with control, b= compared with CP 1, £= compared with CP 2.

4.3.2 Markers of Oxidative Stress

Figure 4.1: There is an increase in protein carbonyl (PC) activity in Cisplatin groups compared with control. PC was reduced in both pretreated and posttreated groups.

Figure 4.2: There is a slight increase in xanthine oxidase in cisplatin groups compared with the control. Both pretreated and posttreated groups show decrease in xanthine oxidase

Figure 4.3: There is a slight increase in advanced oxidative protein products in cisplatin groups compared with the control. Both pretreated and posttreated groups show decrease in advanced oxidative protein products

Figure 4.4: There is a slight increase in hydrogen peroxide in cisplatin groups compared with the control. Both pretreated and posttreated groups show decrease in hydrogen peroxide.

Figure 4.5: There is an increase in MDA activity in cisplatin groups compared with control (grp A). MDA generation was reduced in pretreated and posttreated groups.

Figure 4.6: There is a slight increase in myeloperoxidase in cisplatin groups compared with the control. Both pretreated and posttreated groups show decrease in myeloperoxidase.

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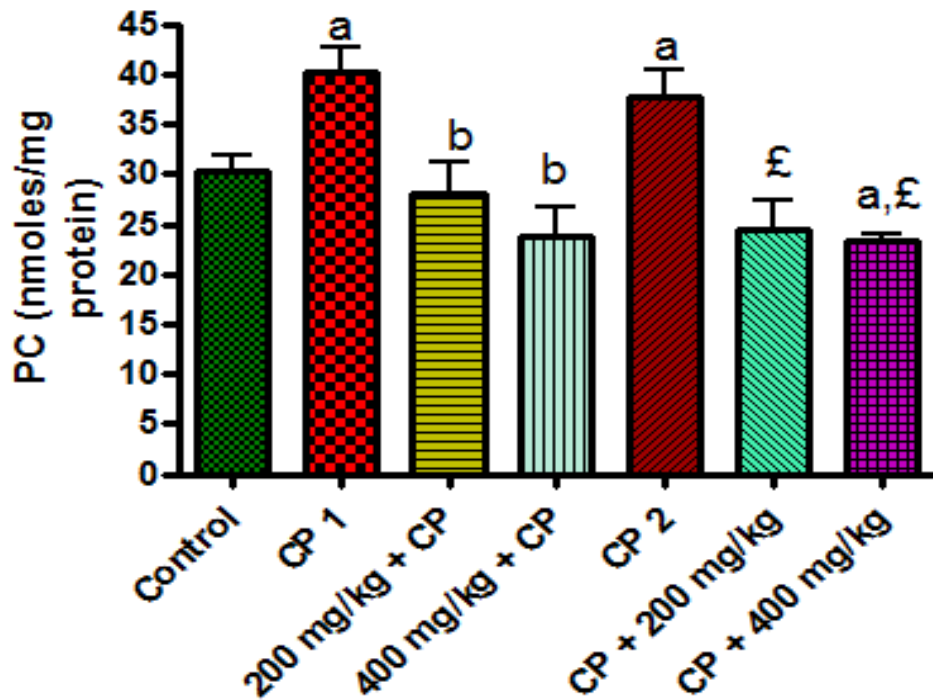
Figure 4.1: *Andrographis paniculata* leaf extract effect on protein carbonyl in CP-induced renal damage

n =5, a - compared with control, b -compared with CP 1, £ - compared with CP 2.
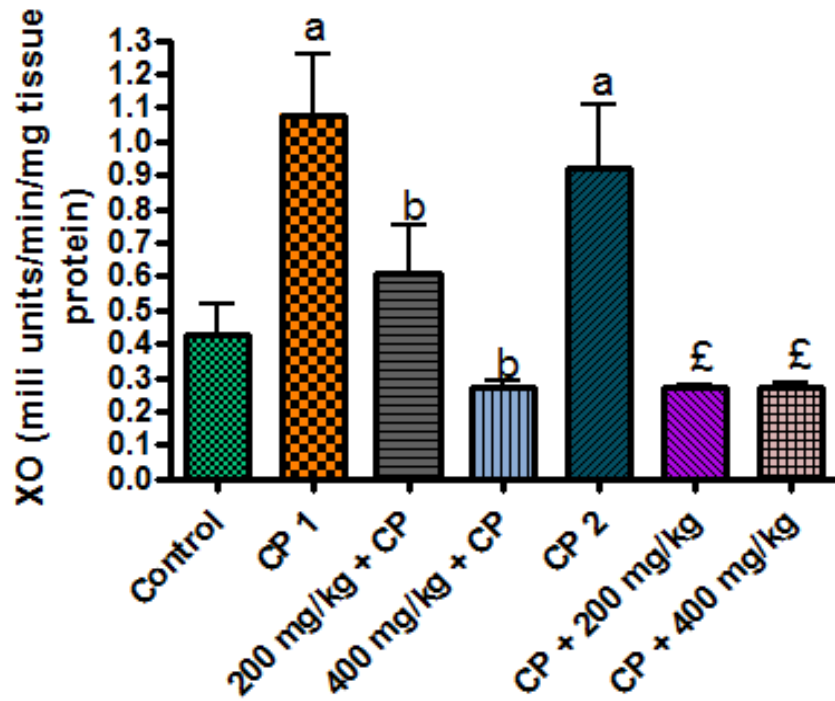
Figure 4.2: *Andrographis paniculata* leaf extract effect on xanthine oxidase in CP-induced renal damage

n =5, a - compared with control, b -compared with CP 1, £ - compared with CP 2.
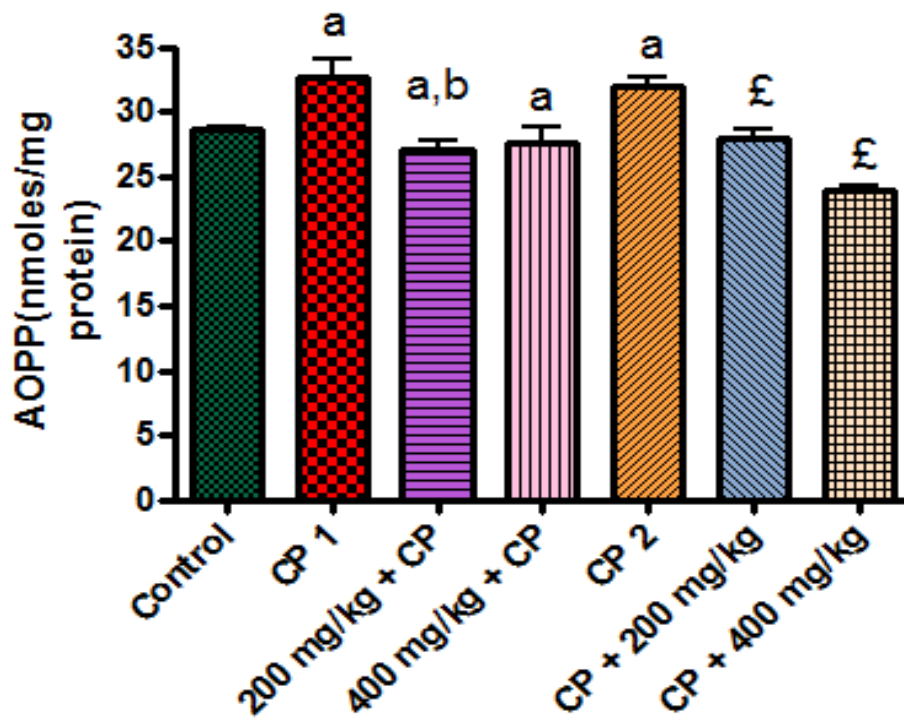
Figure 4.3: *Andrographis paniculata* leaf extract effect on advance oxidative protein products in CP- induced renal damage.

n =5, a - compared with control, b -compared with CP 1, £ - compared with CP 2.
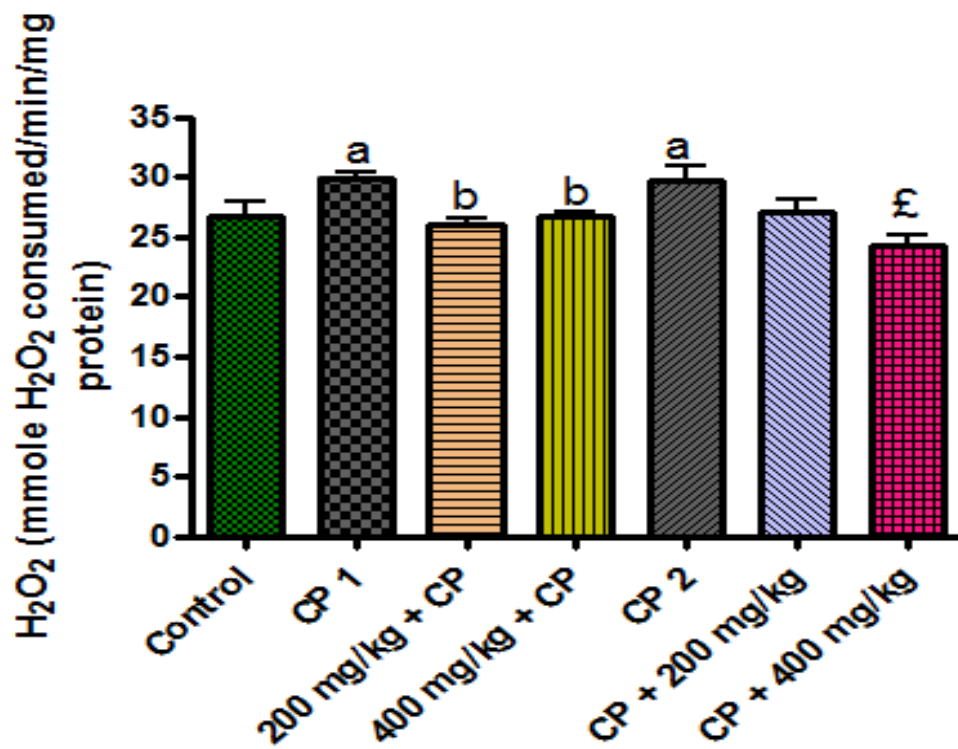
Figure 4.4: *Andrographis paniculata* leaf extract effect on hydrogen peroxide in CP-induced renal damage

n =5, a - compared with control, b -compared with CP 1, £ - compared with CP 2.
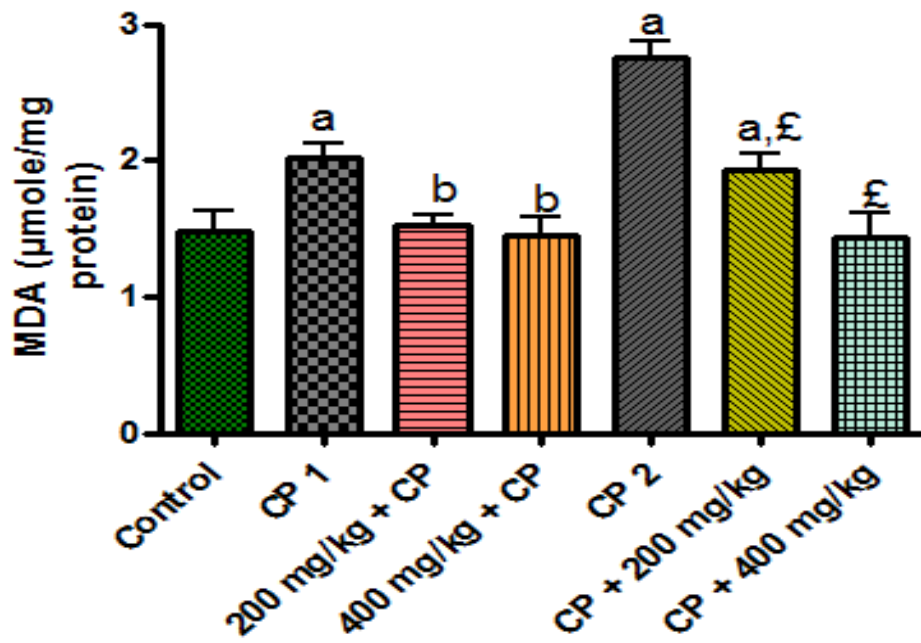
Figure 4.5: *Andrographis paniculata* leaf extract effect on lipid peroxidation (MDA) in CP- induced renal damage

n =5, a - compared with control, b -compared with CP 1, £ - compared with CP 2.
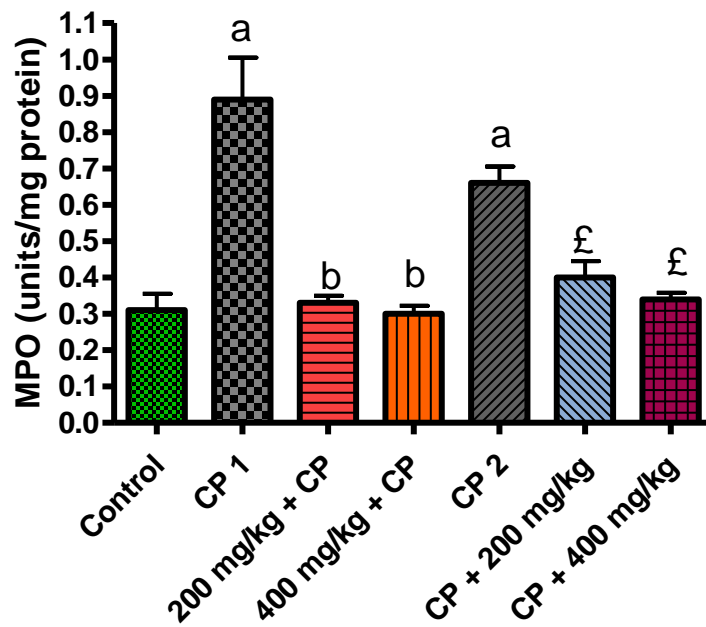
Figure 4.6: *Andrographis paniculata* leaf extract effect on myeloperoxidase in CP-induced renal damage using rats

n =5, a - compared with control, b -compared with CP 1, £ - compared with CP 2.

4.3.3 Antioxidants enzymes

Figure 4.7: There is a decrease in glutathione-s-transferase activity in Cisplatin groups compared with control. There is increase in glutathione-s-transferase in both pretreated and posttreated groups.

Figure 4.8: Show that there is decrease in SOD in the cisplatin groups compared with control. There is increase in SOD in both pretreated and posttreated groups.

Figure 4.9: Show that there is decrease in GPx in the cisplatin groups compared with control. There is increase in GPx in both pretreated and posttreated groups.

Figure 4.10: Show that there is decrease in GSH in the cisplatin groups compared with control. There is increase in GSH in both pretreated and posttreated groups.

Figure 4.11: Show that there is decrease in NPT in the cisplatin groups compared with control. There is increase in NPT in both pretreated and posttreated groups.

Figure 4.12: Show that there is decrease in PT in The cisplatin groups compared with control. There is a significant increase in PT in both pretreated and posttreated groups.
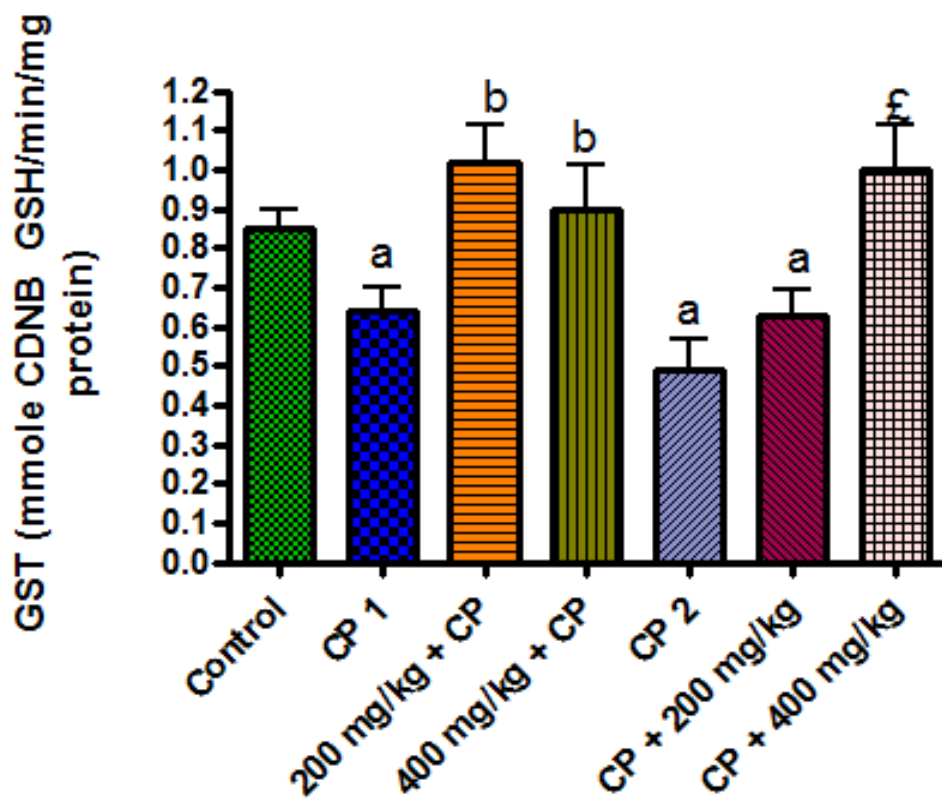
Figure 4.7: *Andrographis paniculata* leaf extract effect on glutathione-S-transferase in CP- induced renal damage

n =5, a - compared with control, b -compared with CP 1, £ - compared with CP 2.
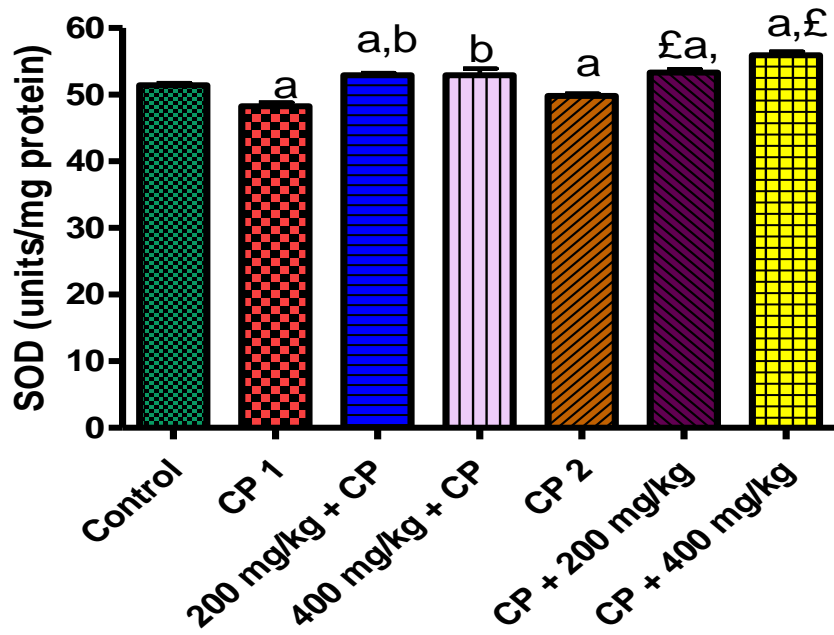
Figure 4.8: *Andrographis paniculata* leaf extract effect on superoxide dismutase in CP- induced renal damage

n =5, a - compared with control, b -compared with CP 1, £ - compared with CP 2.
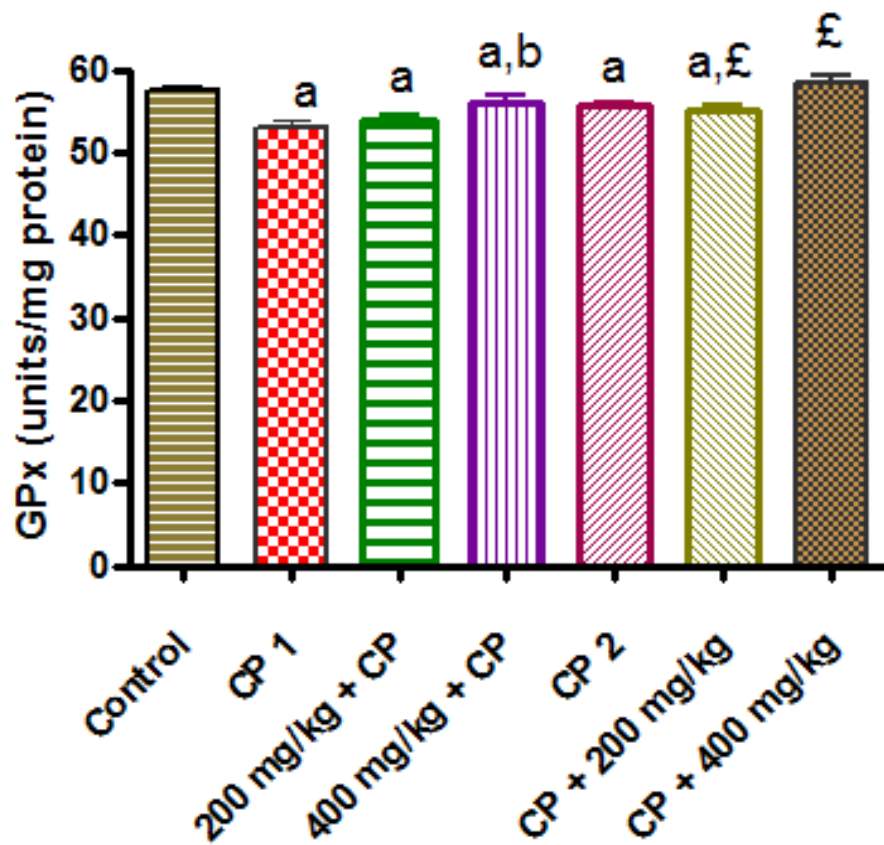
Figure 4.9: *Andrographis paniculata* leaf extract effect on glutathione peroxidase in CP- induced renal damage

n =5, a - compared with control, b -compared with CP 1, £ - compared with CP 2.
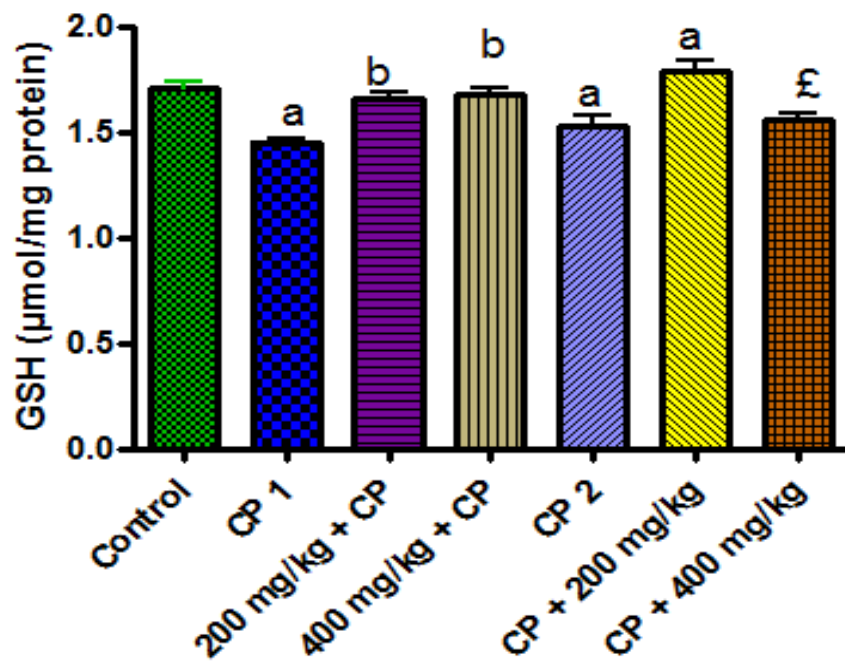
Figure 4.10: *Andrographis paniculata* leaf extract effect on glutathione in CP-induced renal damage

n =5, a - compared with control, b -compared with CP 1, £ - compared with CP 2.
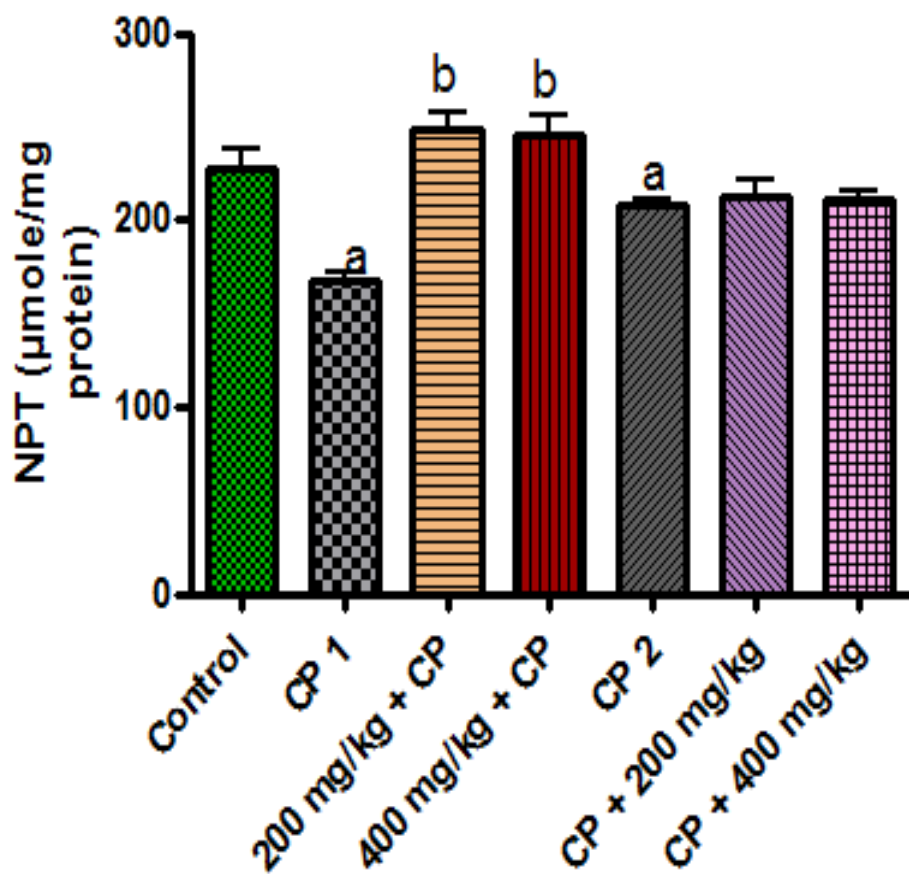
Figure 4.11: *Andrographis paniculata* leaf extract effect on NPT in CP- induced renal damage

n =5, a - compared with control, b -compared with CP 1, £ - compared with CP 2.
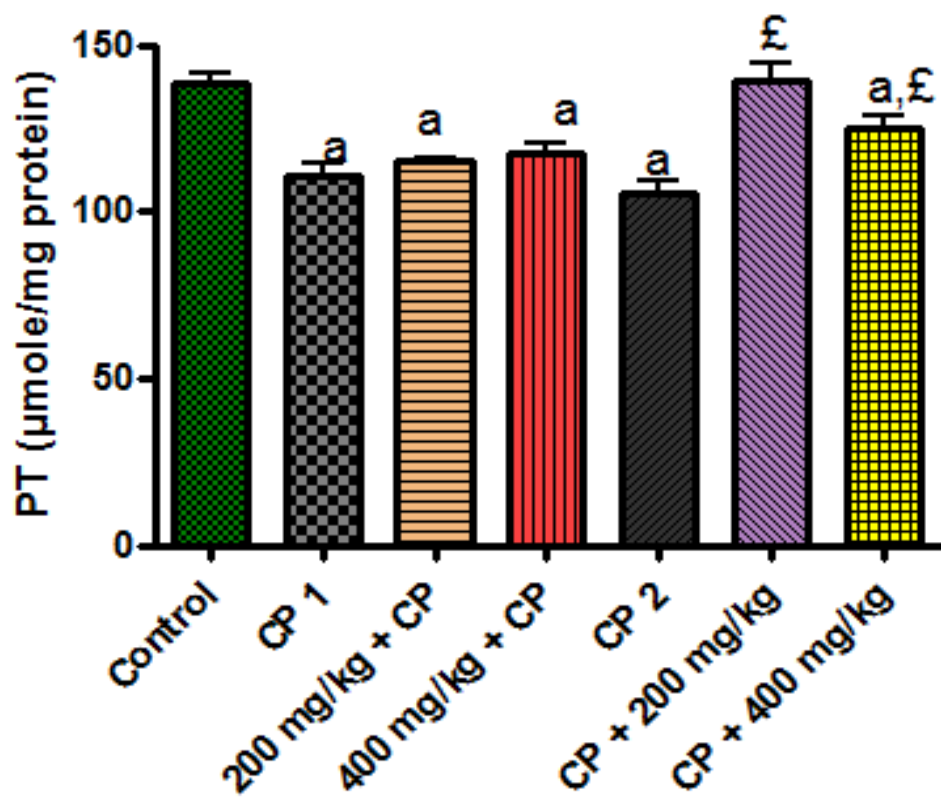
Figure 4.12: *Andrographis paniculata* leaf extract effect on PT in CP- induced renal damage

n =5, a - compared with control, b -compared with CP 1, £ - compared with CP 2.

4.3.4   Serum chemistry and total protein

Table 4.2: Show that there is increase in urea, creatinine, nitric oxide and total protein in the cisplatin groups compared with control. There is a significant decrease in these markers in both pretreated and posttreated groups.

Table 4.2: *Andrographis paniculata* leaves extract effect on serum chemistry and total protein in CP- induced renal damage

	Control	Pre- treated groups			Post-treated groups		
			Extract			Extract	
	Control	CP 1	200 mg/kg	400 mg/kg	CP 2	200 mg/kg	400 mg/kg
CRT (mg/dL)	2.21±0.70	3.75±0.40^a	2.89±0.92	2.68±0.21^b	3.27±0.46^a	1.87±0.35$^£$	1.58±0.43$^£$
UREA (mg/dL)	28.83±2.64	49.97±3.78^a	46.34±4.00^a	42.55±7.14^a	41.75±6.36^a	25.04±7.35$^£$	26.32±1.68$^£$
NO (μmol/L)	1.08±0.14	2.69±0.71^a	1.86±0.74^a	1.34±0.40^{ab}	4.32±0.87^a	2.24±0.21$^{a£}$	1.73±0.24$^{a£}$
TP urine (mg/dL)	2.01±0.08	4.07±0.42^a	1.46±0.04^{ab}	2.71±0.23^{ab}	9.43±0.55^a	8.47±1.58^a	5.57±0.36$^{a£}$

n =5, a - compared with control, b -compared with CP 1, £ - compared with CP 2.

NO= nitric oxide. TP= total protein

4.3.5 Histopathology

The results of the histopathology of rat kidney of cisplatin-induced renal damage are shown in   figure 4.13 a-g.
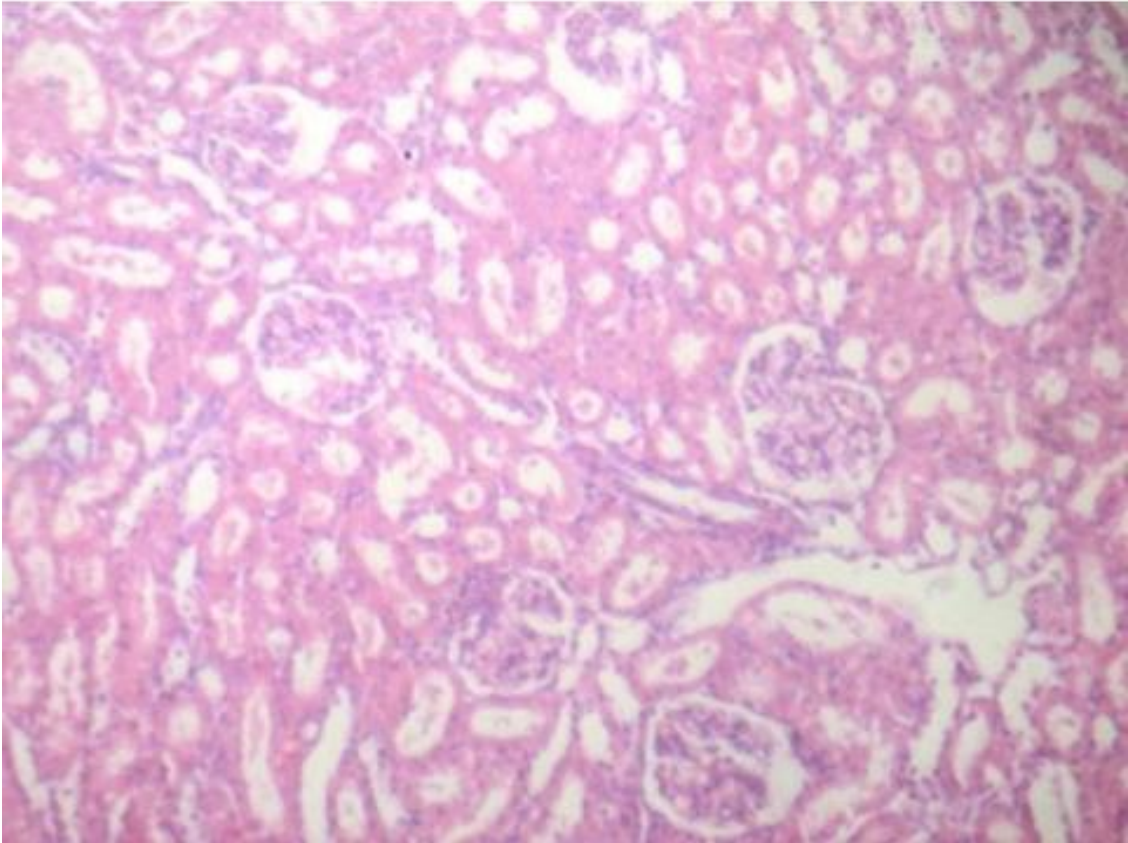
Figure 4.13a: The kidney section of rat in the control group showing no visible lesion (mag x 100)
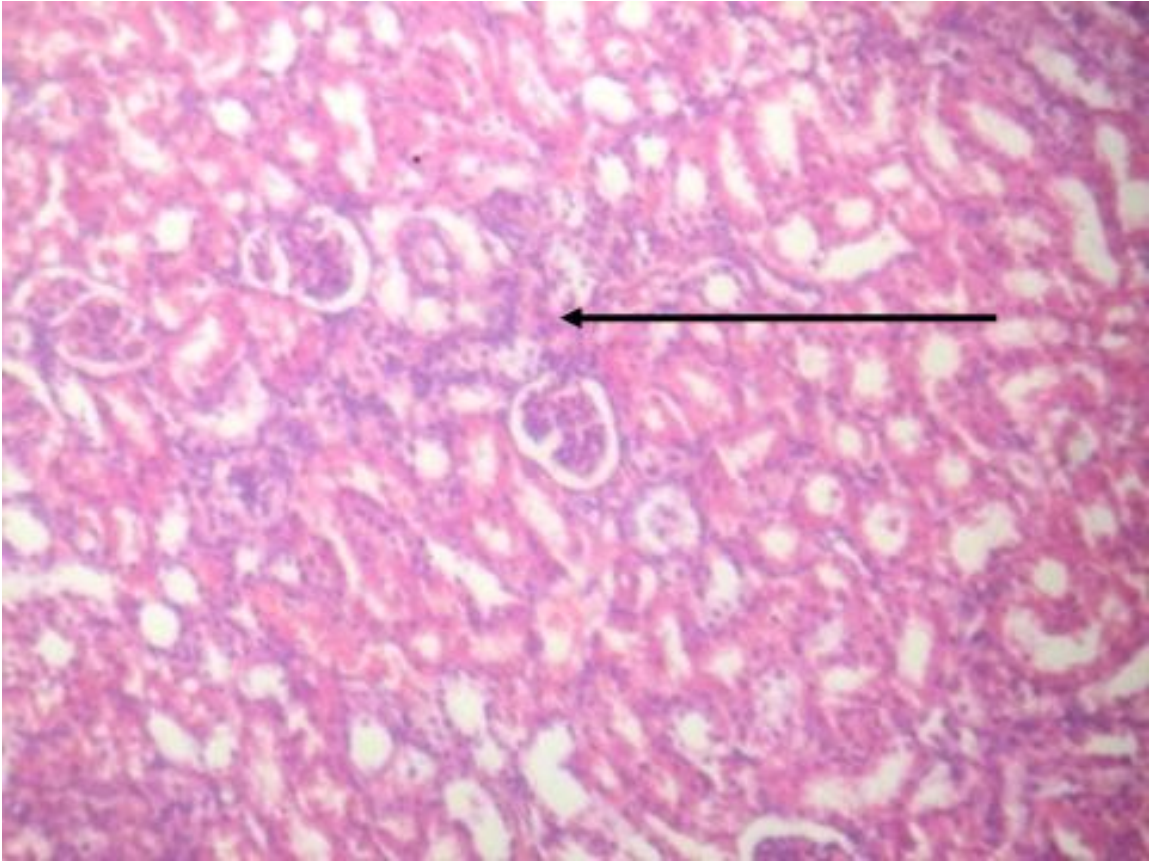
Figure 4.13b: The kidney section of rat in the cisplatin 1 group

showing glomerular degeneration and severe infiltration of inflammatory cells (mag x 100)
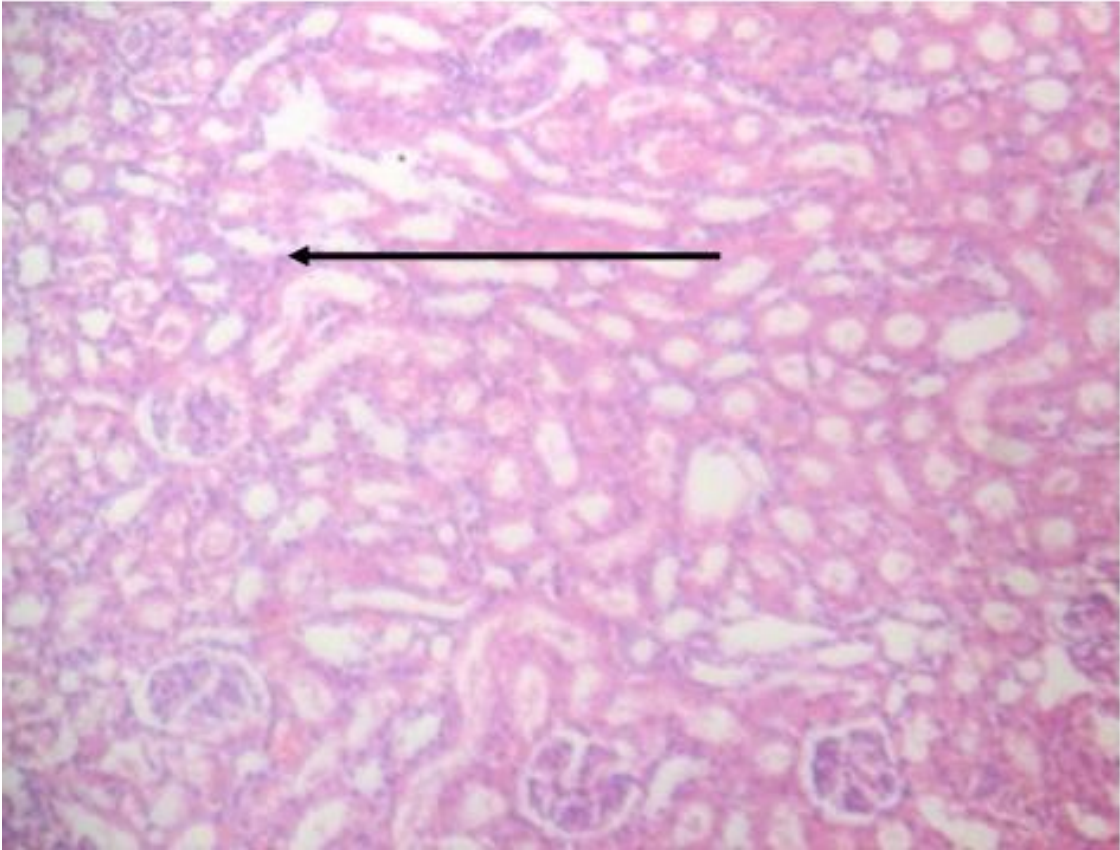
Figure 4.13c: The kidney section of rat in the 200 mg/kg EEAP+CP group

showing mild glomerular degeneration and infiltration of inflammatory cells (mag x 100)
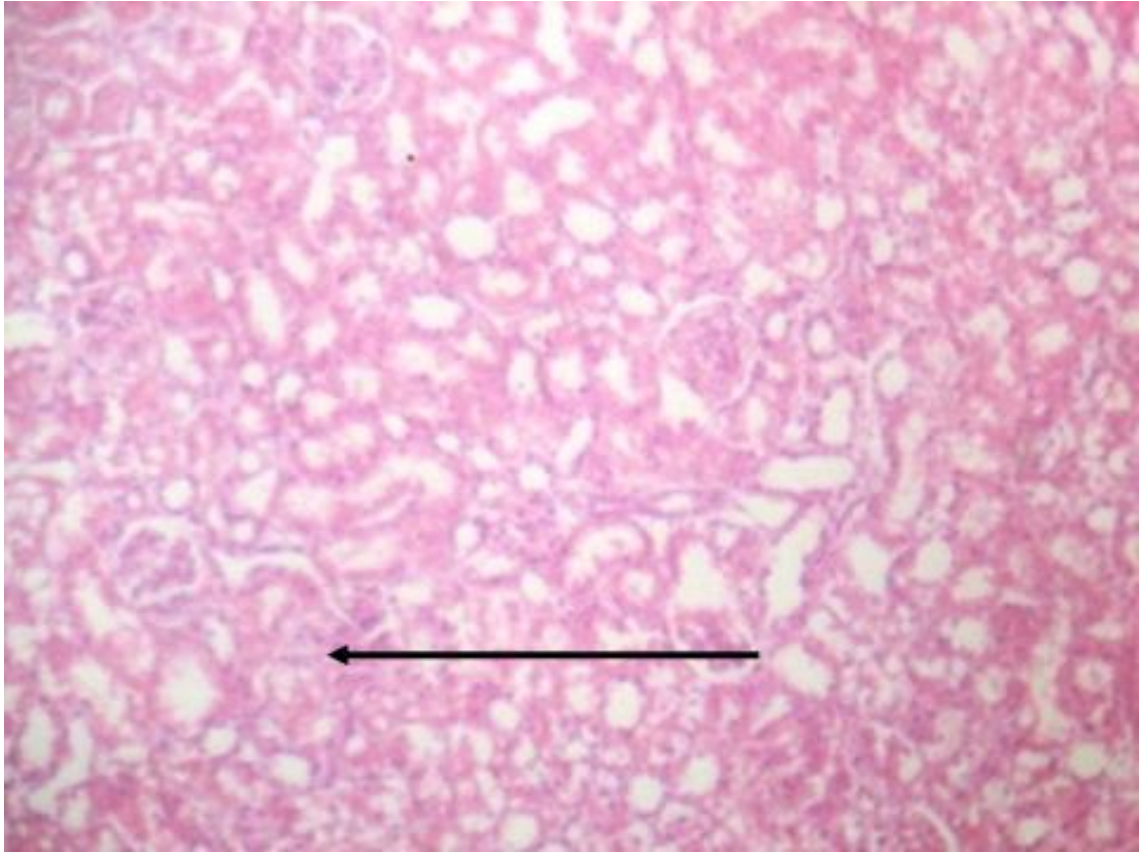
Figure 4.13d: The kidney section of rat in the 400 mg/kg EEAP+CP group

showing mild glomerular degeneration and infiltration of inflammatory cells (mag x 100)
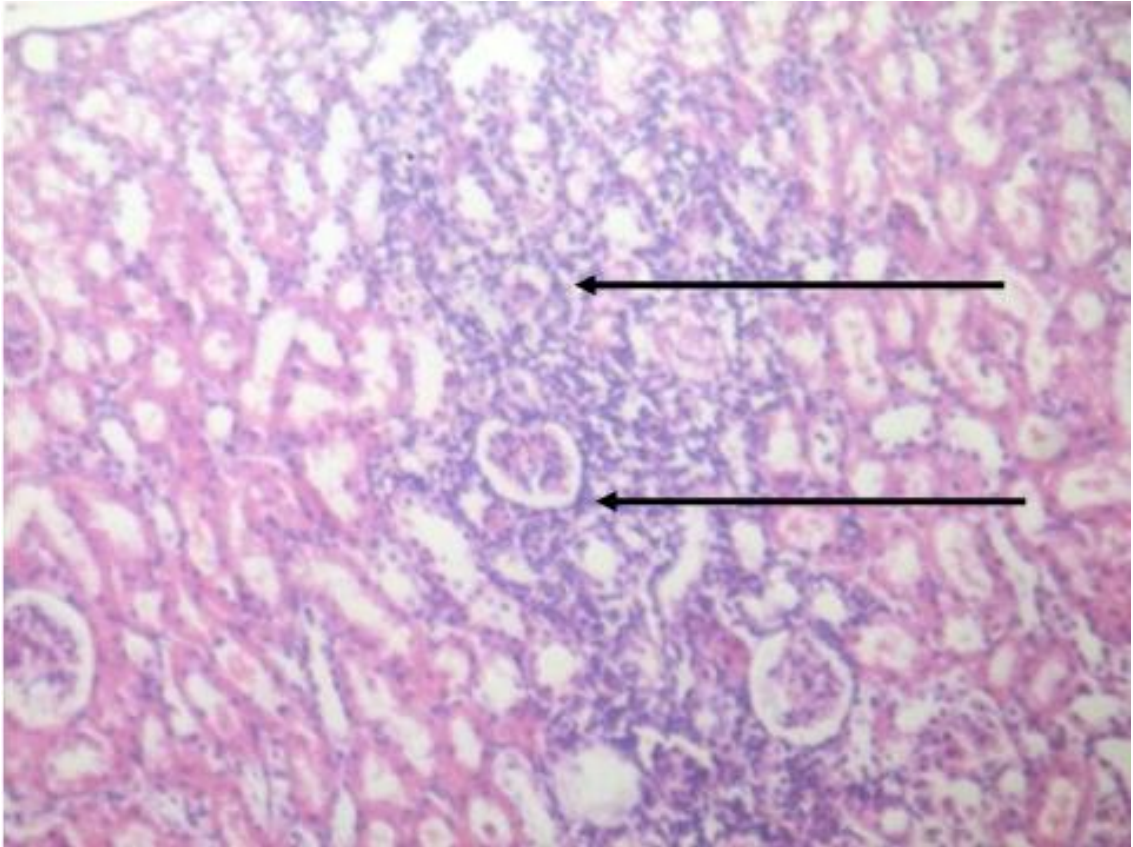
Figure 4.13e: The kidney section of rat in the cisplatin 2 group

showing glomerular degeneration and severe infiltration of inflammatory cells (mag x 100)

Figure 4.13f: The kidney section of rat in the 200 mg/kg EEAP+CP group

showing mild glomerular degeneration and infiltration of inflammatory cells (mag x 100)
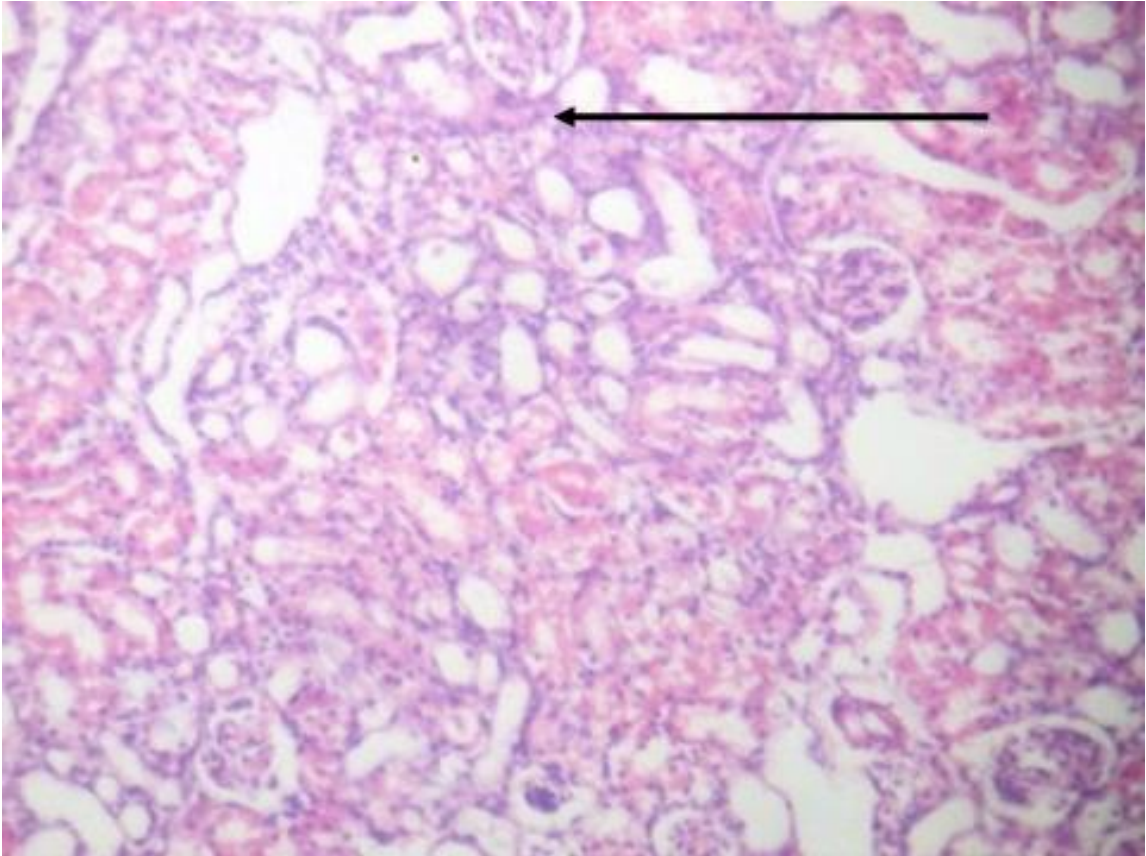
Figure 4.13g: The kidney section of rat in the 400 mg/kg EEAP+CP group

showing mild glomerular degeneration and infiltration of inflammatory cells (mag x 100)

4.3.6   Effect of KIM-1 in the kidney of cisplatin caused renal damage

The results of the kidney injury molecule - 1 was shown in Figure 4.14 a-g
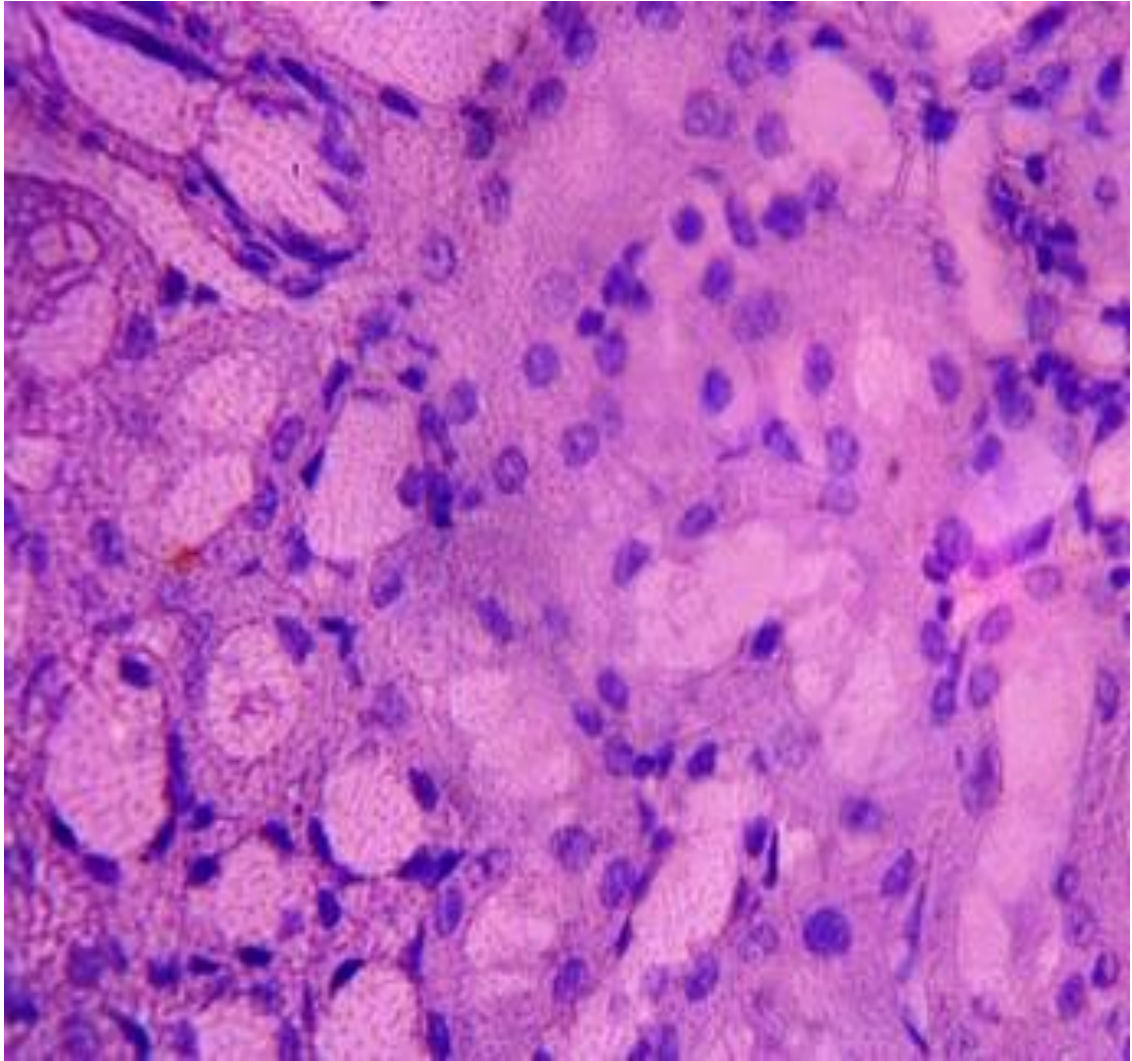
Figure 4.14a: Effect of KIM-1 in kidney of cisplatin caused renal damage

Control: low expression of kidney injury molecule-1. Mag x100
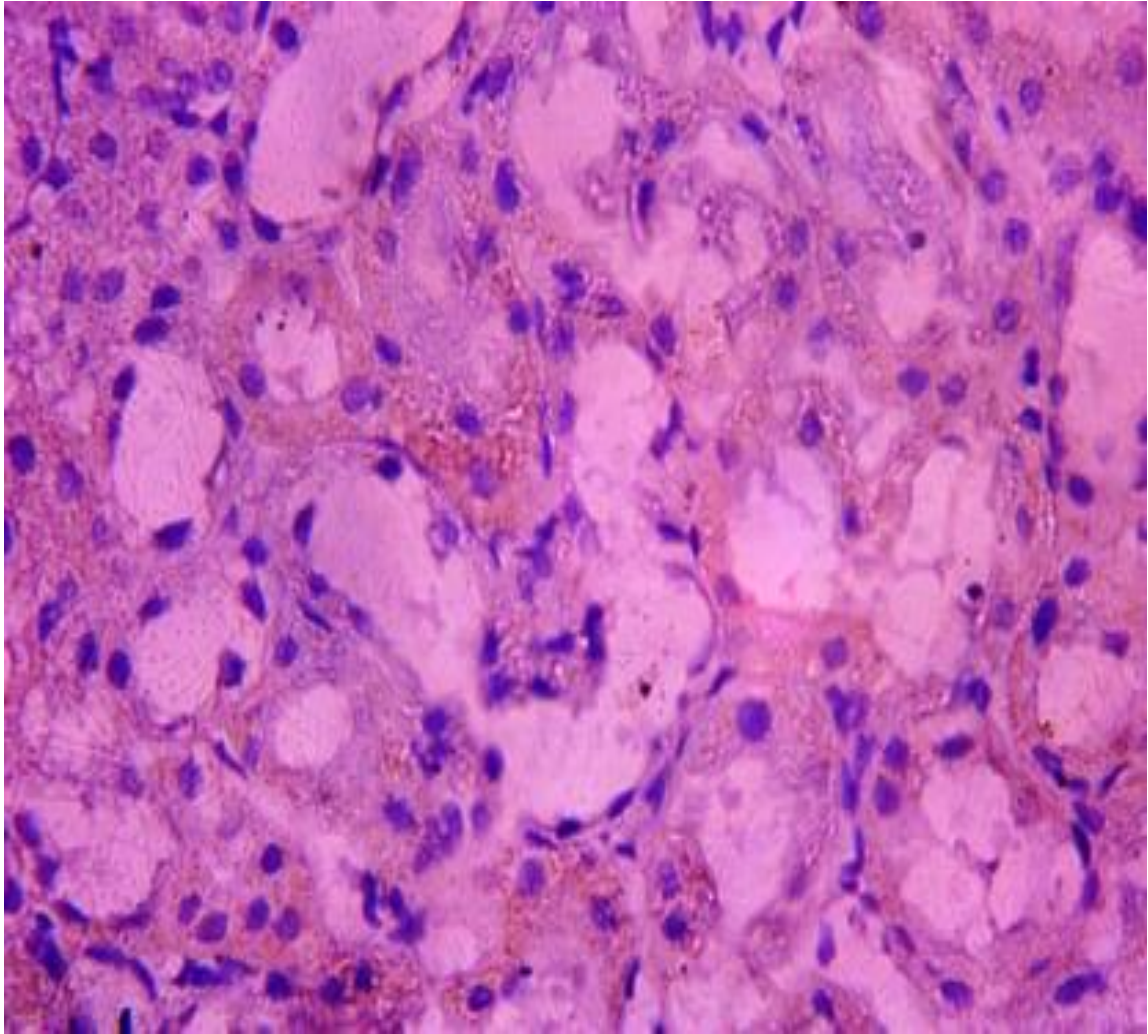
Figure 4.14b: Effect of KIM-1 in kidney of cisplatin caused renal damage.

CP 1: there is more expression of kidney injury molecule-1 than control.
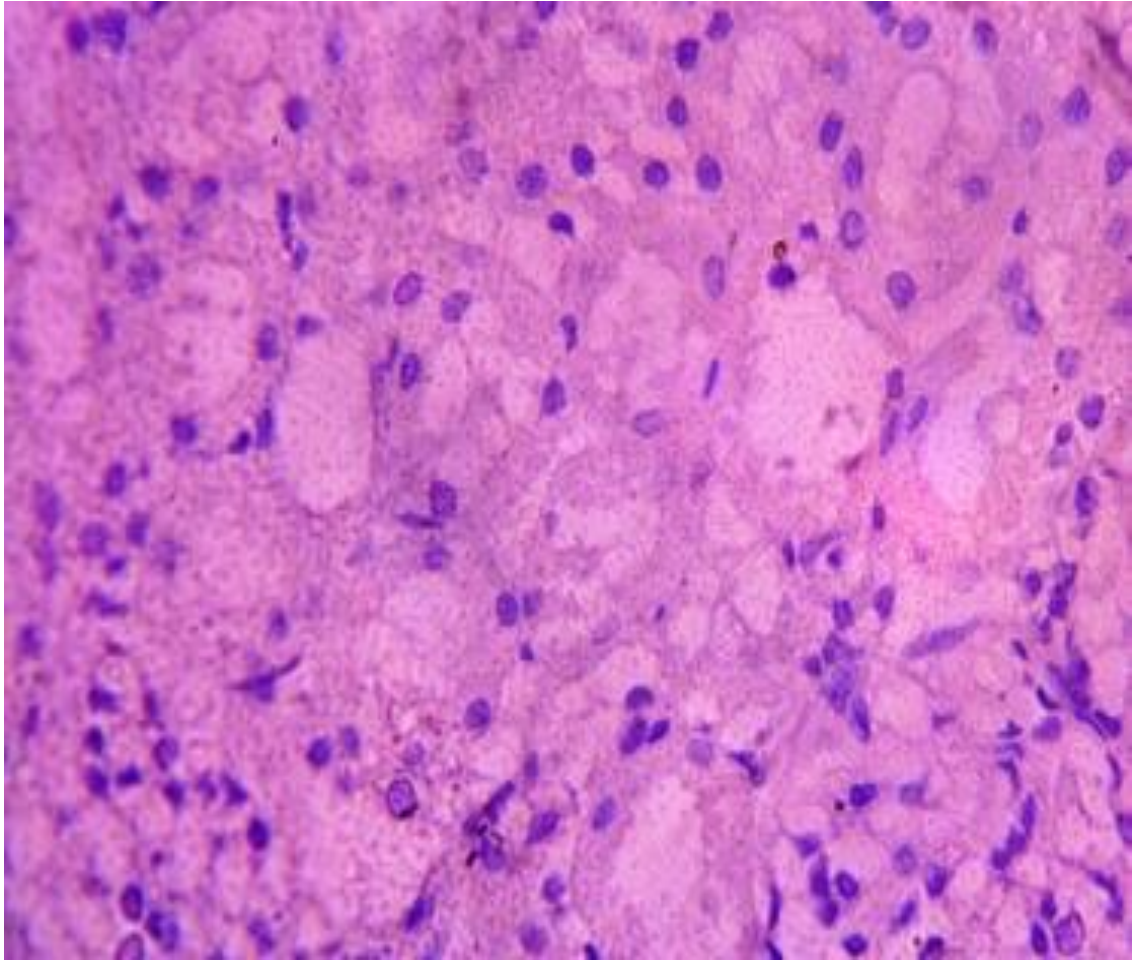
Figure 4.14c: Effect of KIM-1 in kidney of cisplatin caused renal damage

200 mg/kg + CP: lesser expression of kidney injury molecule-1 than CP 1.
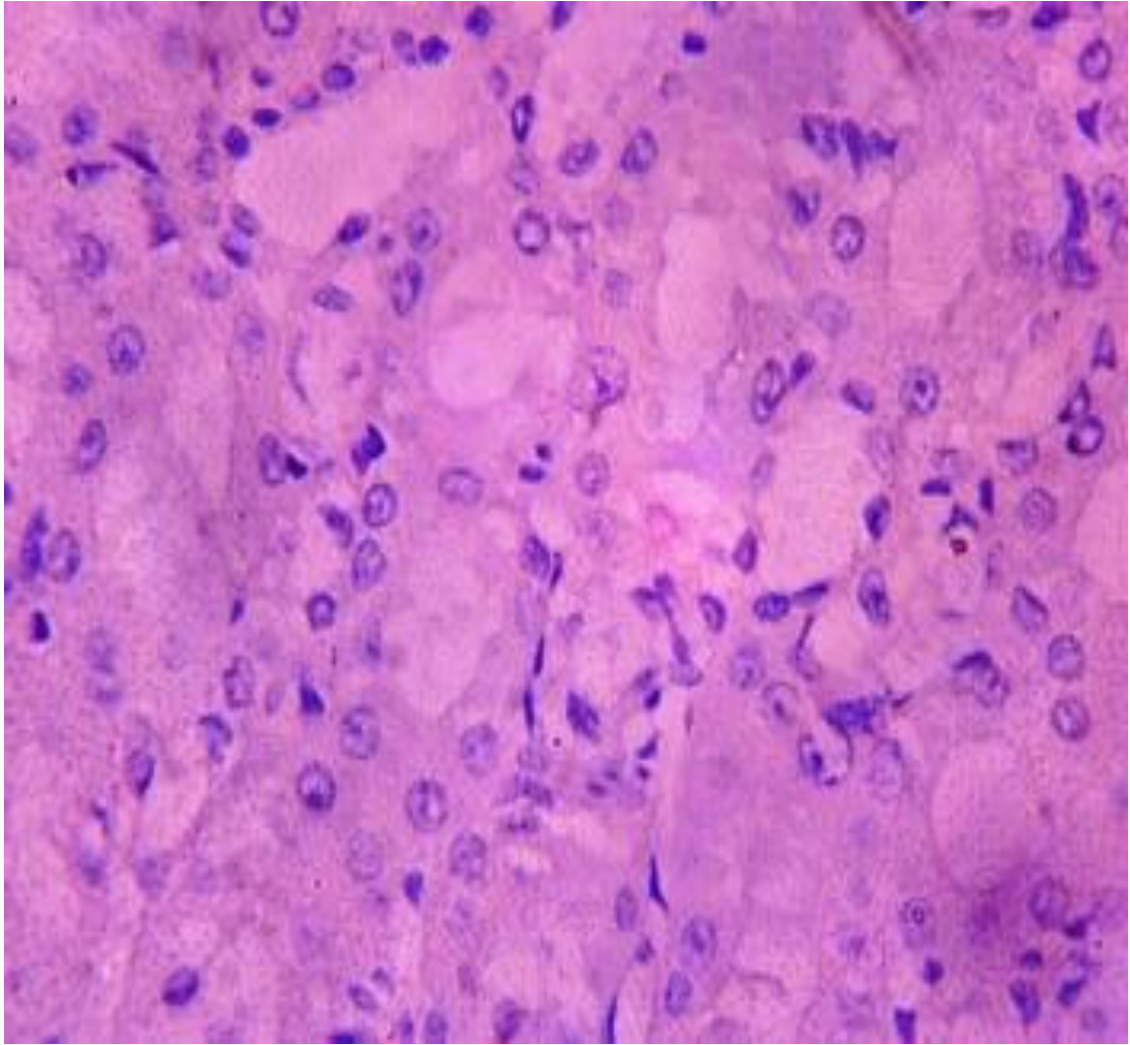
Figure 4.14d: Effect of KIM-1 in kidney of cisplatin caused renal damage

400 mg/kg + CP: lesser expression of kidney injury molecule-1 than CP 1
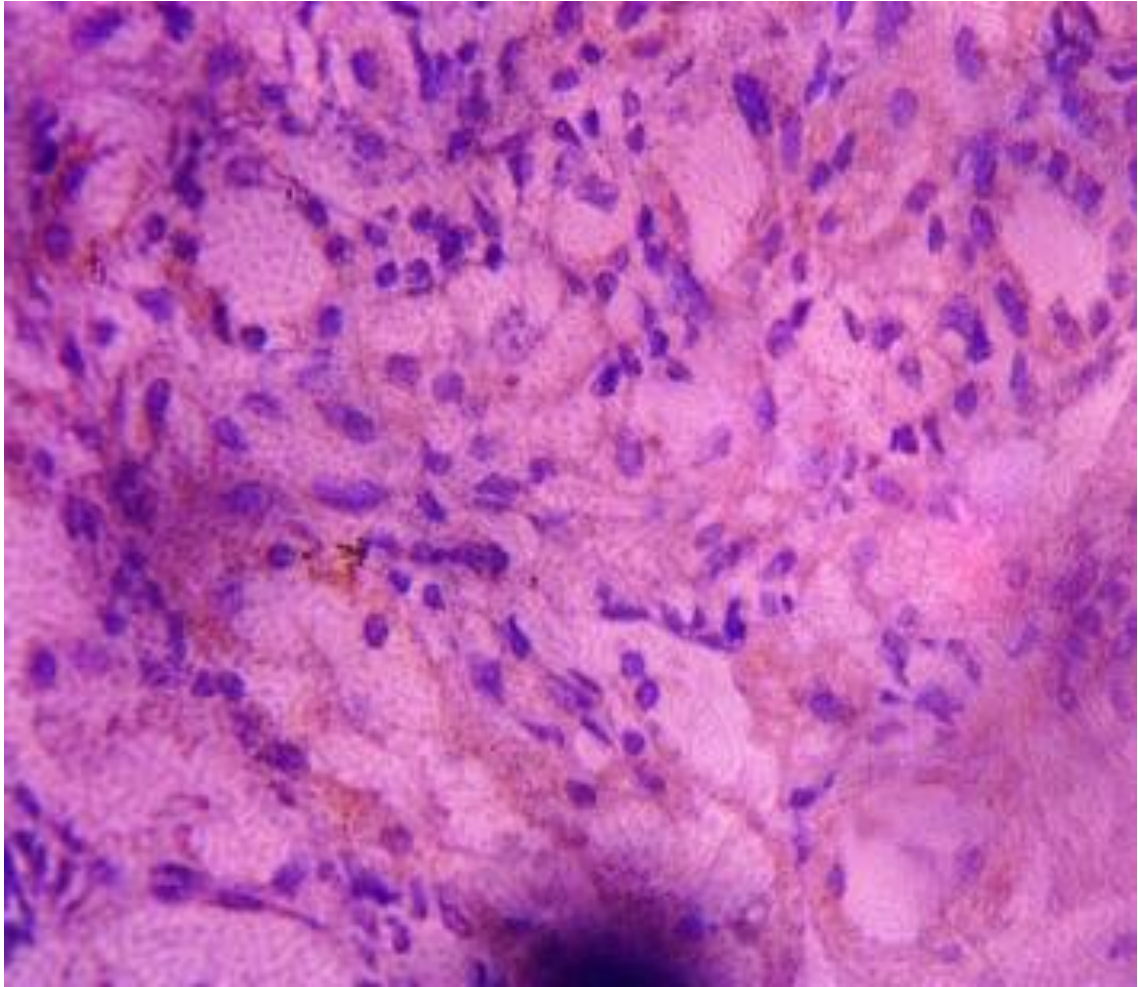
Figure 4.14e: Effect of KIM-1 in kidney of cisplatin caused renal damage

CP 2: more expression of kidney injury molecule-1 than contol.
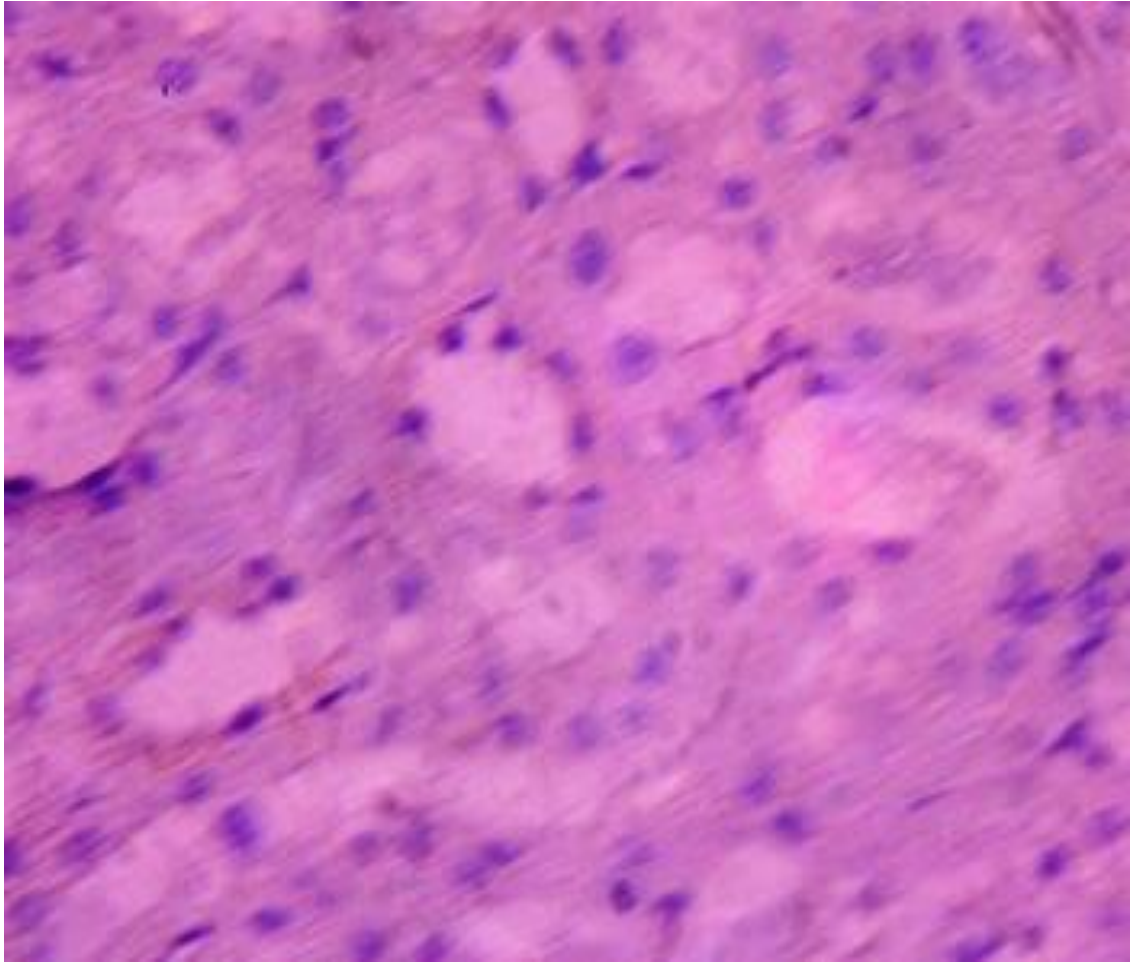
Figure 4.14f: Effect of KIM-1 in kidney of cisplatin caused renal damage

CP + 200 mg/kg: lesser expression of kidney injury molecule-1 than CP 2.
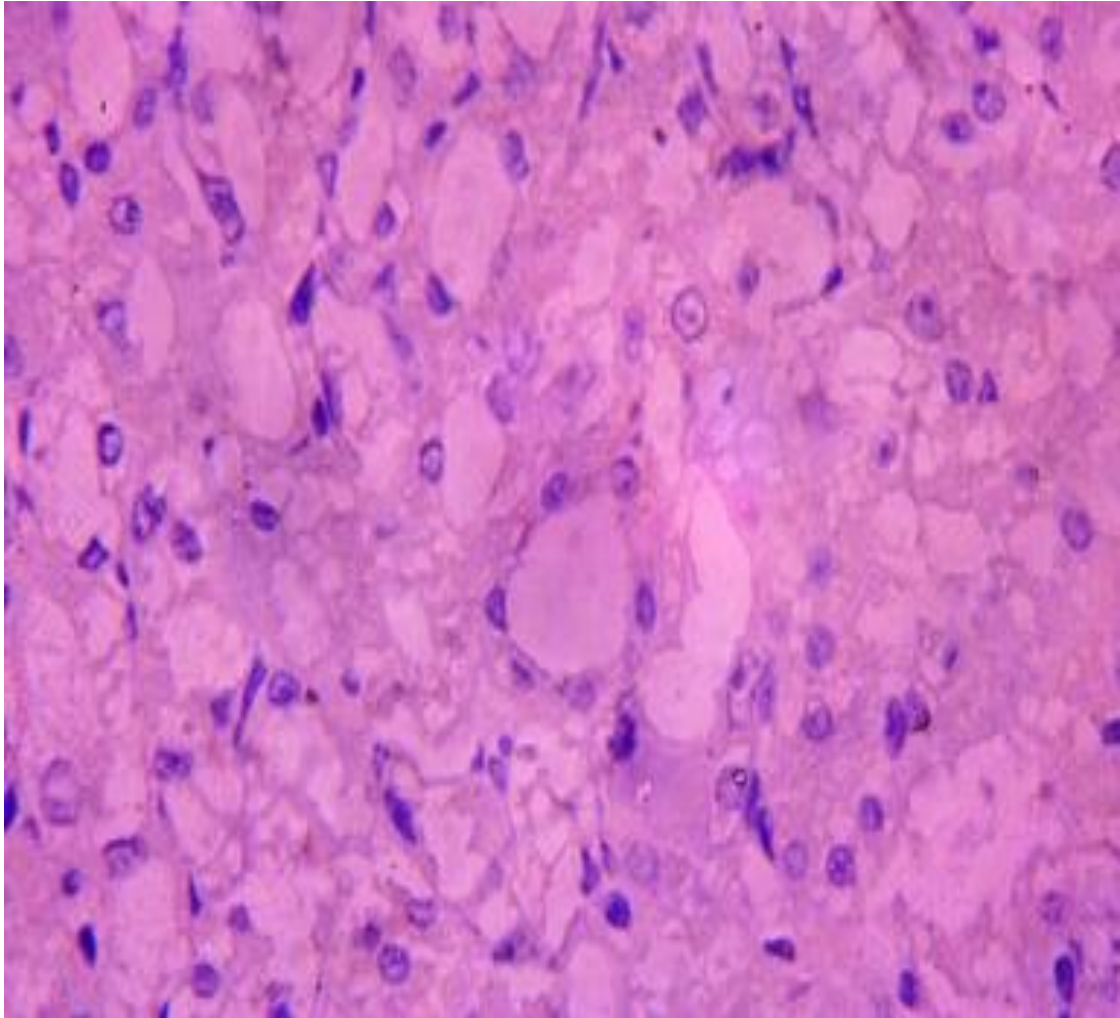
Figure 4.14g: Effect of KIM-1 in kidney of cisplatin caused renal damage

CP + 400 mg/kg: lesser expression of kidney injury molecule-1 than CP 2.

4.3.7 Effect NrF 2 in the kidney of cisplatin caused renal damage.

The results of the nuclear factor erythroid-2- related factor was shown in Figure (Figure 4.15 a-g)
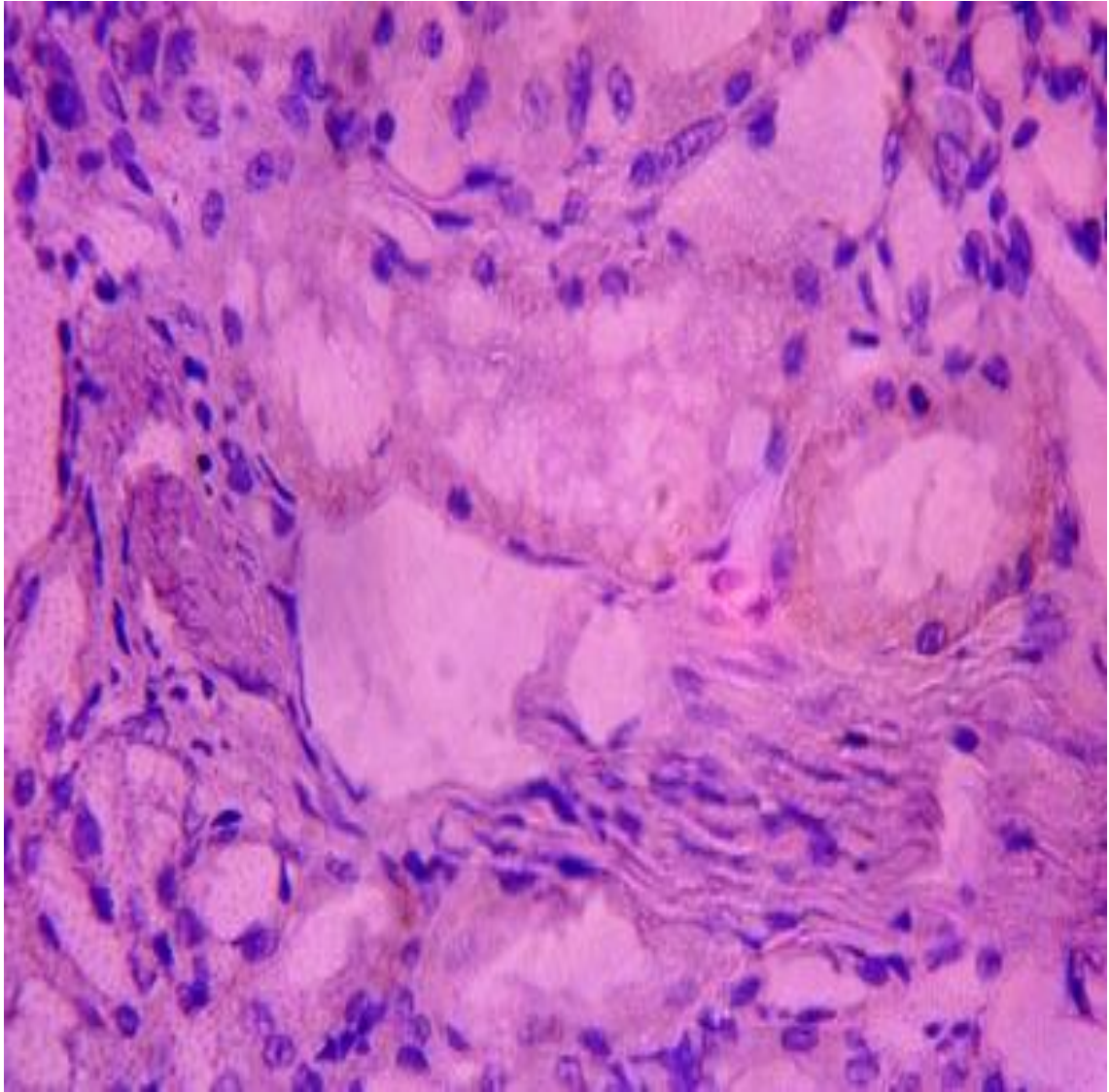
Figure 4.15a: Immunohistochemistry of NrF 2 in the kidney of cisplatin caused renal damage

Control (group A) shows positive and higher expression of Nrf$_2$.
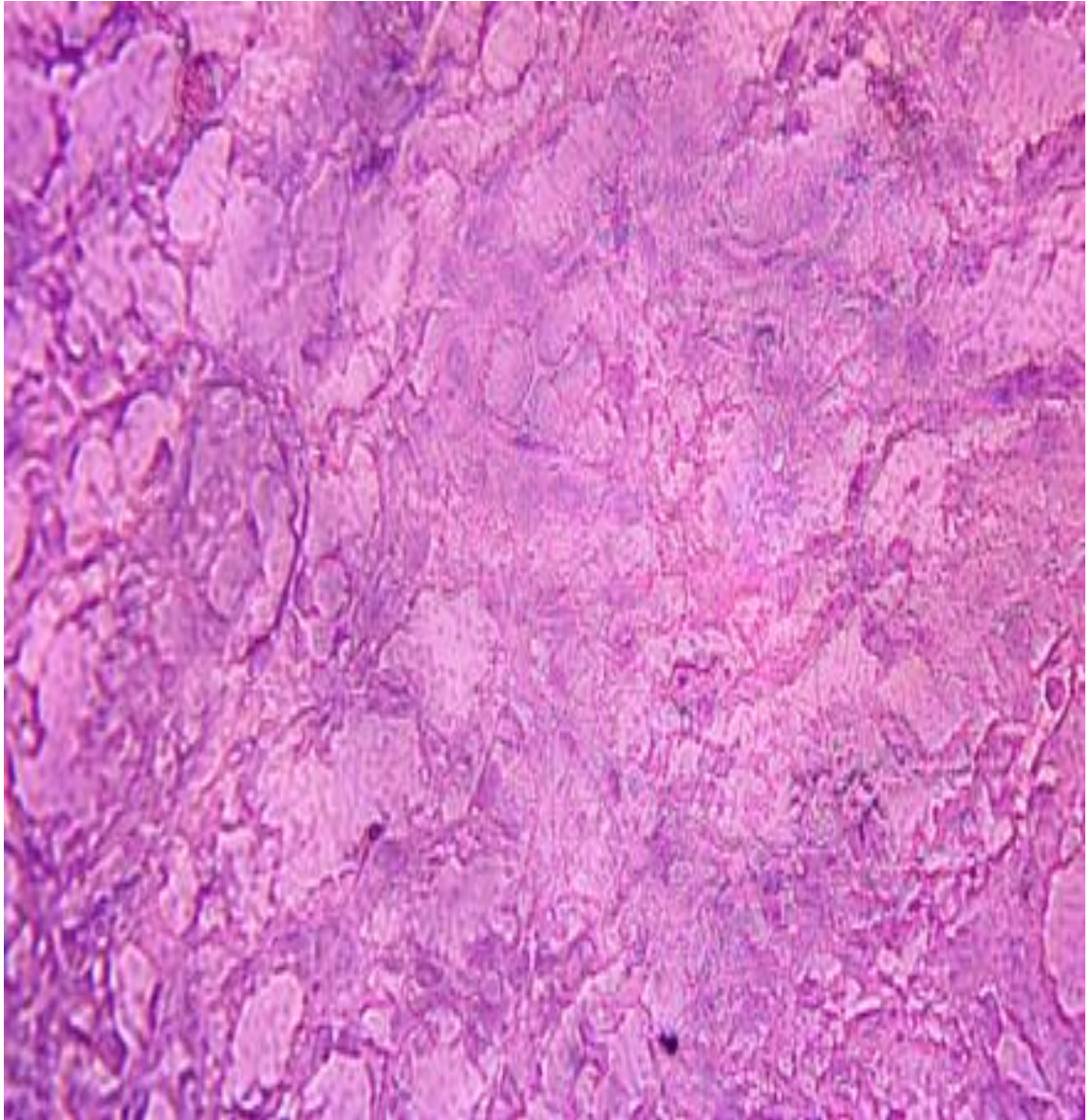
Figure 4.15b: Immunohistochemistry of NrF 2 in the kidney of cisplatin caused renal injury.

Cisplatin 1 (group B): lesser expression of Nrf2 than control.
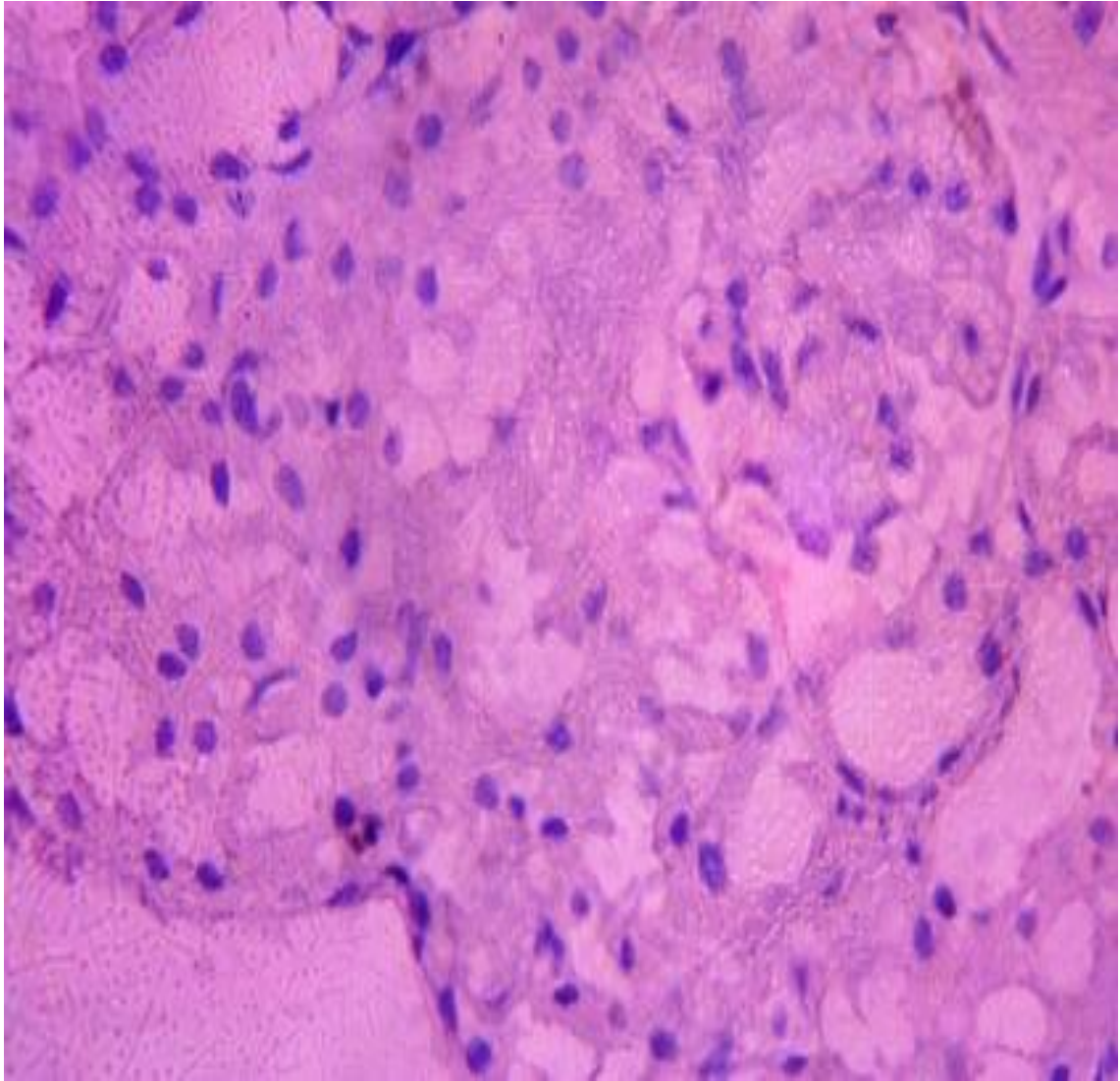
Figure 4.15c: Immunohistochemistry of NrF 2 in the kidney of cisplatin caused renal injury.

200 mg/kg EEAP+CP (group C) :more expression of Nrf2.
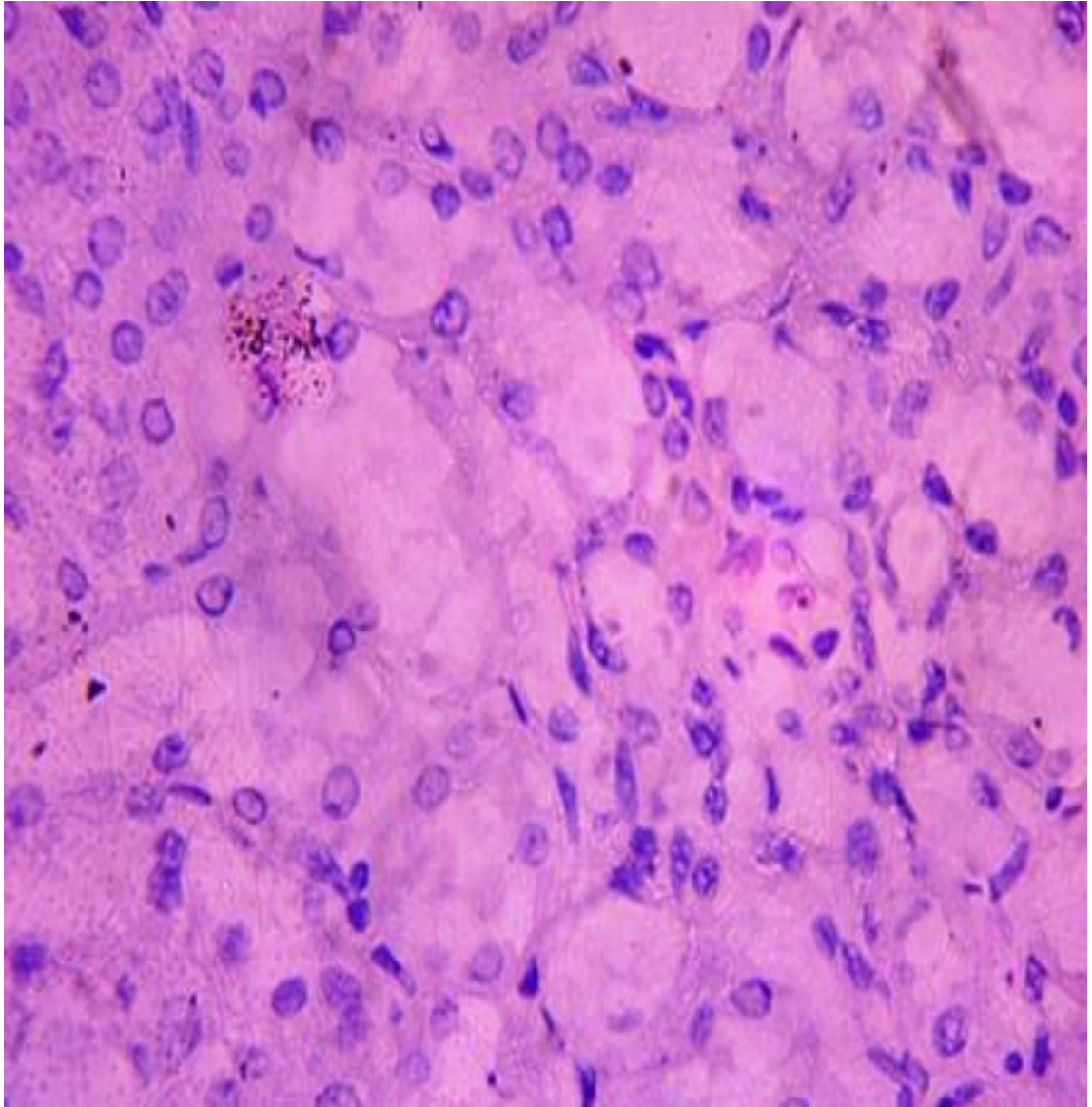
Figure 4.15d: Immunohistochemistry of NrF 2 in the kidney of cisplatin caused renal injury**.**

400 mg/kg EEAP+CP): more expression of Nrf2.
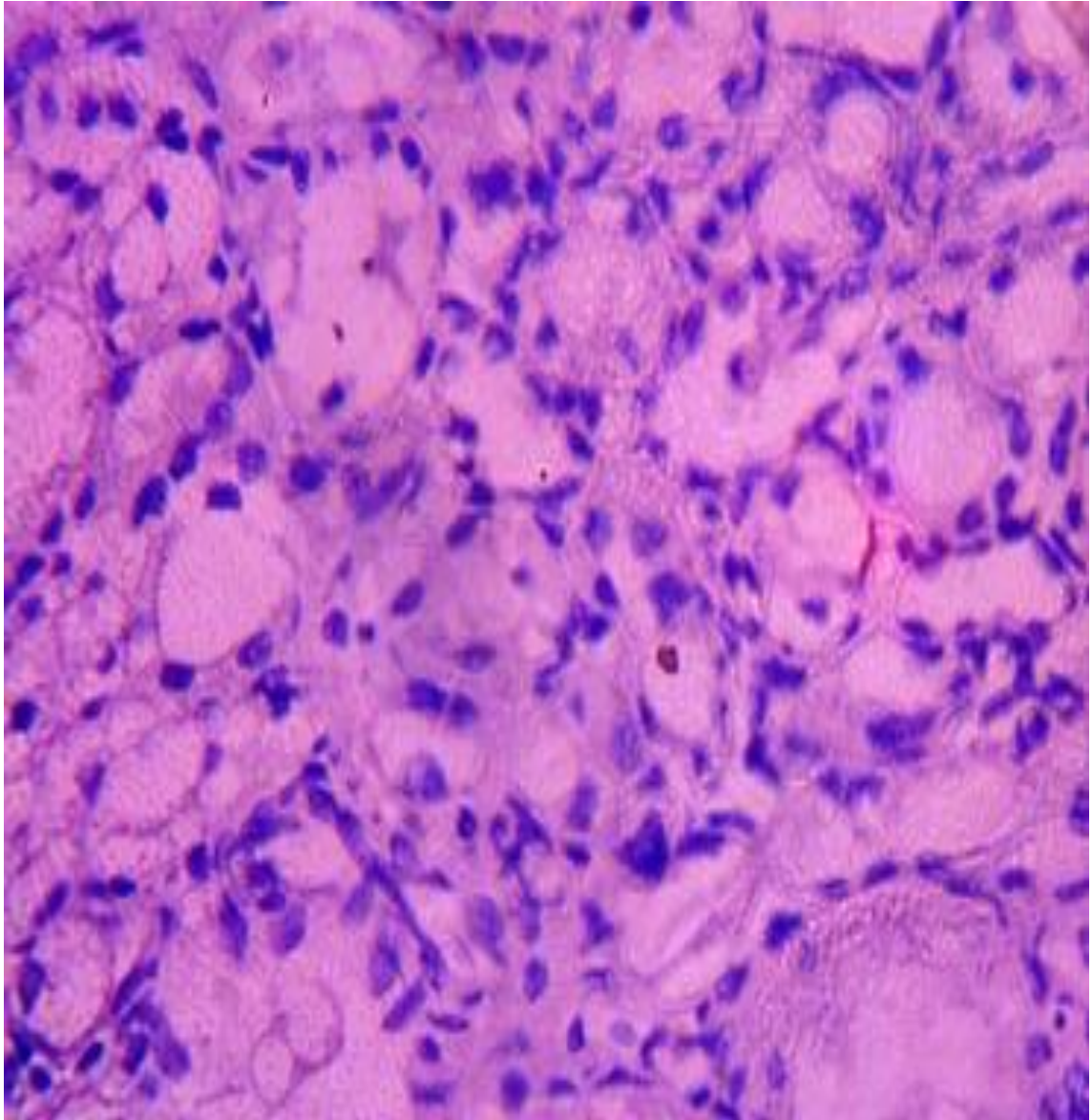
Figure 4.15e: Immunohistochemistry of NrF 2 in the kidney of cisplatin caused renal injury.

CP 2 only (group E): lesser expression of Nrf2.
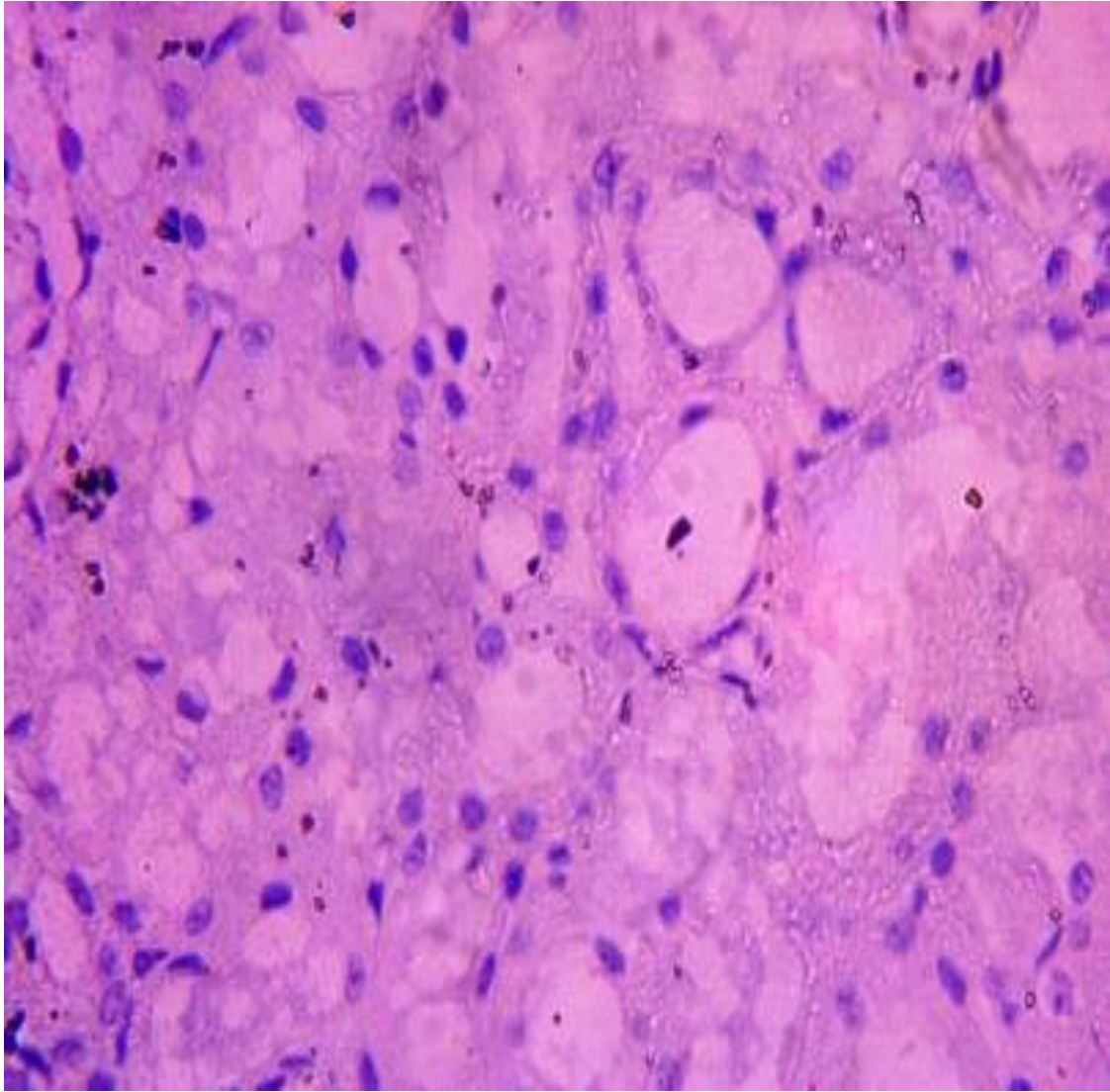
Figure 4.15f: Immunohistochemistry of NrF 2 in the kidney of cisplatin caused renal injury**.**

 CP+200 mg/kg EEAP (group F): more expression of Nrf2.
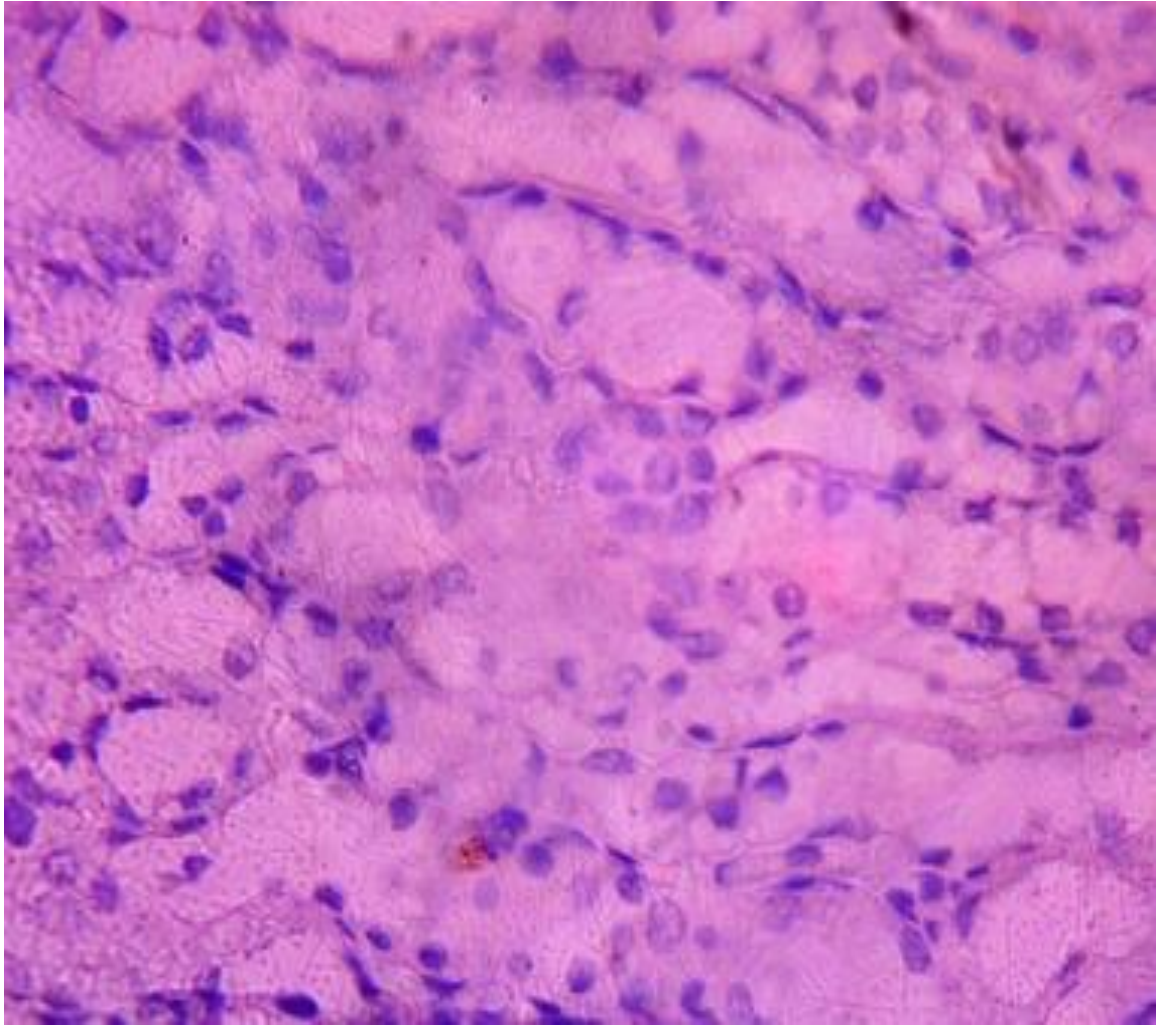
Figure 4.15g: Immunohistochemistry of NrF 2 in the kidney of cisplatin caused renal injury.

CP+400 mg/kg EEAP: more expression of Nrf2

4.4   Discussion

Kidney is the most vulnerable target organs for drug associated toxicity due to its high perfusion rate and ability for drugs uptake and metabolism (Kim *et al*., 2015). Cisplatin, a platinum drug is the most extensively used in the treatment of cancer. It is highly effective in the treatment of many solid tumors like breast, testis, head and neck cancer; however, its major limitation is the nephrotoxicity which is dosing limiting (Osman *et al*., 2015). Many experimental researches have shown that oxidative pressure, apoptosis and inflammation are involved with renal toxicity caused by cisplatin (Sahu *et al*., 2013; Baradaran *et al*., 2016). Therefore, new approaches like excellent combination regimens, is needed to ameliorate cisplatin side effects.

Treatment with cisplatin has been associated with leukopenia (Chan *et al*., 2015). In this study low level of WBC in cisplatin groups reported compared to control and increase in EEAP post treated groups as it was reported by Geyikoglu *et al*., (2016).

In this research, there is increase in RBC, HB and PCV, similar to the findings of Soni *et al.,* (2016), where RBC, Hb and PCV levels increased in rats after cisplatin administration due to increase erythropoietin. However EEAP mitigate this effect.

Cisplatin groups show decrease in GPx, SOD and GST levels compare to control with down regulation of Nrf2. These findings is in accordance with prior studies of Hassan *et al.,* (2017) and Karwasra *et al*., (2016) who reported significant decrease in antioxidant enzyme level in cisplatin-induced nephrotoxicity. In this experiment, treatment with EEAP increase the SOD, GPx and GST level in accordance with Verma and Vinayak (2008) who reported that *Andrographis paniculata* caused elevation of antioxidant activities and this revealed the antioxidant action of *Andrographis paniculata*. Also, antioxidant effect of *Andrographis paniculata* has been reported by Qader *et al*., (2011). *Andrographis paniculata* therapeutic effect shown against cisplatin-induced toxicity may be due to antioxidant property of the plant.

GSH, protein thiols and non-protein thiols are line of defense, also use as marker of oxidative stress. GSH is well known as an essential intracellular reducing agent that helps in the preservation of thiol groups on intracellular proteins and antioxidant molecules in living organism either by converting the toxic radicals to nontoxic end products or by scavenging free radicals. GSH and protein thiols are used up because of

their ability to react with reactive oxygen species. In this study, GSH, PT and NPT were significantly decreased in cisplatin groups. This is in agreement with Karwasra *et al*., (2016) that reported same result in cisplatin-induced renal toxicity. GSH, NPT and PT increase in both pre and post treatment with EEAP compare with cisplatin groups in agreement with Abdellatief *et al.,* (2017).

Furthermore, xanthine oxidase increase significantly in this study in cisplatin treated groups. Similar increase level of xanthine oxidase in cisplatin-induced kidney damage was earlier observed by Yilmax *et al*., (2005) and Gulec *et al.,* (2016). As previously shown, free radicals, including superoxide anion are generated by cisplatin, and are closely related to nephrotoxicity (Farooqui *et al*., 2017). Xanthine oxidase activities increased in the cisplatin treated groups. There is reduction in xanthine oxidase in EEAP treated groups and this can be attributed to radical scavenging activity of *Andrographis paniculata.*

In addition there is significant decrease in AOPP in pre-treatment and post treatment with EEAP groups compare to the cisplatin-induced groups and earlier report by Ilic *et al*., (2016) find increase in AOPP in cisplatin-induced renal toxicity and Kimoto *et al.,* (2011) who suggested that expressions of AOPP were associated with the severity of renal injury

Reactive oxygen species have been suggested as an essential component of cisplatin-induced nephrotoxicity *in vivo*. In this study there is increase in H_2O_2 in cisplatin treated groups compare to control in harmony with Fernández-Rojas *et al.,* (2014) who reported that C-Phycocyanin prevents renal toxicity initiated by cisplatin via reduction of oxidative stress. Meanwhile, pre-treatment and post treatment with EEAP caused a decrease in H_2O_2 compare to the cisplatin-induced groups in accordance with previous study by Sivakumar and Rajeshkumar (2015) who reported protective ability of AP in high blood sugar initiated by oxidative damage in kidney tissues of diabetic rats.

Oxidative modification of proteins may result in the structural and functional changes of some enzyme proteins (Jung *et al.*, 2014). In this study, significantly increased PC levels in cisplatin groups was significantly reduced in EEAP treated groups and this may be as a result of *Andrographis paniculata* ability to scavenge free radicals. This result runs parallel with the report from Neogy *et al.,* (2008) who reported that

Andrographis paniculata meaningfully lower protein carbonyl in nicotine induced oxidative stress.

Hagar *et al.,* (2015) and Hassan *et al*., (2017) reported that in cisplatin-induced nephrotoxicity ROS play a vital part. Renal damage in cisplatin groups may be due to peroxidation of poly unsaturated fatty acid, leading to increase in lipid peroxidation products like malondialdehyde (MDA) that is significantly increase in this experiment. This is in harmony with earlier report by Hassan *et al.,* (2017) that indicate oxidative stress as vital influence in cisplatin caused renal injury.

This study shows that cisplatin caused increase in MPO activity, a marker of oxidative stress and inflammation. This indicates the presence of enhanced polymorphonuclear leucocyte recruitment in the damage kidney under the effect of oxidative stress. EEAP mitigated the cisplatin-induced increase in MPO activity, exhibiting the anti-inflammatory potential of *Andrographis paniculata*. This is in harmony with Chao *et al.,* (2011) and Adedapo *et al.,* (2015) who proposed anti-inflammatory potential of *Andrographis paniculata.*

Severity of kidney injury can be measure by serum creatinine and urea biomarkers in the labouratory. Increase in serum creatinine and serum urea levels are the major pathological lesions seen in renal toxicity caused by cisplatin (Malik *et al*., 2015). Also, increase in nitric oxide level leads to interactions with superoxide anions to form peroxynitrite that cause tissue damage. In this study, cisplatin-induced groups showed increase level of creatinine, urea and nitric oxide levels compare with control group. This is in consonance with Abdel *et al.,* (2014) earlier study show the same significant increase after cisplatin-induced renal toxicity. *Andrographis paniculata* significantly reduced the levels of creatinine, urea and nitric oxide compare to cisplatin groups which suggest the protective role of *Andrographis paniculata* on the kidney. This result is in consonance with Chao *et al.,* (2011) findings that *Andrographis paniculata* significantly reduced serum nitric oxide due to it anti-inflammatory potential. Also, in a study using gentamicin to induced renal damage, Singh *et al.,* (2009) find out that *Andrographis paniculata* had a noteworthy reduction in the levels of serum urea and creatinine.

In this experiment deterioration of renal function was further characterized by increase excretion of urinary protein. This was significantly increased in cisplatin groups

causing proteinuria in agreement with earlier report Ahmed *et al.,* (2014) that tempol was effective against cisplatin-induced increase level of protein in urine. Pre-treatment and post treatment with EEAP caused significant reduction of urine protein compared to cisplatin group, indicating enhancement of glomerular and tubular activities. This is similar to study of Kumara and Prasad (2013), who reported that *Andrographis paniculata* significantly, reduced urinary protein level.

In this experiment, molecular mechanism of antioxidant action of *Andrographis paniculata* was evaluated by finding the effect of kidney injury molecule-1 and a primary transcription factor Nrf2. Kidney injury molecule-1 is highly expressed protein in renal tissue in any disease condition involving kidney (Bonventre, 2009). KIM-1 mediates phagocytosis of oxidized fatty acids and apoptotic bodies (Ichimura *et al.*, 2008). Over expression of this molecule caused progressive kidney fibrosis that result to, chronic renal failure. According to Dugbartey *et al.,* (2016) it was stated that kidney injury molecule-1 is a definite biological marker for analysing nephrotoxicity in patients on cisplatin and in this experiment *Andrographis paniculata* reduced the expression of kidney injury molecule-1 showing its potential by keeping the renal tissue from cisplating-induced damage. End-stage renal failure occurs when acute renal injury persists without treatment and lead to chronic renal injury. Kidney injury molecule-1 progress kidney fibrosis, it's critically uprgulated in fibrotic kidney condition (Humphreys *et al*., 2013). Kidney injury molecule-1 was down regulated in this study hence elucidating the potential effect of *Andrographis paniculata* against renal damage.

Nrf-2 is Transcriptional factor that is responsible for the release of antioxidant enzyme by binding to the antioxidant responsive element find in enhancer region of antioxidant in the nucleus. This results to stimulation of antioxidant proteins and phase II purifying enzymes (Itoh *et al.,* 1997). In cisplatin only treated groups there is decrease in Nrf2 expression. In this study, it was observed that EEAP upregulate Nrf2 expression thus cisplatin-induced renal damage was prevented by inhibition of oxidative stress and inflammatory responses. Wong *et al.,* (2016) find out in a research that andrographolide, stimulated Nrf2 causing release of antioxidant enzymes and upregulated heme oxygenase-1 expression in primary astrocytes thereby protecting the cells against oxidative damage respectively.

Also in this study the program cell death rate was in harmony with damages of renal tissue shown in pathological examination of the kidney tissue using H & E staining. Histopathological result shows extensive inflammatory cells infiltration into kidney of rat administered cisplatin alone while EEAP treated rat tissue show moderate glomerular degeneration. Potential tubular, vascular factors and inflammatory process are involved in the pathogenesis of acute kidney damage. In the pathophysiological of acute kidney there is increase in inflammatory reaction (Friedewald and Rabb, 2004) *Andrographis paniculata* protect against cisplatin-induced nephrotoxicity in this study

4.5   Conclusion

It could be established from this study that *Andrographis paniculata* has anti-inflammatory and antioxidant potential thus it protects against cisplatin-induced renal damage. It thus implies that concurrent administration of *Andrographis paniculata* along with cisplatin in oncology patients may go a long way in relieving the nephrotoxic properties associated with this anticancer agent.

CHAPTER FIVE
STUDY 3: *IN VITRO* ANTIOXIDANT, PHYTOCHEMICAL AND GC-MS ANALYSIS OF *ANDROGRAPHIS PANICULATA*

5.1 Introduction

Several phytochemicals components known to have broad activities which help protect against lipid oxidation and by extension chronic conditions such as cancer, osteomyelitis or cardiovascular conditions (Ghosh *et al.*, 2016).

Main function of antioxidants enzymes is to remove or prevent oxidative stress (Carocho and Ferreira, 2013). Presently, researchers have shown keen interest in the pharmacology potential of botanicals as antioxidants. Artificial antioxidant are known to offer protection against oxidative stress, but they are often too costly and have been implicated in causing negative health effects such as radio-sensitization, increased toxicity of other chemicals, mutagenic activity, and tumor yield from chemical carcinogens (Mallawaarachchi *et al.,* 2015). Therefore, strong restrictions placed on their usage have prompted the need to search for naturally occurring antioxidants. Consequently, many plants have been investigated and found to contain varying amounts of antioxidant. Still antioxidant potentials of plant species information cannot be over emphasized.

Gas chromatography together with mass spectrometry is used to analyse components of botanicals directly. This method is becoming more resourceful in the analysis of essential oil, alkaloids and non-polar components of medicinal plants by many (Sermakkani and Thangapandian, 2012)

Andrographis paniculata is applied in herbal medicine to treat diseases (Ojha *et al.,* 2012). This study is designed to analyse *in vitro* antioxidant properties of *Andrographis paniculata* using FRAP, DPPH, ABTS and NO. This study also carried

out analysis of phytochemical compounds of this plant and subjecting the leaves to GC-MS analysis.

5.2 Materials and Methods

5.2.1 Collection and processing of plant

Andrographis paniculata leaves was collected, identified and authenticated as described in first study.

5.2.2 Extract preparation

Andrographis paniculata leaves extract was prepared using method of Panovska *et al.* (2005) as described in first study.

5.2.3 Separation of the extract

The leaves of *Andrographis paniculata* was extracted with ethanol as already described in first study.

5.2.4 Chemicals and Reagents

All chemicals were bought from Merck, Gauteng, South Africa. Rutin, FeCl2 (Ferric Chloride), DPPH (1,1Diphenyl-2 PicrylHydrazyl), CH3CO2K (Potassium Acetate), FeCl2 (Ferric Chloride), vitamin E, Potassium $K_3Fe(CN)_6$ (Ferricyanide), TCA, CH3COOH, Sodium Nitroprusside and Folin-Ciocalteu reagent. All chemicals used were of analytical grade.

5.2.5 Determination of tannins

Andrographis paniculata leaf extract tannin content was measured using association of official analytical chemists (1990) method with little adjustment. Twenty ml of fifty percent ethanol was added to 0.02 g of the extract. Shake methodically and put in water bath (80°C) for 60 minutes (to mix equally). Then the mixture was strained into volumetric flask, 10 ml of 17% aq. Na2CO3, twenty ml of DW and 2500 microliter of Folin-Denis reagent were added. It was painstakingly mixed and distilled water was used to increase the mixture to 100 ml, then kept for another 20 mins and read at 760 nm as milligram/g of tannic acid equivalent. $Y = 154.45x – 0.0485$, $R^2 = 0.9585$, where Y = tannic acid equivalent. x = absorbance

5.2.6 Estimation of flavonoids

Method of Ordon Ez *et al*., (2006) was used. 500 microliter of the sample and 500 microliter of 2% ethanol based Aluminum chloride liquid were added together. Kept for 60 minutes at 25^0C and read at 420 nm. Presence of yellow color specified that flavonoids were present. *Andrographis paniculata* leaf extract were quantified at 0.1 mg/ml. standard curve: $Y = x11.922$, $R^2 = 0.9955$. x = absorbance. Y = quercetin equivalent as mg/g of quercetin.

5.2.7 Estimation of total phenol

Using Wolfe *et al*., (2003) method with Folin-Ciocalteu reagent 1:10 v/v of Folin-Ciocalteu reagent was prepared with water and 5000 microliter of this was mixed with a portion of the sample and 4000 microliter (75 g/l) of Na Carbonate was added. Vortexed (15 seconds), kept for 30 min at 40°C and read at 765 nm using spectrophotometer. Using the standard curve: $Y = x14.885$, $R^2 = 0.9961$. x = absorbance, Y = gallic acid equivalent as milligram/g of gallic acid equivalent.

5.2.8 Total flavonols

Kumaran and Karunakaran (2007) method was used. 2000 microliter of ethanol based Aluminum chloride; 3.0 ml of fifty gram per liter of Na acetate liquid and 2.0 ml of the sample were added together. Kept for 150 minutes at 20°C and read at 440 nm. Using a formula according to standard curve $Y = 13.128x$, $R^2 = 0.9990$. x = absorbance, Y = quercetin equivalent and calculated as milligram/gram of quercetin equivalent.

5.2.9 Estimation of total proanthocyanidin

Oyedemi *et al*., (2010) method was used. 3000 microliter of vanillin-ethanol (4% v/v), 1500 microliter of HCL and 500 microliter of 1000 microgram per ml of the extract was put together. Vortexed allowed standing for 15 minutes at 25^0C and read at 500 nm. 0.1 mg/ml concentration was used for estimation. Then expressed using the standard curve formula is: $Y = 0.5825x$, $R^2 = 0.9277$ as mg/g of catechin equivalent.Y = catechin equivalent, x = absorbance

5.2.10 Nitric oxide scavenging assay

Andrographis paniculata leaf extract nitric oxide assay was done using Oyedemi *et al*., (2010) method. Different concentration 0.025 to 0.05 mg per ml of BHT, extract and

gallic acid was prepared. 2000 microliter of 10 mM of Na nitroprusside (0.5 mM PBS pH 7.4) was put in 500 microliter of each concentration and kept for 2.5 hour at 25°C. 1 ml of Griess reagent was added to 1 ml of incubated solution then incubated at RT for another 30 minutes and read at 540 nm. NO radical scavenging activity (%) = [(Absorbance of control – Absorbance of sample)] divided by (Absorbance of control) then multiplied by 100. Absorbance control is the absorbance of NO radicals + ethanol and Absorbance sample is the absorbance of nitric oxide radical plus extract or standard.

5.2.11 Ferric reducing power determination

Aiyegoro and Okoh (2010) method was used to determine *Andrographis paniculata* leaf extract reducing power. Different concentration of 0.025 to 0.5 of the extract and standard were prepared in DW, 2500 microliter of 0.2 M PBS (pH 6.6) and 2500 microliter of K 3Fe(CN)6 (1% w/v) was put in 1 ml of standard and extract. After which it was kept at 50°C for 20 minutes. 5 ml of 10 % w/v tricholoroacetic acid was put in and centrifuged at 3000 revolutions per minute for 10 minutes. 2500 microliter of the resulting supernatant was added to 2500 microliter of DW and 500 microliter of FeCl 3 (0.1% w/v). Then read at 700 nm against blank sample with spectrophotometer.

5.2.12 ABTS radical scavenging assay

Andrographis paniculata leaf extract ABTS activity was determined by Adedapo *et al*., (2008) method. Two stock solutions of 2.4 mM potassium persulfate and 7 mM ABTS were added together in volume by volume and kept in dark at 25^0C for 12 hour. There is formation of bluish greenish coloration that disappeared in the presence of antioxidant. The solution was diluted with 1000 microliter ABTS and 60 ml ethanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm and read 7 minutes after. 1ml of the solution was added to 1 ml of the extract at different concentration of 0.025 - 0.5 mg per ml and read with spectrophotometer at 734 nm. This formula was then used for the calculation: percentage inhibition = [(Absorbance of control –Absorbance of sample)] divided by [(Absorbance of control)] then multiplied by 100 the percentage inhibition of ABTS was calculated.

5.2.13 DPPH radical scavenging determination

Andrographis paniculata leaf extract was determined by Erdogan-orhan *et al.,* (2010) method. 0.025 to 0.5 mg/ml different concentration of the extract was prepared. 1 ml DPPH (0.135 mM) prepare with ethanol was added to 1 ml each concentration. After vortexing, it was kept dark for thirty minutes at 25^0C and was read using spectrophotometer at 517 nm. Control absorbance is optical density of DPPH plus methanol. Sample absorbance is optical density of DPPH radical plus standard or sample. Thus the formula absorbance control minus absorbance sample, then divided by absorbance of control and multiply by 100 was used to calculate the percentage DPPH scavenging activity.

5.2.14 Hydro-distillation using Clevenger-type apparatus

Andrographis paniculata dried leaf weighing about 100 g was hydro-distillated with the use of Clevenger-type apparatus equipped with a condenser. This is join to 5-L round bottom flask put in boiling water and boil for 180 minutes. Steam was used to transport the oil vapour into condenser for cooling (below 30°C) resulting to formation of two non-mixing liquid phases i.e. the upper layer consisting of the volatile oil and the lower hydrosol portion which was separated with dividing funnel. The extracted oils were harvested into small glass vessels which were sealed.

5.2.15 Gas chromatography/Mass spectrometry (GC-MS) analysis of volatile oils

Agilent 7890 gas chromatography complex fitted with a Zebron-5MS (cross-linked 5%- phenyl methyl polysiloxane) column (ZB-5MS 30 m X 0.25 mm X 0.25 µm) and Agilent 5977A Mass selective sensor system was used. Using 1 µL injections Helium grade serve as a transporter of gas at a movement of 2 mL per minute. Volatile oil extracted was aspirated into a needle and fixed to gas chromatograph opening. Temperature of ion and injector were retained at 280°C and oven at 70°C. After which it was ramped at 15°C/minutes to 120°C, 10°C/minutes to 180°C and 20°C/minutes to 270°C (allowed to stay for 3 minutes). ChemStation was used to gather the data. Retention times and mass spectra were compared with National Institute of Standards and Technology (NIST11.L) library data.

5.3   Results

5.3.1   *Andrographis paniculata* phytochemical compounds

Phytochemical analysis of *Andrographis paniculata* leaf extract revealed tannins, total flavonoids, phenols, total flavonols and total proanthocyanidins (Table 5.1).

Table 5.1: Phytochemical analysis of *Andrographis paniculata* leaves

Phytochemicals (mg/g)	Extract
Tannins	1.33±0.14 [a]
Total flavonoids	6.71±1.82 [b]
Total phenol	13.77±1.22 [c]
Total flavonols	38.16±1.73 [b]
Total proanthocyanidin	46.35±8.58 [d]

a expressed as milligram tannic acid per gram of the extract, b expressed as milligram quercetin per gram of the extract, c expressed as milligram gallic acid per gram of the extract, d expressed as milligram catechin per gram of the extract.

5.3.2 *In vitro* antioxidant activity of *Andrographis paniculata* leaves extract.

Figure 5.1 shows the reducing power of the ethanol leaf extract of *Andrographis paniculata* in evaluation with the rutin and vitamin E. Ferric reducing effect of the extract was lesser than that of vitamin E but higher than that of rutin.

Figure 5.2 shows the DPPH scavenging potential of *Andrographis paniculata* leaf extract The IC50 value of *Andrographis paniculata* leaf extract was < 0.025 mg/ml, Vit E is 0.08 mg/ml and Rutin < 0.025 mg/ml.

Figure 5.3 shows the percentage inhibition of ABTS radical by *Andrographis paniculata* leaf extract. ABTS percentage inhibition by the *Andrographis paniculata* leaf extract likened with the rutin and vitamin E.

Figure 5.4: shows the radical scavenging activity of the *Andrographis paniculata* leaf extract. The IC50 value of *Andrographis paniculata* leaf extract was is 1.05mg/ml. Vit E is 1.2 mg/ml and Rutin < 0.025 mg/ml.
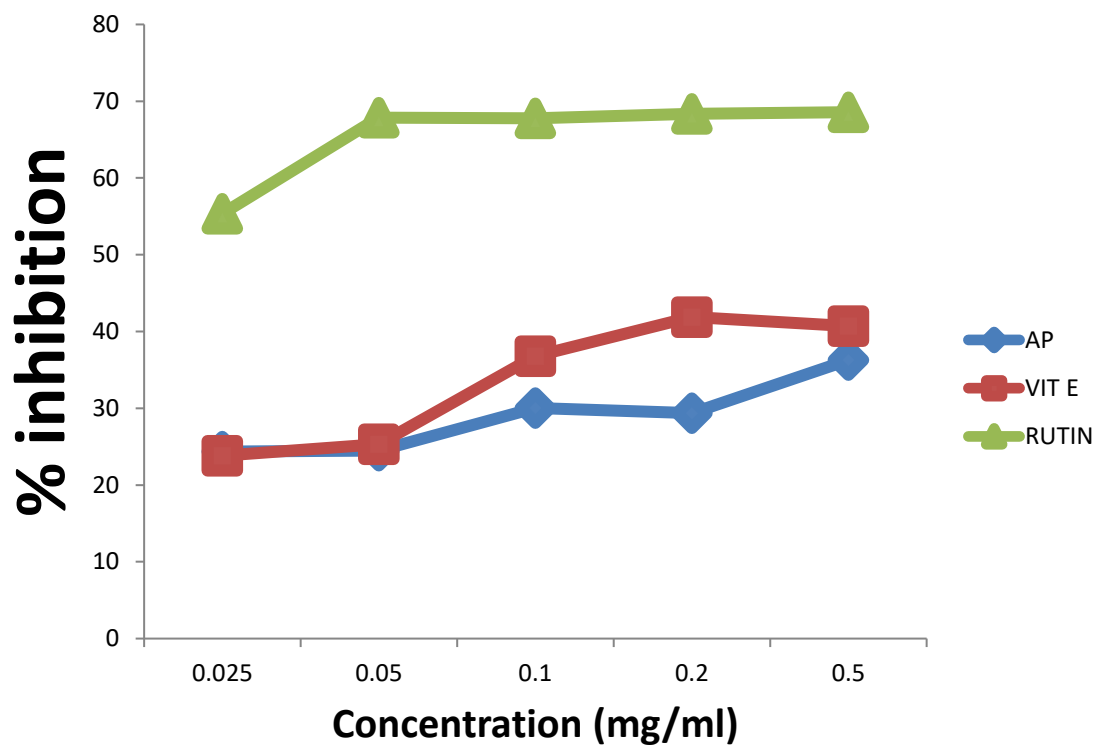
Figure 5.1: Nitric oxide radical scavenging action of *Andrographis paniculata* leaf extract
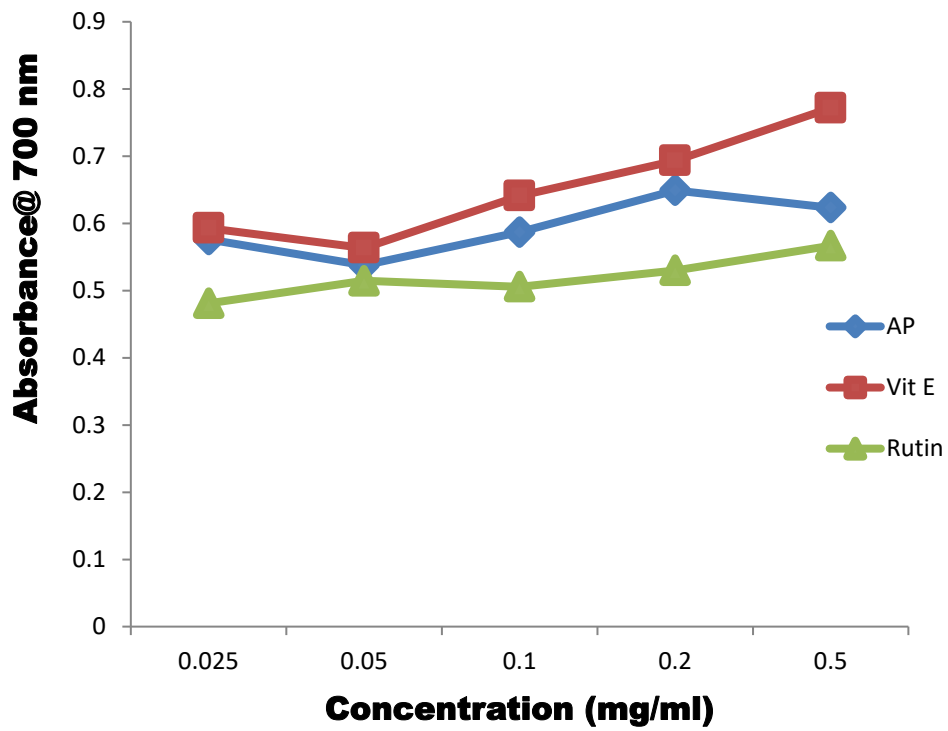
Figure 5.2: Reducing power of *Andrographis paniculata* leaf extract
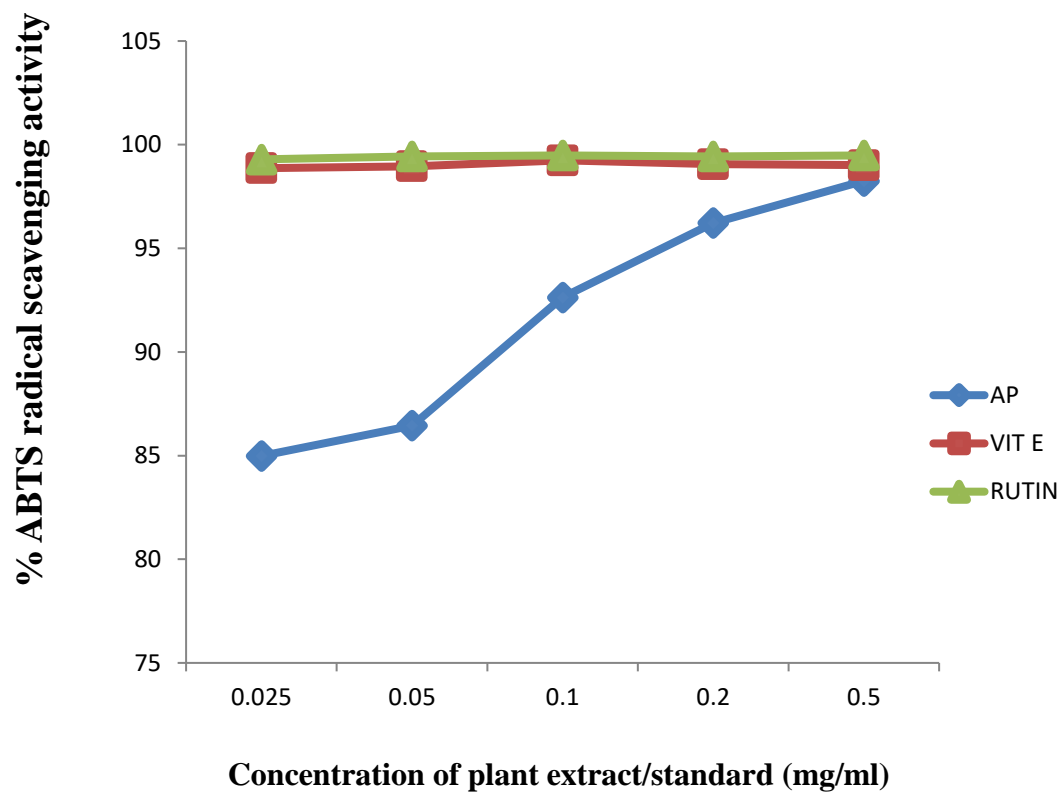
Figure 5.3: ABTS radical scavenging action of *Androgxaphis paniculata* leaf extract
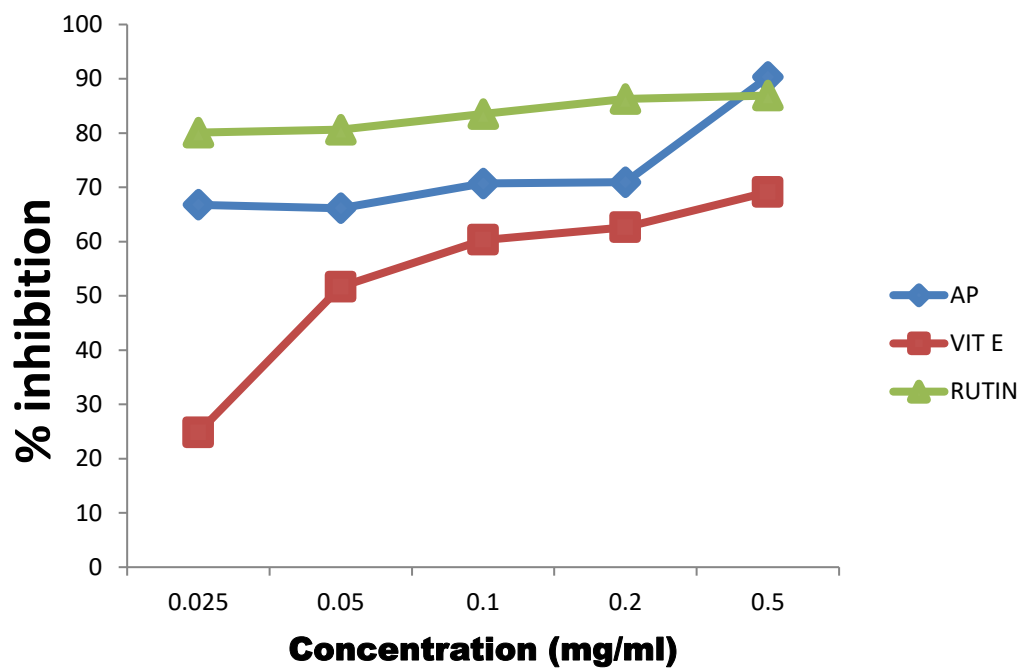
Figure 5.4: DPPH radical scavenging action of *Andrographis paniculata* leaf extract

5.3.3 GC-MS analysis result

The GC-MS analysis of volatile oils derived from *Andrographis paniculata* leaf extract by hydro distillation revealed the presence of 1 major compound benzenepropanoic acid elicited at 3.296 retention time and 0.74 area percentage of compounds present in *Andrographis paniculata*
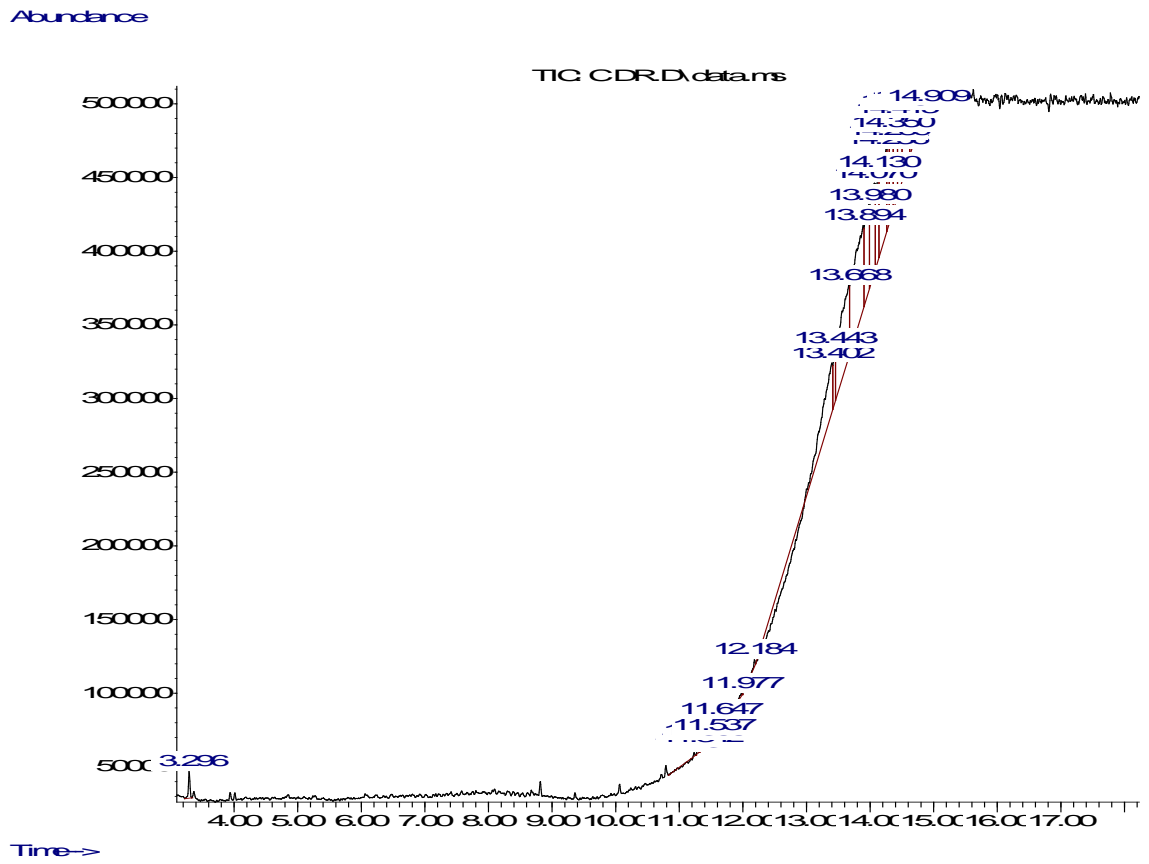
Figure 5.5: GC-MS Chromatogram of Essential Oils of AP

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Table 5.2: GC-MS of Essential Oil of AP

S/N	Retention time	Compound name	Formula	Molecular weight
1	3.296	Benzenepropanoic acid	$C_9H_{10}O_2$	150.1745 g/mol

5.4 Discussion

Medicinal plants are useful for healing and curing of human and animal ailments because of their phytochemical components (Nostro *et al.*, 2000). Phytochemical quantitative estimation of *Andrographis paniculata* leaf extract indicated the presence of Tanins, Total flavonoids, Total phenols, Total flavonols and Total proanthocyanidin. The tanins content in the *Andrographis paniculata* leaf extract was 1.33 mg tannic acid per gram of the extract, total flavonoids and flavonols were 6.71 and 38.16 mg quercetin per gram of the extract respectively, total phenol was 13.77 mg gallic acid per gram of the extract and total proanthocyanidin was 46.45 mg catechin per gram of the extract. The concentrations of these polyphenols vary depending on many environmental factors and the extraction procedures employed (Del-Castillo-Alonso *et al.*, 2016; Mendes Lopes *et al*., 2016; Top *et al.*, 2017). In this study *Andrographis paniculata* was found to be very rich in different polyphenolic components. In medicinal sciences beneficial importance of several phytochemical compounds has been reported. Flavonoids are known to adjust the body's response to virus and allergies. It has anti-allergic, anti-microbial and anti-cancer activities and anti-inflammatory and that is the reason it is called nature's biological response modifiers (Ntchapda *et al*., 2016). Phenol and flavonoid are natural high-level antioxidants that act by removing reactive oxygen species and neutralize free radicals (Jacob and Vigasini, 2017). Flavonoids have been known to be the most diverse and widespread groups of naturally occurring compounds. They are capable of exhibiting *in vitro* antioxidant activities. Proanthrocynidin are known to be a powerful antioxidant.

Reactive oxygen can cause deleterious effects to the general functioning of the system and thus the tests of the ability of a substance to scavenge radical species may be relevant in the assessment of antioxidant action (Sánchez-Moreno, 2002). *In vitro* radical scavenging activities performed showed promising results owing to the rich polyphenol profiles of *Andrographis paniculata*. The results showed that the extracts demonstrated powerful antioxidants activity compared to the standard (Rutin and Vit E) at the concentrations used.

The results of phytochemical of *Andrographis paniculata* revealed high content of polyphenolic compounds which are known to possess reducing ability and up-regulate antioxidant defenses.

DPPH activity of antioxidants may be due to their protonating capability (Marín *et al*., 2016). The result shows that the extract had proton donating potential and could act as free radical removers. Therefore, the extract could be used as therapeutic agent in free radical related diseases.

Nitric oxide is known as a mediator of some physiological process like inhibition of platelet aggregation and smooth muscle relaxation. Nitric oxide produce free radicals that act as a defense but excessive production of the free radicals could exacerbate inflammatory disease process (Jakubowska *et al*., 2016).

Based on these assays, it appears Rutin exhibited the strongest antioxidant activity. The extract however exhibited stronger antioxidant activities than Vit. E. These results corroborate the use of *Andrographis paniculata* for management of several ailments.

The GC-MS analysis of essential oils derived from *Andrographis paniculata* leaf by hydro distillation revealed the presence of one major compound, benzenepropanoic acid elicited at 3.296 retention time and 0.74 area percentage of compounds present in *Andrographis paniculata*. Composition of essential oil of plants growing at different localities largely depends on the geographical conditions. Benzenepropanoic acid are known to Lower fatty acid content in the liver, plasma and also improves tissue insulin sensitivity (Al-lahham *et al.,* 2010).

5.5   Conclusion

This present study revealed that *Andrographis paniculata* possess *in vitro* antioxidant effects and this may be due to the polyphenolics present in the extracts. *Andrographis paniculata* leaf is therefore a potential good source of natural antioxidant. This study has contributed to the validation of the medicinal potential of extracts of the *Andrographis paniculata* leaf.

CHAPTER SIX

6.1   Conclusion and Recommendation

This study suggests that isoproterenol-induced myocardial infarction increased markers of oxidative stress, inhibited enzymatic and non-enzymatic antioxidant enzyme. It also affects the normal electrocardiogram and cause changes in haemodynamic parameters. Meanwhile, treatment with enalapril and EEAP decreased indicators of oxidative stress, increased enzymatic and non-enzymatic antioxidant enzymes, reversed changes in hemodynamics parameters. Thus enalapril and EEAP ameliorated isoproterenol induced oxidative stress through its antioxidant properties.

EEAP has both protective and curative effect on cisplatin-induced nephrotoxicity. Therefore, this plant could be proposed as a therapeutic agent for the reduction of renal toxicity caused by cisplatin.The anticancer effect of the plant has been reported and its concurrent administration with cisplatin could lead to synergism.

These findings indicate that *Andrographis paniculata* could be used as a potential phytochemical plant that could prevent cardiovascular diseases and nephrotoxicity that occur with the use of chemotherapeutic agents.

CONTRIBUTIONS TO KNOWLEDGE

- EEAP has both protective and curative effects on cardiac and renal toxicity

- EEAP has anti-oxidant activities

- This study establish that the plant is more effective in the treatment of an established myocardial infarction (post treatment)

- EEAP is a potential source of drug candidate for cardiovascular diseases

- EEAP blunts CP-induced renal damage hence could serve as adjunct in cancer chemotherapy

- This study provided justification for the use of *Andrographis paniculata* in many communities for therapeutic purpose.

FURTHER STUDIES

1   Anticancer properties of *Andrographis paniculata*

2   Purification and isolation of constituents responsible for cardiac and renal protective properties

3   Patenting of the constituents

4   Cardiac and renal protective properties of the isolates

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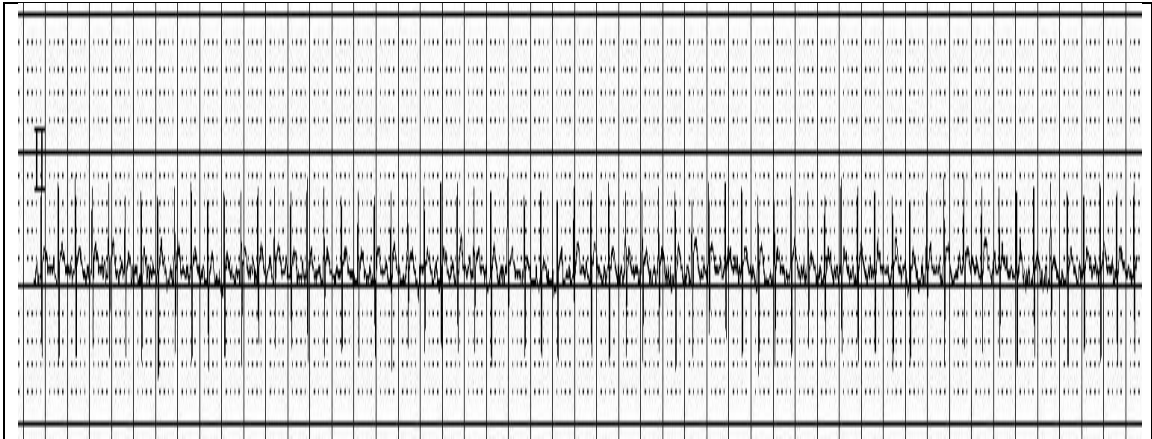
Appendix 1: Effect of *Andrographis paniculata* ethanol leaf extract on QRS wave in isoproterenol induced myocardial infarction using rats as a model

Group A
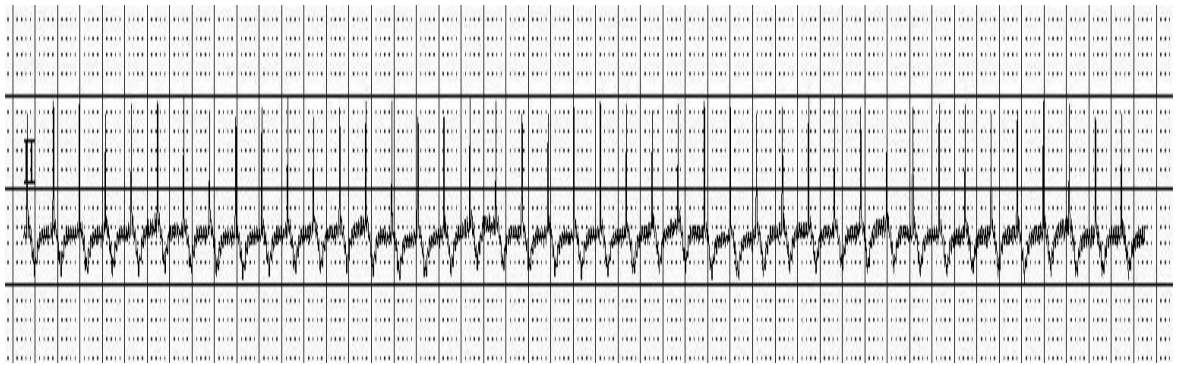
Appendix 2: Effect of *Andrographis paniculata* ethanol leaf extract on QRS wave in isoproterenol induced myocardial infarction using rats as a model

Group B

Appendix 3: Effect of *Andrographis paniculata* ethanol leaf extract on QRS wave in isoproterenol induced myocardial infarction using rats as a model

Group C

Appendix 4: Effect of *Andrographis paniculata* ethanol leaf extract on QRS wave in isoproterenol induced myocardial infarction using rats as a model

Group D
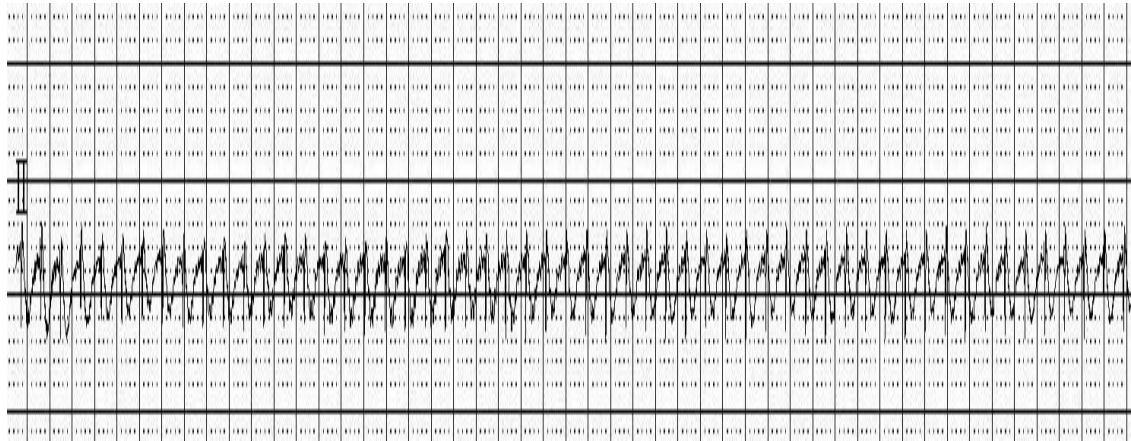
Appendix 5: Effect of *Andrographis paniculata* ethanol leaf extract on QRS wave in isoproterenol induced myocardial infarction using rats as a model

Group E

Appendix 6: Effect of *Andrographis paniculata* ethanol leaf extract on QRS wave in isoproterenol induced myocardial infarction using rats as a model

Group F
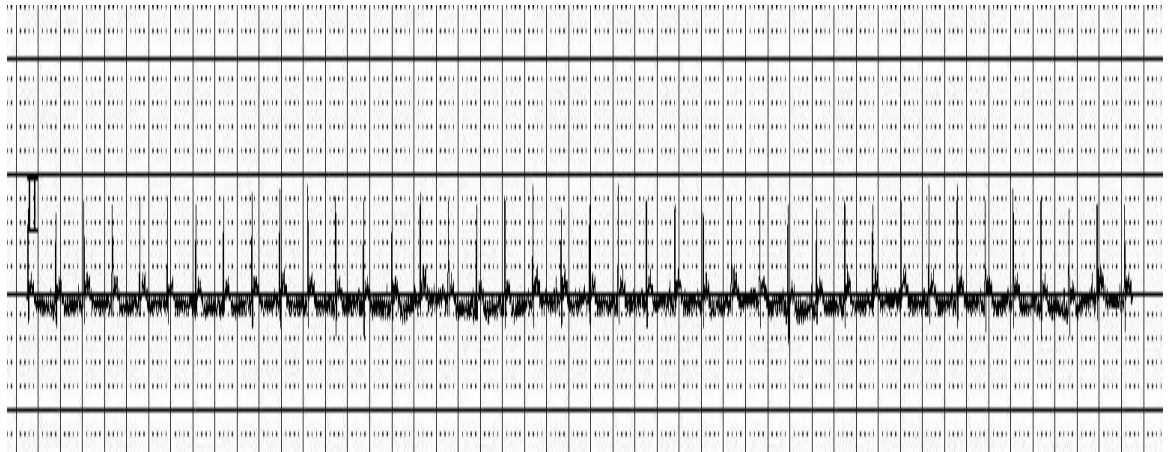
Appendix 7: Effect of *Andrographis paniculata* ethanol leaf extract on QRS wave in isoproterenol induced myocardial infarction using rats as a model

Group G

June 9, 2016

Dr. Adeoye, Bisi Olajumoke,
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Ibadan.

NOTICE OF ETHICAL APPROVAL FOR A RESEARCH PROJECT PROPOSAL

Your letter on the above requesting for Ethical Approval of your proposal titled:"*Cardioproctective, renoprotective, antioxidant and anti-inflammatory effects of ethanolic leaf extract of Andrographis paniculata on isoproterenol induced myocardial injury and cisplatin induced renal injury in Rats.*" *refers*: strictly as outlined in your proposal submitted for assessment.

Please quote **UI-ACUREC/App/2016/030** as reference for this approval.

You are to note that **UI-ACUREC** reserves the right to monitor and conduct compliance visit to your research site without previous notification.

Thank you.

09/06/2016

Prof. V.O. Taiwo
Chairperson, UI-ACUREC

Cc: Dean, FVM
            Director, Research Management Office

Appendix 8: Ethical approval.

Enalapril Confers Protective Effect on Isoproterenol-Induced Myocardial Infarction in Rats through Downregulation of Cardiac Troponin, C-reactive Protein, Upregulation of IL-10β as Well as Anti-Oxidant and Anti-inflammatory Activities

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Authors' contributions

This work was carried out in collaboration between all authors. Authors BOA, ERA and AAO carried out the experiment. Authors TOA, OAA and TOO measured the ECG and blood pressure while author AAA designed and drafted the final manuscript. All authors read and approved the final manuscript before its submission.

Article Information

Original Research Article

ABSTRACT

Myocardial infarction is an irreversible death of heart muscle secondary due to prolonged lack of oxygen supply. The present study was designed to evaluate the protective effect of enalapril in isoproterenol-induced myocardial infarction in rats using changes in haemodynamic, biochemical, histopathological and immunohistochemistry parameters. Twenty-one male Wistar rats divided into

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three groups were used where the control (group A) was administered for normal saline which continued for 7 days, group B animals received normal saline for 7 days and thereafter isoproterenol (ISO) at 85 mg/kg on day 8 and 9. Group C animals were pretreated with enalapril (10 mg/kg) for 7 days and thereafter received ISO on day 8 and 9. On day 10, the blood pressure change in the animals were measured and thereafter sacrificed by cervical dislocation. The heart of each rat was removed, homogenized and used to assay for some oxidative stress markers and some antioxidant parameters. In this study, ISO caused myocardial infarction as seen by significant decrease in systolic, diastolic and mean arterial pressure but was corrected by enalapril. Enalapril caused significant increase in the levels of SOD, GPx, GST and GSH but significant decrease in MDA content and H_2O_2 generation. But reverse was the case for group B animals. Immunohistochemistry showed that ISO caused higher expressions of cardiac C-reactive protein (CRP) and cardiac troponins 1 (CTn1) and decrease in IL-10β but vice-versa for enalapril. No histopathological changes were recorded for enalapril. The study thus showed that enalapril significantly exhibits cardioprotective effects.

1. INTRODUCTION

Human health is being seriously threatened by cardiovascular diseases (CVD), which have been regarded as the main cause of death throughout the world [1-3]. Myocardial infarction (MI) is a common presentation of ischemic heart disease (IHD) and remains the major cause of death in the developed world. Though rapid advancements have been made in the treatment of coronary artery diseases (CAD), MI is still a major pathological issue worldwide [4]. Increased myocardial metabolic demand and decreased supply of oxygen as well as nutrients via the coronary circulation to the myocardium brings about myocardial infarction hence leading to cell injury. This pathological heart condition is one of the most lethal manifestations of cardiovascular diseases. Acute myocardial infarction or heart attack occurs when blood stops flowing to part of the heart leading to injury to the heart muscle due to the fact the heart is not receiving enough oxygen [5-9].

Isoproterenol [1–(3, 4–dihydroxyphenyl)–2–isopropylaminoethanol hydrochloride] (ISO) a synthetic catecholamine is a β-adrenergic agonist that is very important in the regulation of myocardial contractility and metabolism. It serves as a standard model for the study of potentially beneficial effects of numerous drugs on cardiac function [10,11]. ISO induces myocardial injury in rat because of the alteration in the physiological balance between production of free radicals and antioxidative defence system [12]. It thus causes the acute condition of myocardial necrosis, which can lead to cardiac dysfunctions, increased lipid peroxidation, altered activities of cardiac enzymes and antioxidants [13]. It has been observed that the pathophysiological and morphological changes observed in ISO-treated rats are similar to those observed in human MI [14].

Enalapril an Angiotensin-converting-enzyme inhibitor (ACE inhibitor) is a drug used primarily for the treatment of high blood pressure and congestive heart failure where it can be used alone or in combination with other antihypertensive agents. ACE inhibitors have also been found to be useful for other cardiovascular and kidney diseases including acute myocardial infarction, diabetic nephropathy, and cardiac failure [15]. The mechanism of action of ACE inhibitors involves reduction of the activity of the renin-angiotensin-aldosterone system (RAAS) [16].

In recent times, a novel strategy has been employed in drug discovery. It is the use of known and approved drugs and compounds for newer indications. This is termed drug repurposing. In this study, Isoproterenol was used to induce acute myocardial infarction and enalapril was then used to ameliorate this and then to see if it could serve as a repurposed drug for myocardial infarction.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Isopreterenol, enalapril, Tween 80, Biurett's reagent, hydrogen peroxide, hydrochloric acid, sulphuric acid, xylenol orange, potassium dichromate, O-diasinidine, sodium potassium tartrate, copper sulphate, ethanol, sodium azide, 2-dichloro-4-nitrobenzene (CDNB) Greiss

reagent, phosphoric acid, sodium hydroxide, N 1-naphthyl ethylenediamine, sulphanilamide, distilled water, phosphate buffer saline, creatinine reagent, copper sulphate, tri chloro acetate, reduced glutathione (GSH), thiobarbituric Acid (TBA), trichloroacetic acid (TCA), ammonium ferrous sulphate, glacial acetic acid, potassium iodide, sorbitol, Ellman's reagent (DTNB), ethanol, urea reagent. All other chemicals used were of analytical grade and obtained from British Drug Houses (Poole, Dorset, UK). All other chemicals, reagents and drugs used were of analytical grade.

2.2 Experimental Animals

All experiments and protocols described in present study were approved by the UI-ACUREC. Twenty one (21) male Wistar rats weighing between 90 to160 g were obtained from the Experimental animal unit of the Faculty of Veterinary Medicine, University of Ibadan for the experiment. They were allowed free access to standard rat pellets and fresh water *ad libitum*. The rats were housed in the animal house unit of the Department of Veterinary Pharmacology and Toxicology, University of Ibadan with a 12 hour light duration. Pre-conditioning of the rats was done for two weeks before commencement of the experiment. The institutional approval was given to this study and the number is UI-ACUREC/App/2016/030

2.3 Cardioprotective Study

The animals were randomly divided into three (3) groups with seven (7) animals in each group, and the treatment was as follow: Animals in the control (group A) were administered normal saline, group B; isoproterenol at 85 mg/kg, while group C animals were pretreated with enalapril orally (10 mg/kg) for 7 days and thereafter administered ISO (85 mg/kg) subcutaneously on day 8 and 9. Blood pressure values of all the animals were carried out on day 10. At the end of the experimental period, blood samples were collected for haematology and serum chemistry before the rats were sacrificed by cervical dislocation. The serum in plain bottles was rapidly centrifuged at 4000 revolutions per minute (rpm) for fifteen (15) minutes and processed for determination of serum myeloperoxidase, total protein, and xanthine oxidase, AST, ALT and nitric oxide. The heart of each rat was carefully removed and homogenized on ice and then used to assay for

some oxidative stress markers and antioxidant parameters. Baseline cardiovascular parameters were obtained prior to the commencement of the experiment. The equipment used was a non-invasive tail cuff BP monitor, the 6-channel CODA blood pressure monitor for rats and mice. The haemodynamic parameters assessed were: the systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) and were determined indirectly in nonanaesthesised rats, by tail plethysmography with the use of an electrosphygnomanometer (CODA, Kent Scientific, USA). The average of at least nine most consistent readings, taken in the quiescent state, following acclimatization, was recorded per animal.

Blood samples for serum chemistry were collected from the rats through retro-orbital vein after which the animals were sacrificed by cervical dislocation.

2.4 Preparation of Tissue Homogenate

The heart tissues of the rats were harvested on ice, rinsed with normal saline and homogenized in aqueous potassium buffer (0.1 M, pH 7.4) and the homogenate centrifuged at 12,000 rpm (4°C) for 15 min to obtain the supernatant fraction.

2.5 Determination of Biochemical Assay

Biuret method as described by Gornal et al. [17] was used to determine the protein concentrations of the various samples with a slight modification. To prevent precipitation of Cu^{2+} ions as cuprous oxide potassium iodide was added to the reagent. To determine the concentration of reduced glutathione the method of Beutler et al. [18] was used while glutathione peroxidase (GPX) activity was measured by the method of Rotruck et al. [19]. In this case, hydrogen peroxide was used as substrate to oxidize reduced glutathione to oxidized glutathione (GSSG). Estimation of Glutathione S-transferase (GST) was by the method of Habig et al. [20] using 1-chloro-2, 4-dinitrobenzene as substrate. Superoxide dismutase (SOD) assay on the other hand was carried out by the method of Misra and Fridovich [21]. MDA content was measured in the heart as an index of lipid peroxidation [22]. Hydrogen peroxide generation was measured using Wolff's [23] method while the determination of Sulfhydryl (Thiol) content was by-the method of Ellman [24]. Nitric oxide was quantified as previously described [25].

2.6 Histopathology

Small slices of the heart were collected in 10% buffered formalin for proper fixation and after the tissues have been processed and embedded in paraffin wax, sections that were about 5-6 μm thick were made and stained with haematoxylin and eosin for histopathological examination [26].

2.7 Immunohistochemistry of Cardiac Troponins-1, CRP and IL-10

The heart tissues obtained from the rats were paraffin embedded and then used for immunohistochemistry. Paraffin sections were melted at 60℃ in the oven but the dewaxing of the samples in xylene was followed by passage through ethanol of decreasing concentration (100-80%). Peroxidase quenching in 3% H_2O_2/methanol was carried out with subsequent antigen retrieval performed by microwave heating in 0.01 M citrate buffer (pH 6.0) to boil. All the sections were blocked in normal goat serum (10%, HistoMark®, KPL, Gaithersburg MD, USA) and probed with cardiac troponins 1, CRP antibody and I IL-10β (Abclonal®), 1:375 for 16 h in a refrigerator. 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®, KPL, Gaithersburg MD, USA).

Diaminobenzidine (DAB, Amresco®, USA) was used to enhance the reaction product for 6 – 10 min and counterstained with high definition haematoxylin (Enzo®, NY - USA), and was thereafter dehydrated in ethanol. Once the slides were covered with cover slips, they were sealed with resinous solution. The immunoreactive positive expression of CRP, cardiac troponin and IL-10β 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®, Touptek Photonics, Zhejiang, China).

2.8 Statistical Analysis

All values were expressed as mean ± standard deviation (SD). The test of significance between two groups was estimated by Student's t-test. One-way Analysis of Variance (ANOVA) with Tukey's post-hoc test using Graph pad prism 5.0 was also performed with p-values < 0.05 considered statistically significant.

3. RESULTS

In this study, ISO caused significant decreases in the levels of SBP, DBP and MAP while enalapril (ENA) caused significant increase though not to the same extent as the control (Figs. 1-3). The results of haematological analysis showed that ISO caused significant increases in the levels of WBC, PCV, MCV and MCH while ENA caused significant decrease in WBC and no changes relative to ISO (Table 1). ISO also caused significant increases in the levels of AST and ALT while ENA caused significant decreases in the levels of these enzymes. On the other hand, while ISO caused significant decrease in the level of NO, ENA caused significant increase (Table 2). ISO caused significant increases in the levels of oxidative markers such as MDA, H_2O_2 and MPO while ENA caused significant decreases in the levels of these markers in a similar fashion to the control (Figs. 4-6). Again, while ISO caused significant decrease in the levels of protein thiols and non-protein thiols, ENA caused a significant increase in the levels of these molecules (Figs. 7 and 8). The result also showed that ISO caused significant decrease in the levels of anti-oxidant markers such as SOD, GPx, GST and GSH but reverse is the case for ENA (Figs. 9-12). Histopathological examinations showed that while there is severe infiltration of inflammatory cells into the cardiac tissue, there was no visible lesion seen in the ENA and control groups (Fig. 13). The immunohistochemical analysis showed that there were high expressions of cardiac troponin and CRP in ISO group but lower expression of these proteins in ENA and control group (Figs. 14 and 15). In the case of IL-10β, there was low expression of this protein in ISO group but higher expression in ENA and control group (Fig. 16).

4. DISCUSSION

Myocardial infarction (MI), one of the main causes of death from cardiovascular disease is defined as an acute condition of necrosis of the myocardium and it occurs as a result of imbalance between coronary blood supply and myocardial demand [27]. MI is known to cause local inflammation and apoptosis and this can result in cardiomyocyte damage [28]. ISO induces cardiac necrosis by several mechanisms, including increased oxygen consumption, poor oxygen utilization, increased calcium overload and accumulation, altered myocardial cell metabolism, increased myocardial cAMP levels, deranged electrolyte

milieu, altered membrane permeability, intracellular acidosis, and increased levels of lipid peroxides [11]. The pathophysiological changes that occurred in heart following isoproterenol administration in rats are comparable to those taking place in human myocardial infarction [29].
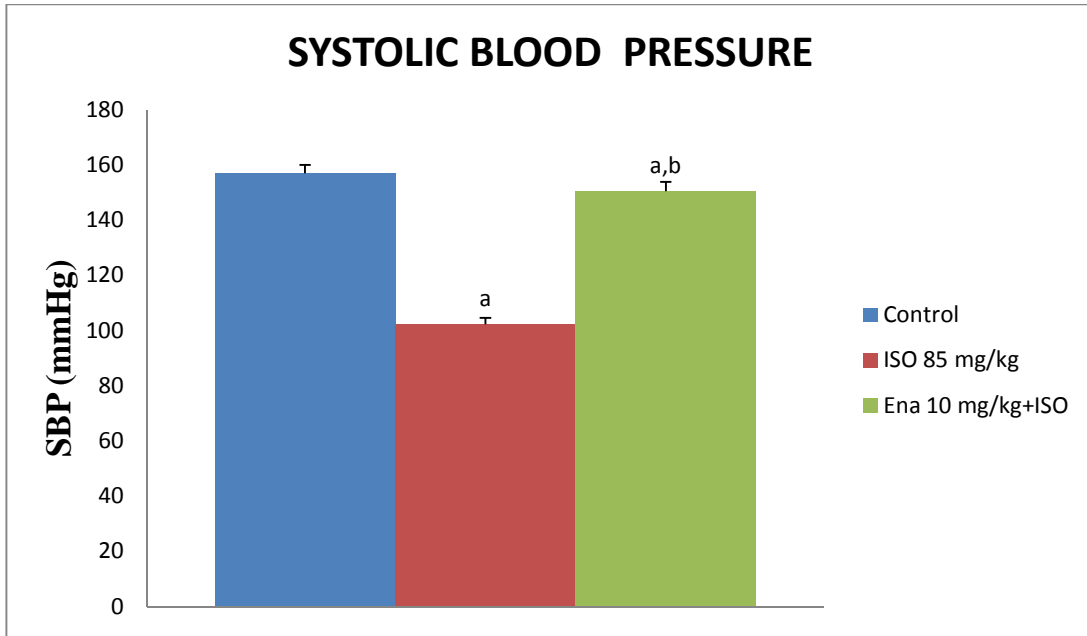
Fig. 1. Effect of enalapril on SBP in isoproterenol induced myocardial infarction using rats as a model. The superscript 'a' showed that ISO caused significant decrease when compared to control while superscript 'b' showed significant decrease when compared with ENA (n=7)
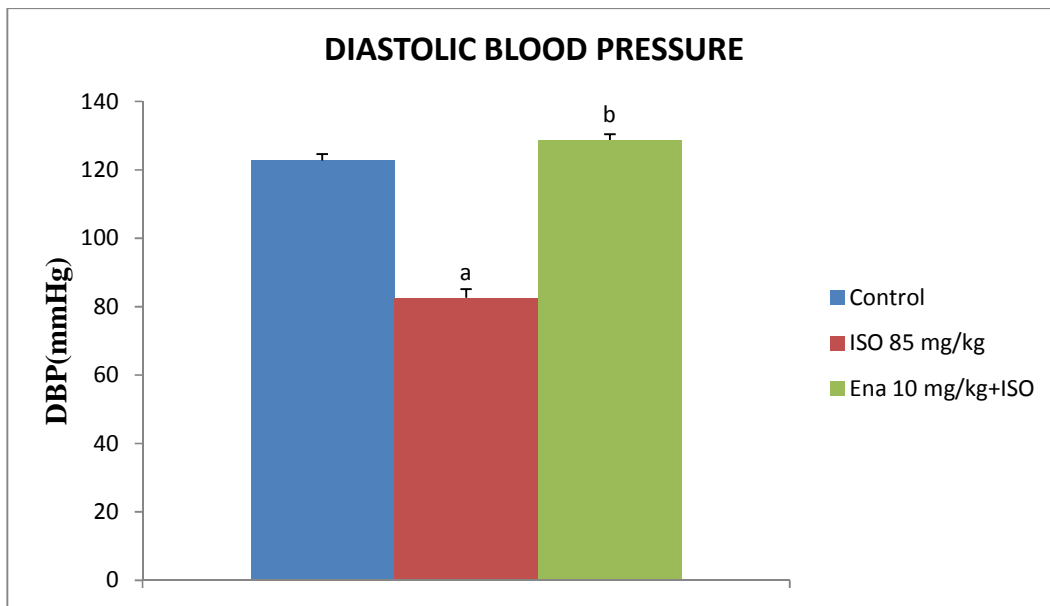
Fig. 2. Effect of enalapril on DBP in isoproterenol induced myocardial infarction using rats as a model. The superscript 'a' showed that ISO caused significant decrease in the level of this parameter compared to control while 'b' showed that ENA caused significant increase relative to control and ISO groups (n=7)

Fig. 3. Effect of enalapril MAP in isoproterenol-induced myocardial infarction using rats as a model. The superscripts showed that ISO caused significant decrease relative to ENA and control groups (n=7)

MDA

Fig. 4. Effect of enalapril on lipid peroxidation in isoproterenol-induced myocardial infarction using rats as a model (n=5). Values are presented as mean ± standard deviation. Grp A (Control), Grp B (ISO treated only), Grp C (ENA+ 85 mg/kg ISO). The superscript (a) showed that ISO caused significant decrease in the level of this parameter compared to control while (b) showed that ENA caused significant increase relative to control and ISO groups

Table 1. Effects of enalapril on RBC, WBC, HB, PCV, MCV, MCH and MCHC in isoproterenol-induced myocardial infarction using rats as a model (n = 7)

Parameters	Control	ISO	Enalapril
RBC (×1012/L)	4.75±0.90	4.96±0.43	5.03±0.69
WBC (103/µL)	5.47±0.38	6.71±1.13[a]	4.68±1.68 [b]
HB (g/dl)	13.33±1.40	15.15±1.84	14.95±1.62
PCV (%)	45.75±4.65	54.25±4.25 [a]	50.25±3.10
MCV (fl)	83.88±9.03	127.33±30.12 [a]	98.87±22.76
MCH (pg)	26.41±3.48	38.64±8.08 [a]	26.05±2.25
MCHC (%)	29.97±2.05	27.41±2.38	30.79±2.37

Values are mean ± SD, n =5, [a] - p < 0.05 compared with control, [b] - p < 0.05 compared with ISO.
The superscript (a) showed that ISO caused significant decrease in the level of this parameter compared to control while (b) showed that ENA caused significant increase relative to control and ISO groups

Table 2. Effects of enalapril on ALT, AST and NO in isoproterenol-induced myocardial infarction using rats as a model (n=7)

Parameters	Control	ISO	Enalapril
ALT	14.51±0.02	14.67±0.05[a]	14.41±0.05[ab]
AST	19.91±0.01	19.97±0.02[a]	19.87±0.02[ab]
NO	4.11±0.68	1.72±0.47[a]	2.67±0.71[ab]

Values are mean ± SD, n =5, [a] - p< 0.05 compared with control, [ab] - p< 0.05 compared with ISO.
The superscript 'a' showed that ISO caused significant decrease in the level of this parameter compared to control while 'b' showed that ENA caused significant increase relative to control and ISO groups

Fig. 5. Effect of enalapril on hydrogen peroxide generation in isoproterenol-induced myocardial infarction using rats as a model (n=5). Values are presented as mean ± standard deviation. Superscript 'a' indicates significant difference (p< 0.05) when compared with control (Grp A), whereas superscript 'b' indicates significant difference (p < 0.05) when compared with ISO treated only (Grp B). Grp A (Control), Grp B (ISO treated only), Grp C (ENA+ 85 mg/kg ISO)
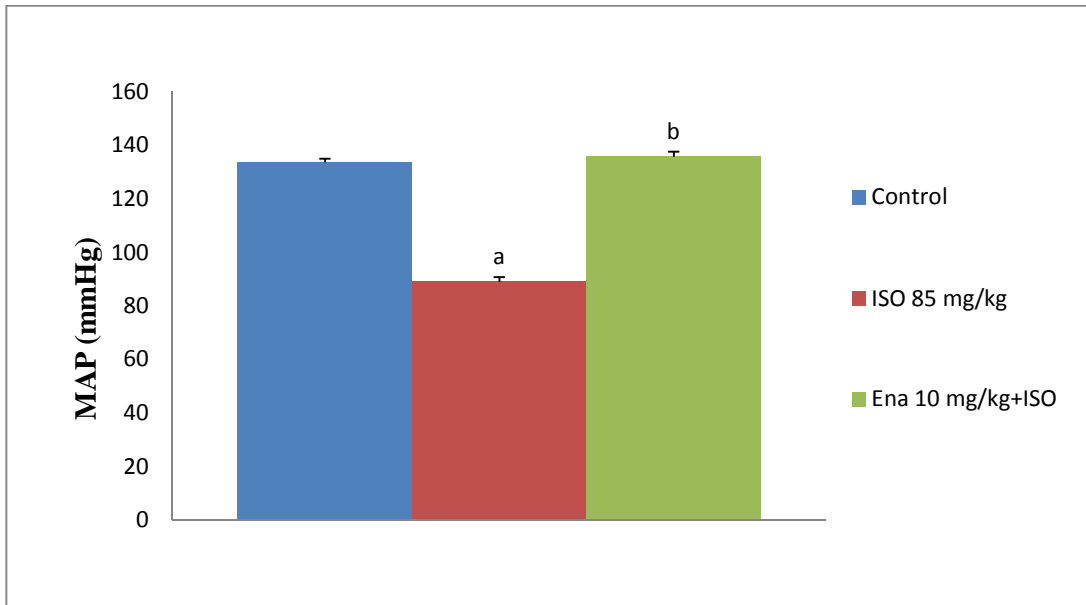
Fig. 6. Effect of enalapril on myeloperoxidase in isoproterenol-induced myocardial infarction using rats as a model (n=5). The superscript '*a*' showed that ISO caused significant increase in the level of this parameter when compared to the control and ENA groups
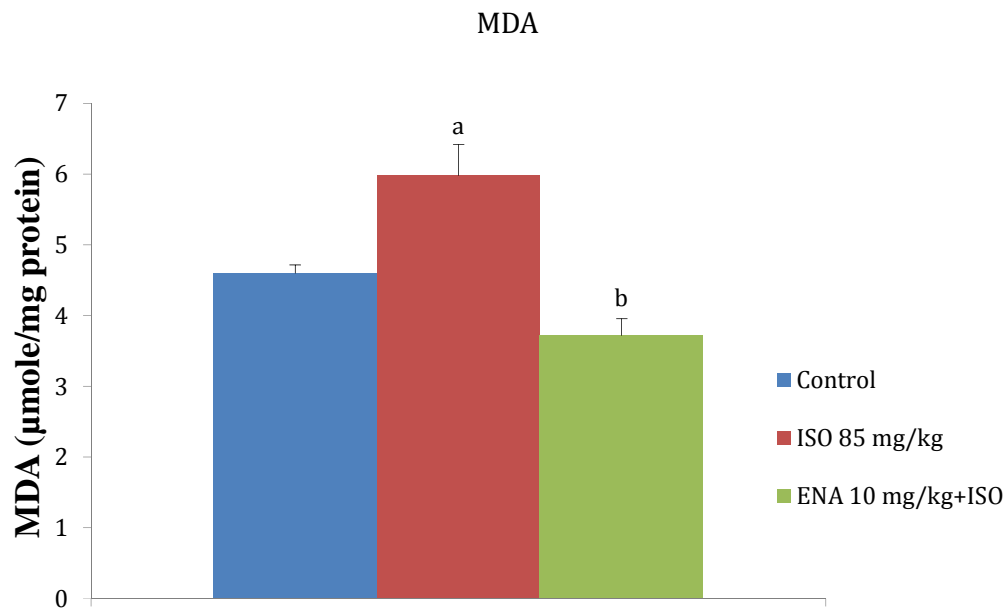

Fig. 7. Effect of enalapril on protein thiol in isoproterenol-induced myocardial infarction using rats as a model (n=5). Values are presented as mean ± standard deviation. Superscript 'a' indicates significant difference (p<0.05) when compared with control (Grp A), whereas superscript 'b' indicates significant difference (p<0.05) when compared with ISO treated only (Grp B). Grp A (Control), Grp B (ISO treated only), Grp C (ENA+ 85 mg/kg ISO)
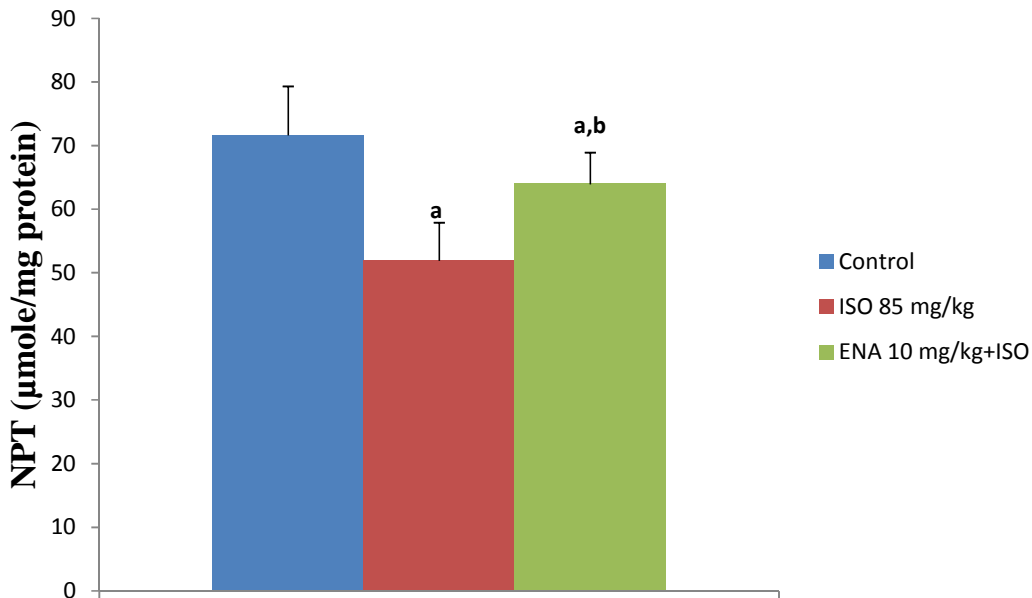
Fig. 8. Effect of enalapril on non-protein thiol in isoproterenol-induced myocardial infarction using rats as a model (n=5). Values are presented as mean ± standard deviation. Superscript 'a' indicates significant difference (p<0.05) when compared with control (Grp A), whereas superscript 'b' indicates significant difference (p<0.05) when compared with ISO treated only (Grp B). Grp A (Control), Grp B (ISO treated only), Grp C (ENA+ 85 mg/kg ISO)
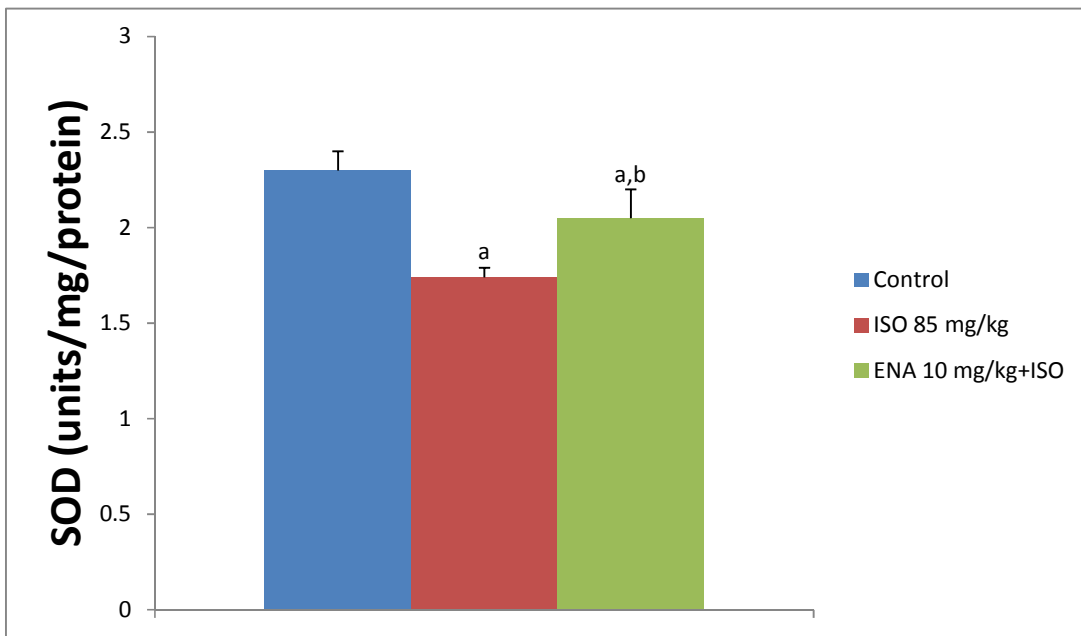
Fig. 9. Effect of enalapril on superoxide dismutase enzyme in isoproterenol-induced myocardial infarction (n=5). Values are presented as mean ± standard deviation. Superscript 'a' indicates significant difference (p<0.05) when compared with control (Grp A), whereas superscript 'b' indicates significant difference (p<0.05) when compared with ISO treated only (Grp B). Grp A (Control), Grp B (ISO treated only), Grp C (ENA+ 85 mg/kg ISO)
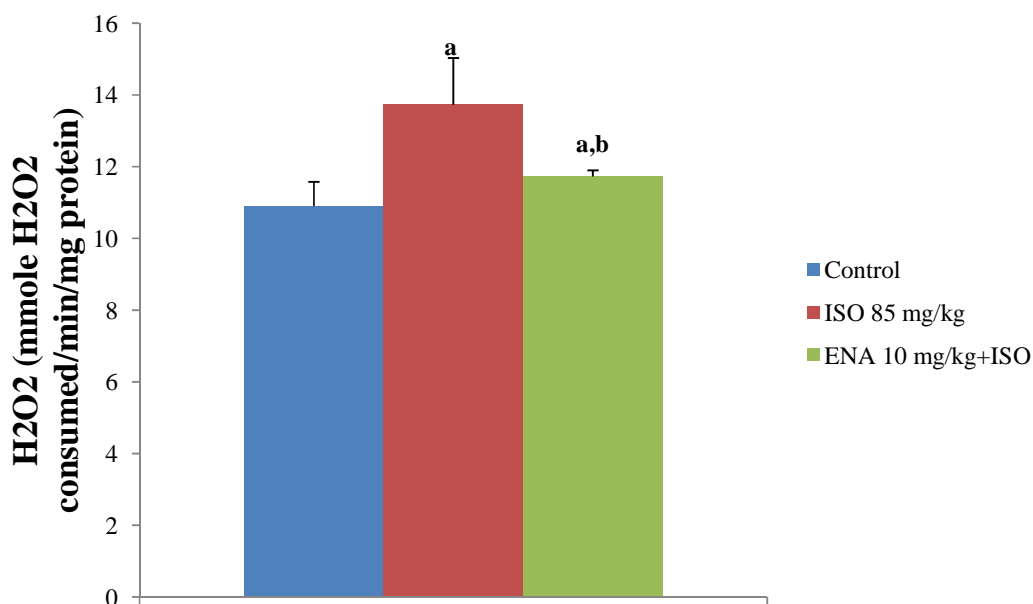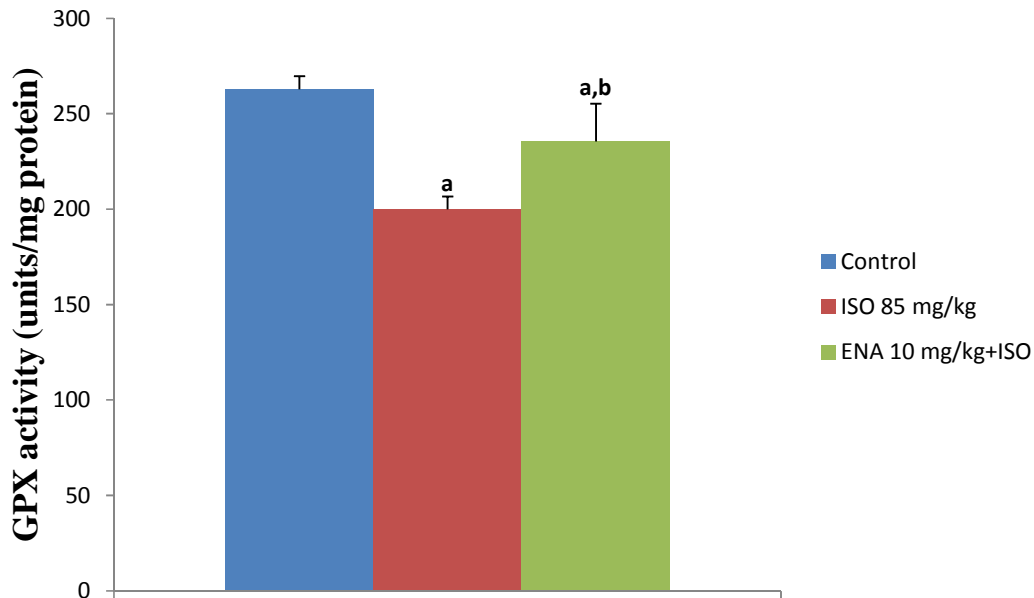
Fig. 10. Effect of enalapril on glutathione peroxidase enzyme in isoproterenol-induced myocardial infarction using rats as a model (n=5). Values are presented as mean ± standard deviation. Superscript 'a' indicates significant difference (p< 0.05) when compared with control (Grp A), whereas superscript 'b' indicates significant difference (p<0.05) when compared with ISO treated only (Grp B). Grp A (Control), Grp B (ISO treated only), Grp C (ENA+ 85 mg/kg ISO)
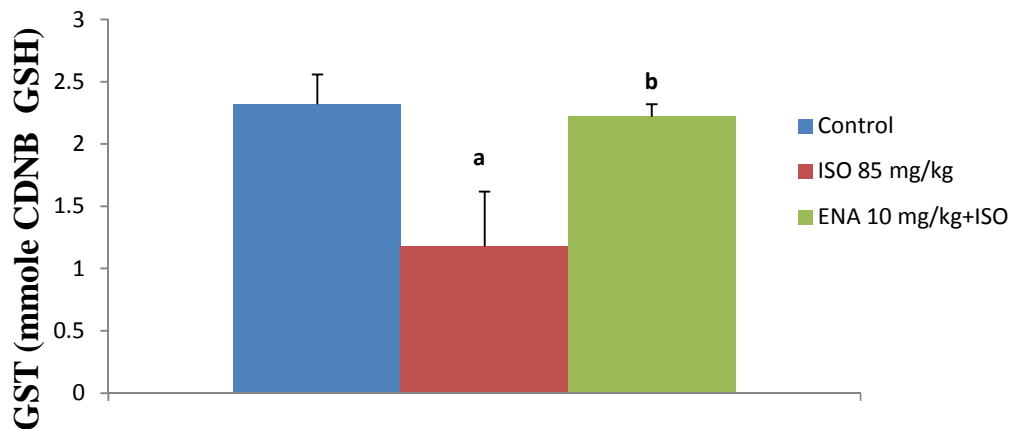
Fig. 11. Effect of enalapril on glutathione-s- transferase enzyme in isoproterenol-induced myocardial infarction using rats as a model (n=5). Values are presented as mean ± standard deviation. Superscript 'a' indicates significant difference (p<0.05) when compared with control (Grp A), whereas superscript 'b' indicates significant difference (p<0.05) when compared with ISO treated only (Grp B). Grp A (Control), Grp B (ISO treated only), Grp C (ENA+ 85 mg/kg ISO)
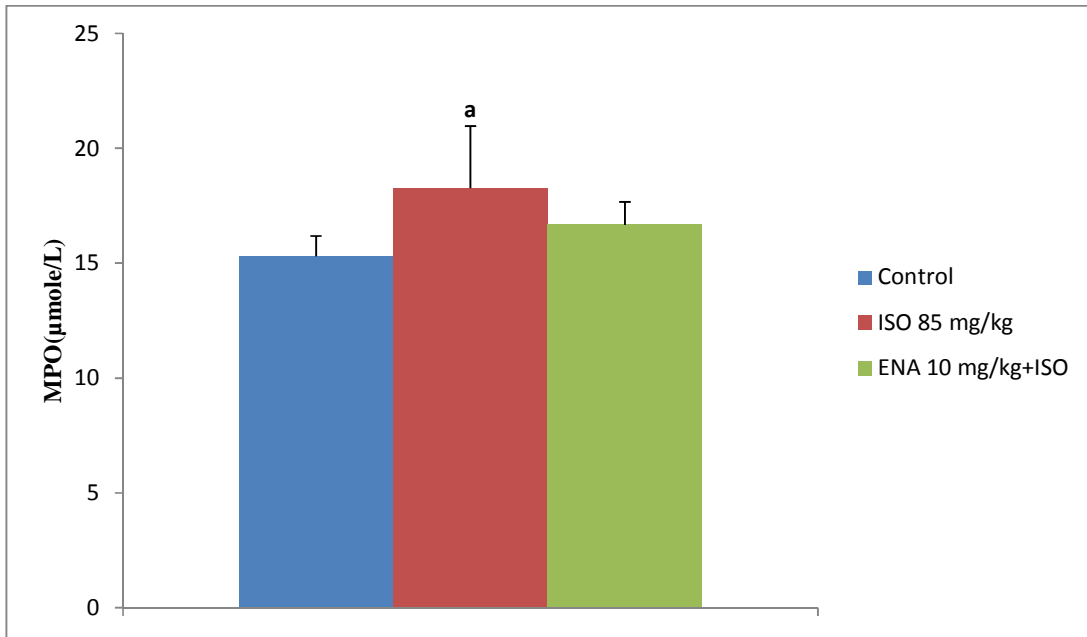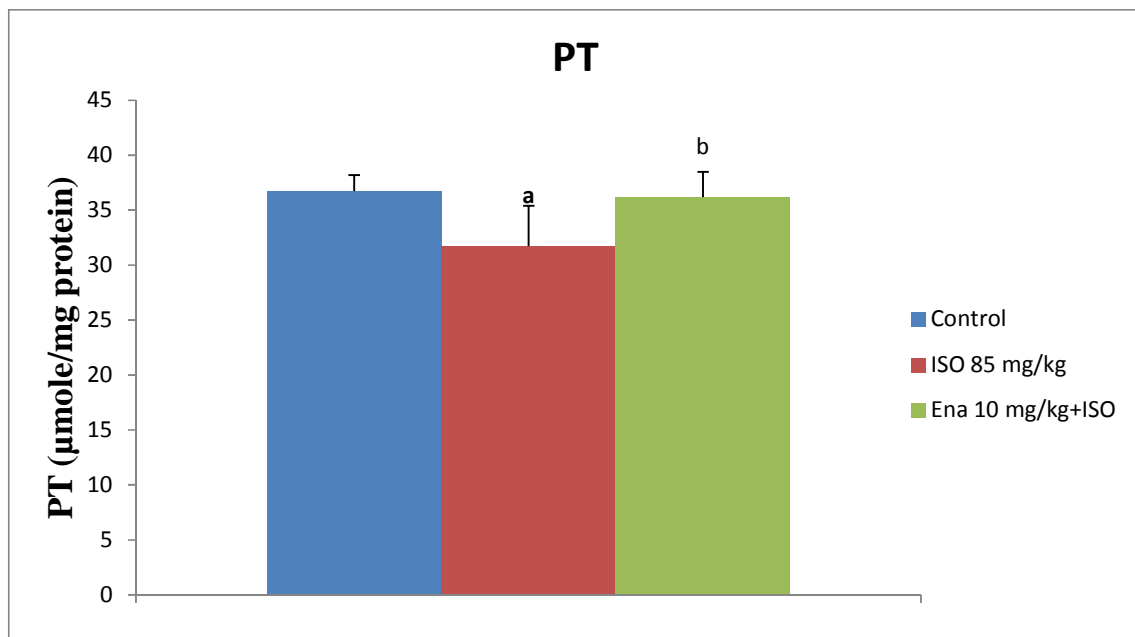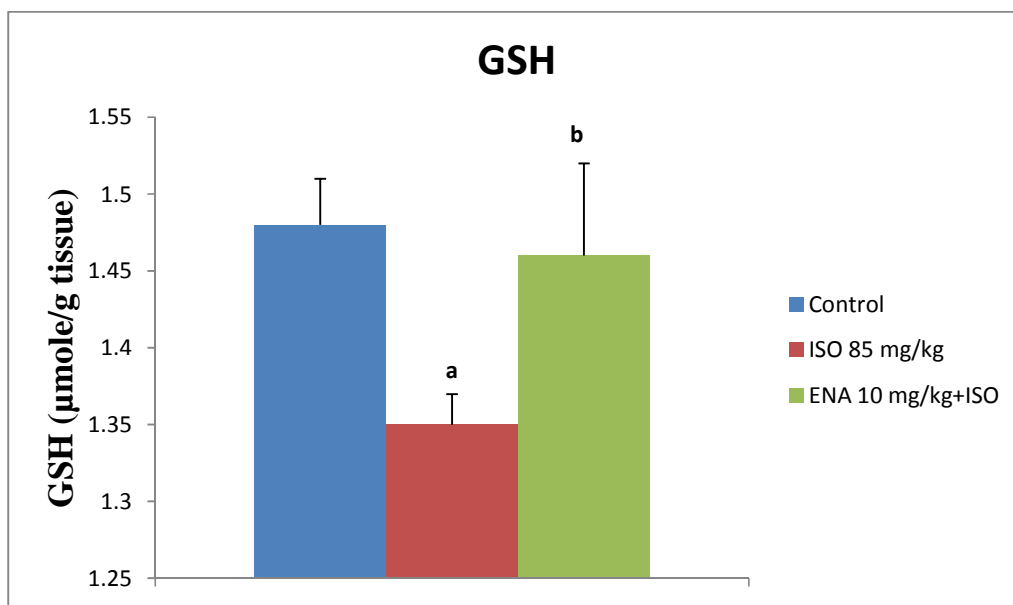
Fig. 12. Effect of enalapril on reduced glutathione in isoproterenol-induced myocardial infarction using rats as a model (n=5). Values are presented as mean ± standard deviation. Superscript 'a' indicates significant difference (p<0.05) when compared with control (Grp A), whereas superscript 'b' indicates significant difference (p<0.05) when compared with ISO treated only (Grp B). Grp A (Control), Grp B (ISO treated only), Grp C (ENA+ 85 mg/kg ISO)
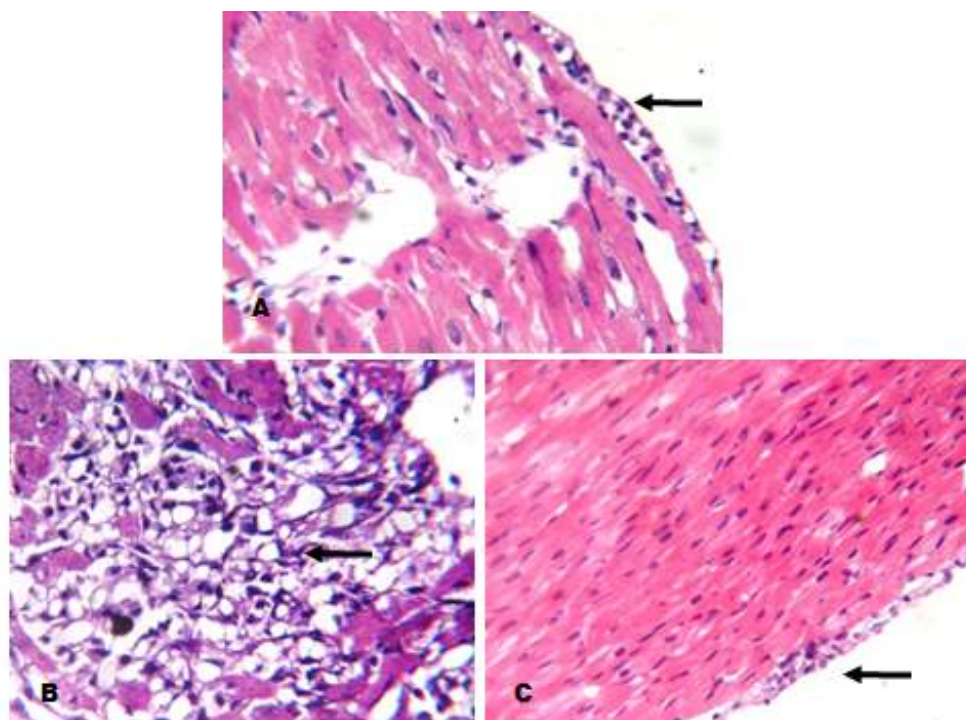
Fig. 13. The photomicrograph of heart from isoproterenol-induced myocardial infarction using rats as a model. A (Control) shows no visible lesion. B (ISO): shows severe infiltration of inflammatory cells. C (enalapril) shows no visible lesion. The slides were with H & E. Mag. ×400
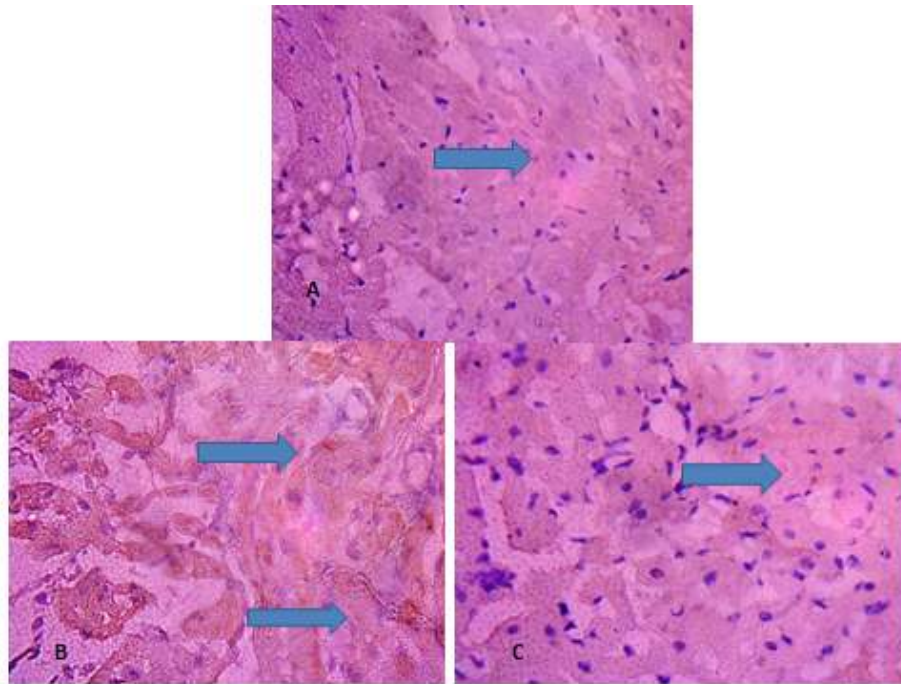
Fig. 14. Immunohistochemistry of cardiac troponin in heart of isoproterenol induced myocardial infarction rats. A (Control): show positive and low expression of CTnI, B (ISO): shows higher expression of CTnI than control, C (enalapril) shows lower expression of CTnI than B (ISO). The slides were counterstained with high definition haematoxylin. Mag. x100
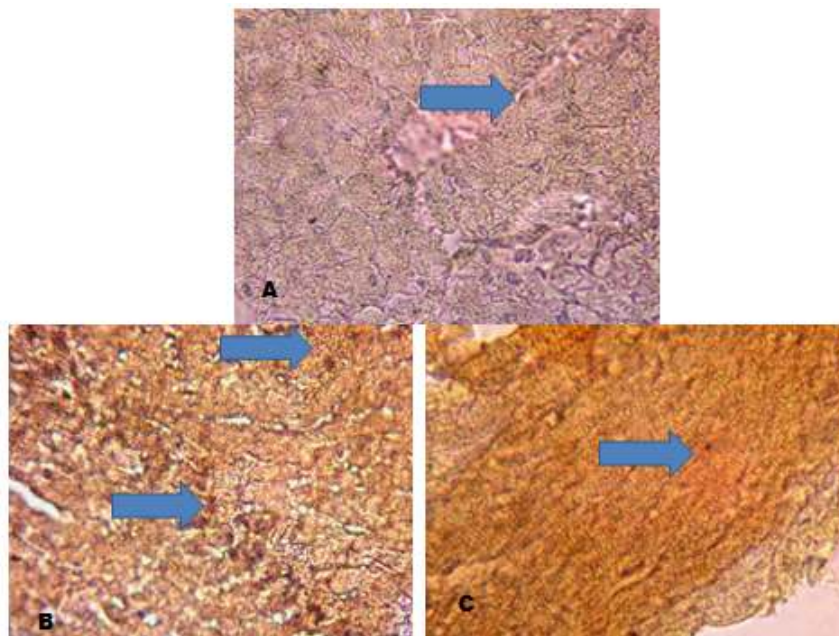
Fig. 15. Immunohistochemistry of c- reacting protein in heart of isoproterenol induced myocardial infarction rats. A (Control): show positive and low expression of CRP, B (ISO): shows higher expression of CRP than control, C (enalapril) shows lower expression of CRP than B (ISO). The slides were counterstained with high definition haematoxylin. Mag. x100
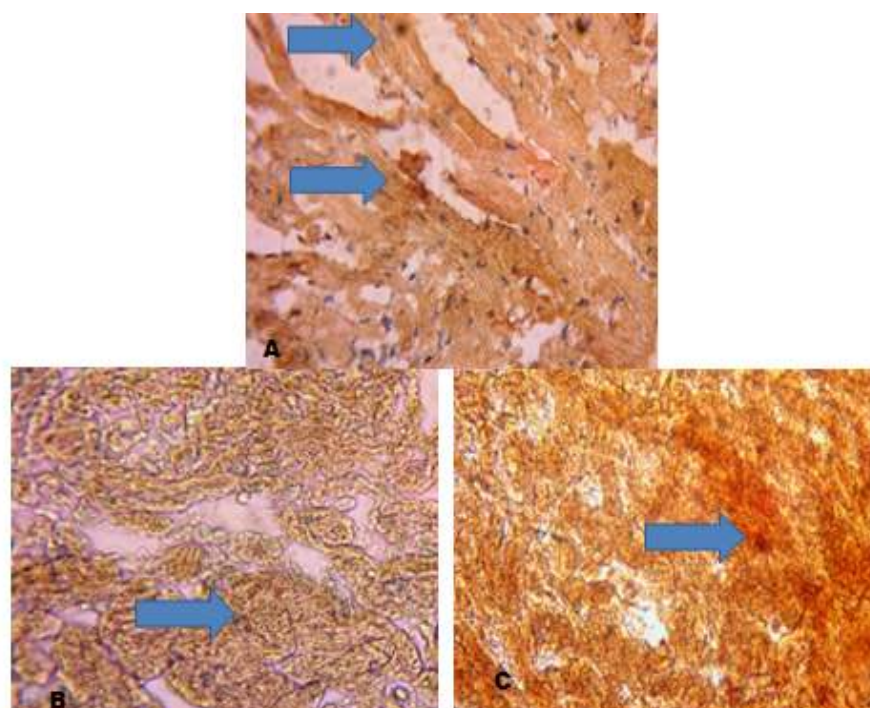
Fig. 16. Immunohistochemistry of interleukin-10 in heart of isoproterenol induced myocardial infarction rats. A (Control): show positive and higher expression of IL-10, B (ISO): shows lower expression of IL-10 than control, C (enalapril) shows higher expression of IL-10 than B (ISO). The slides were counterstained with high definition haematoxylin. Mag. x100

Angiotensin converting enzyme inhibitors are known to prevent both the generation of the potent vasoconstrictor angiotensin II and degradation of the powerful vasodilator bradykinin, which promotes endothelial cell release of NO [30]. In this study, rats treated with ISO had significant decreases in blood pressure parameters (SBD, DBP and MAP) when compared with the controls. This was however prevented in the ENA-treated group. There have been earlier reports of hypotension in subjects with acute myocardial infarction [31,32]. From this study, it was interesting to observe that ENA, a known antihypertensive drug, was able to preserve the blood pressure measurements of ISO-treated rats comparable to the controls. This might have been a consequence of its ability to prevent myocardial infarction. Studies have actually shown that ACEIs have been used in the management of myocardial infarction [33,34,35]. Isoproterenol, a β-adrenergic agonist is known to produce stress in the myocardium due to the generation of free radicals by its auto-oxidation. Some of the mechanisms proposed to explain its damage to cardiac myocytes include coronary hypotension, calcium overload, hypoxia, energy depletion and excessive production of free radicals as a result of catecholamine autoxidation [36,37,38]. The significant decrease in the levels of systolic, diastolic and mean arterial pressure may lead to coronary hypotension as seen in this study. In a study by Owens and O'Brien [39], it was concluded that in patients suffering with ischaemic heart disease and hypotension, symptomatic and silent ischaemia occurred in a temporally causal relation with hypotension, particularly for diastolic pressures. It thus suggests that patients with coronary disease may be susceptible to ischaemic events that could be incurred as a result of low blood pressure. The enalapril used in this study was able to restore the haemodynamic changes caused by isoproterenol indicating its ability to protect against establishment of myocardial infarction.

In this study, the results of haematological analysis showed that ISO caused significant increase in the levels of WBC, PCV, MCV and MCH while ENA caused significant decrease in WBC and no changes in the erythrocyte indices relative to control. The increase in the level of WBC could be explained in terms of necrosis

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caused by ISO leading to white blood cell mobilization [11]. The significant reduction in the level of this parameter by enalapril could also be seen as its ability to counteract the toxic effect of isoproterenol.

The toxicant also caused significant increase in the levels of AST and ALT while ENA caused significant decrease in the levels of these enzymes. In heart failure, the heart has an impaired ability to deliver blood to the body and may in the process affects the kidney and liver. The liver can become dysfunctional, and liver enzymes can be released into the blood [40]. It thus means that the increases noted for the liver enzymes in this study implied that isoproterenol could impair liver functions and this was counteracted by enalapril indicating that enalapril has beneficial effect beyond being an ACE inhibitor.

It was also observed that ISO caused significant decrease in the level of NO while ENA caused significant increase. Nitric oxide (NO) is known to play important functional roles in a variety of physiological systems. For instance within the vasculature, NO induces vasodilation, inhibits platelet aggregation, prevents neutrophil/platelet adhesion to endothelial cells, inhibits smooth muscle cell proliferation and migration, regulates programmed cell death (apoptosis) and maintains endothelial cell barrier function [41]. Nitric oxide (NO) is known to be deficient in chronic progressive renal disease (CRD) and in end-stage renal disease (ESRD) [42,43] and this could result from arginine deficiency [44] which may be caused by a loss of functional renal mass, increased endogenous NO synthase (NOS) inhibitors that accumulate in renal failure [44], and/or other causes, such as increased oxidant stress [45]. Low NO production may also contribute to and/or exacerbate the progression of CRD by both hemodynamic and renal growth-promoting actions [46]. It should also be noted that NO blockade can lead to increased blood pressure and attenuated or delayed the hypotensive effect of all ACE inhibitors [47]. ACE inhibitors such as enalapril also augment the hemodynamic vasodilator action of bradykinin [48]. The increased level of NO in this study due to enalapril may further affirm its antihypertensive property and hence cardioprotective effect.

ISO caused significant increase in the levels of oxidative stress markers such as MDA, H_2O_2 and MPO while ENA caused significant decrease in

the levels of these markers in a similar fashion to the control. Oxidative stress constitutes an alteration produced by disequilibrium between generation of free radicals (FR) and the antioxidant system, which can lead to a damage state, in particular of the biomolecules [49,50,51, 52,53]. FR generates the lipid peroxidation process in an organism with malondialdehyde (MDA) level used as a marker of oxidative stress [54]. Myeloperoxidase (MPO) is abundant in the granules of inflammatory cells and it is an important enzyme in the generation of reactive oxygen species (ROS) [55,56,57]. Hydrogen peroxide (H_2O_2), an ROS marker has been suggested as a mediator of vascular structural and functional alterations observed in hypertension [58,59,60,61,62]. The reduction of these oxidative markers by enalapril is a pointer to its ability to scavenge the radicals generated by the toxicant and it thus showed that enalapril has anti-oxidant activity. In fact, De Cavanagh et al. [63] reported that enalapril inhibits free radical formation and attenuates oxidative stress and also prevents damage to the liver and kidney. This was further confirmed by the ability of this ACE inhibitor to increase the levels of antioxidant enzymes such as SOD, GPx, GST and GSH evaluated in this study. This view is clearly supported by a study carried out by Chandra et al. [64], where it was concluded that enalapril has anti-oxidative property and this may have been responsible for its cardioprotective property. As a matter of fact, ENA caused a significant increase in the levels of protein thiols and non-protein thiols further confirming its anti-oxidant property. The thiol compounds function in the maintenance of cellular redox balance and their play important role in controlling oxidative stress [65,66,67].

Cells have evolved several antioxidant strategies aimed at the detoxification of ROS with glutathione redox cycle as one of the major protective systems against oxidant damage. This cycle composed of the enzymes glutathione peroxidase (GPx) and glutathione reductase (GSSG-Rd) and the co-substrates glutathione and NADPH [68]. Glutathione is the most abundant non-protein intracellular thiol, and has a multiple role as an antioxidant agent [69]. Though the mechanism(s) underlying the enhancement of glutathione and glutathione-related enzymes by ACEI remains unknown, however, tissue glutathione levels and GSSG-Rd and GPx activities have been shown to increase in response to experimentally induced oxidative stress [70].

In this study, histopathological examinations showed that while there was severe infiltration of inflammatory cells into the cardiac tissue of the ISO group, there was no visible lesion seen in the ENA and control groups (Fig. 13). This increase in the inflammatory cells may have been responsible for the increase in the levels of WBC noted in this study (Table 1). It should be noted that the isoproterenol-induced myocardial alterations are similar in certain respects to those occurring in human beings following a myocardial infarction [71]. It is thought that the β-adrenergic cardiostimulatory activity exerted by ISO increases cardiac oxidative metabolism to a level that exceeds the amount of oxygen available to the myocytes through the unobstructed coronary circulation. The area of the heart most susceptible to hypoxia caused by tachycardia appears to be the left ventricular subendocardium [72,73]. Myocyte damage observed following exposure to ISO includes both apoptosis and necrosis [74]. In the study on the isoproterenol-induced myocardial damage, it was discovered that the cardiac lesions varied with treatment duration and doses and that numerous macrophages were observed in the necrotic areas [75]. In our study, enalapril did not show any visible cardiac tissue damage possibly through its ability to prevent cell infiltration thus preventing apoptosis and necrosis.

The immunohistochemical analysis showed that there were high expressions of cardiac troponin and CRP in ISO group but lower expression of these proteins in ENA and control groups (Figs. 14 and 15). In the case of IL-10B, there was low expression of this protein in ISO group but higher expression in ENA and control groups (Fig. 16). Cardiac troponins are regulatory proteins within the myocardium that are released into the circulation when damage to the myocyte has occurred. Therefore, serum troponin is an exquisitely sensitive marker of myocardial injury and is necessary for establishing the diagnosis of MI [76,77,78]. This study has shown that ISO caused myocardial injury with upregulation of this biomarker. On the other hand, the down regulation of cardiac troponin by ENA also showed that this drug has ability to protect against myocardial injury in rats.

C-reactive protein (CRP) has the capacity to precipitate the somatic C-polysaccharide of *Streptococcus pneumonia*. It was the first acute-phase protein to be described and is an exquisitely sensitive systemic marker of

inflammation and tissue damage [79]. It is a known fact that tissue necrosis is a potent acute-phase stimulus. In myocardial infarction, there is a major CRP response with the magnitude of this response indicating the extent of myocardial necrosis [80]. In all acute myocardial infarcts, CRP is co-deposited with activated complement [81,82], and research findings have shown that the CRP response did not only reflects tissue damage in this context but also may actually contribute significantly to the severity of ischemic myocardial injury [83]. The lowering of the level of CRP in this study by ENA is a pointer to its ability to halt cardiovascular disease hence cardioprotective effect through its anti-oxidant and anti-inflammatory properties.

Immunohistochemistry in this study further showed that ENA caused increased level of IL-10B. IL-10B is a Th$_2$-type cytokine that is produced by a wide range of immunological cell types, including monocytes/macrophages, and it is a potent inhibitor of the proinflammatory cytokines and chemokines [84]. Studies have shown that endogenous IL-10 limits angiotensin II (ANG II)-mediated oxidative stress, inflammation and vascular dysfunction both *in vivo* and *in vitro*, indicating a protective action of IL-10 in vascular diseases such as arterial hypertension [85]. As a matter of fact, IL-10 attenuates the increases in vascular superoxide and endothelial dysfunction during diabetes and atherosclerosis [86,87]. In the same way, it could be suggested that IL-10 might be a mediator of cardiac protection against arterial hypertension. It thus shows that the cardioprotective effect of enalapril may also be linked to its anti-inflammatory property as shown by the up regulation of IL-10.

5. CONCLUSION

In conclusion, this study has shown that enalapril, an ACE inhibitor has cardioprotective properties, which it exhibited through its anti-oxidant, anti-inflammatory and anti-apoptotic effects. Its antihypertensive property is also exhibited through its nitric oxide increasing ability leading to vasodilation and hence decreases in peripheral resistance.

ETHICAL APPROVAL

As per international standard or university standard ethical approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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The Protective Effect of the Ethanol Leaf Extract of Andrographis Paniculata on Cisplatin-Induced Acute Kidney Injury in Rats Through nrf2/KIM-1 Signalling Pathway

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ABSTRACT

The ethanol leaf extract of Andrographis paniculata was used to ameliorate the renal toxicity induced by cisplatin in 28 rats divided into four groups of seven rats per group. Group A received normal saline for the duration of the experiment. Group B animals were treated with cisplatin (10 mg/kg i.p) on day 1 and 3 days after received normal saline for the next 7 days while groups C and D animals also received 10 mg/kg dose of cisplatin on day 1 but after 3 days were then respectively treated with 200 and 400 mg/kg doses of the extract of Andrographis paniculata for the remaining 7 days through oral administration. Serum chemistry was used for the determination of markers of oxidative stress, anti-oxidant enzymes, serum biomarkers etc. Histopathology and immunohistochemistry were also carried out. Results showed that all oxidative stress markers assayed were significantly increased in group B animals but reverse is the case for groups C and D. On the other hand, antioxidant enzymes assayed experienced significant increase for groups C and D while these parameters experienced significant decrease for group B animals. Histopathology showed severe infiltration of inflammatory cells into renal tissues of group B animals whereas for groups C and D animals, only moderate glomerular degeneration was noted. In immunohistochemistry, while there is higher expression of KIM-1 for group B, there was a lower expression in groups C and D. Again, there was lower expression of Nrf2 for group B but higher expressions in groups C and D animals.

Introduction

Cisplatin, or cis-diamminedichloroplatinum (CP) which has its molecular formula as cis-[Pt(NH3)2Cl2], is a platinum-based drug used in the treatment of cancer [1]. Its major drawback is that nephrotoxicity is one of its side effects. As a matter of fact, patients receiving high dose CP have severe renal dysfunction [2]. While Cisplatin-DNA cross links causes cytotoxic lesions in tumours and other dividing cells, this DNA damaging agent usually have less toxicity in non-proliferating cells [3]. However, the quiescent proximal tubule cells of the kidney are selectively damaged by CP which accumulates in the kidney to a greater degree than in other organs [4]. Studies show that the CP concentration in proximal tubular epithelial cells is about 5 times the serum concentration and it is proba-

ble that this disproportionate accumulation of CP in kidney tissue may have contributed to CP-induced nephrotoxicity [5, 6]. Though the mechanism for this renal cell injury has been the focus of intense investigation for many years, recent studies suggest that inflammation, oxidative stress injury, and apoptosis may probably have been responsible for some of the toxic changes observed [7]. Studies have also shown that CP causes generation of reactive oxygen species (ROS), which in turn deplete glutathione levels and inhibit the activity of antioxidant enzymes in renal tissue. ROS may also produce cellular injury and necrosis via several mechanisms including lipid peroxidation, DNA damage, and even protein denaturation [8]. ROS are known to be produced via the xanthine-xanthine oxidase system, mitochondria, and NADPH oxidase in cells

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and in the presence of CP; thereby implicated in renal injury [9]. Increased free radical generation and decrease antioxidant defence system also occur when CP induces glucose-6-phosphate dehydrogenase and hexokinase activity [10].

Plants with diverse medicinal properties especially those rich in anti-oxidant, anticarcinogenic and probably anti-inflammatory effects have come under much research activities and Andrographis paniculata (AP) is one of such [11, 12]. AP has been reported to have a broad range of pharmacological effects including antioxidant [13, 14], hepatoprotective [15], anticancer [16, 17] anti-inflammatory [18], cardioprotective [19], antidiarrheal [20], antihyperglycemic [21], antihepatitis [22], anti-HIV [23], antimicrobial, antimalarial [24], cytotoxic [23] and immunostimulatory [25]. Several phytochemicals including 55 ent-labdane diterpenoids, 30 flavonoids, 8 quinic acids, 4 xanthones, andrographidoids A, B, C, D, and E as well as reported 3 new ent-labdane diterpenoids, namely, 19-norandrographolides A, B, and C have been reported in the plant [26–28].

The aim of the present study was to evaluate the possible protective effects of ethanol leaf extract of Andrographis paniculata on CP-induced nephrotoxicity and oxidative stress.

Materials and Methods

Plant collection, processing and extraction

The leaves of the plant were collected from the Botanical Garden of the University of Ibadan, Nigeria. The leaves were identified and authenticated by the herbarium curator, Department of Botany, University of Ibadan with the voucher numbers 2846. The leaves were cleaned with distilled water and air dried in a well ventilated shady room and the dried leaves were grinded to powder using blender. The powdered material was extracted in cold ethanol in a screw-capped flask and shaken at room temperature. The solvent was filtered, squeezed using Whatman filter paper and evaporated off under reduced pressure in a rotatory evaporator at 40 °C to obtain semi-solid crude extract which was store at 4 °C. The stored ethanol extract of Andrographis paniculata (AP) was then used for the studies.

Experimental animals

Twenty eight healthy adult Wistar male rats weighing between (100 and 150 g) at the commencement of experiments were obtained from the Animal House of the Faculty of Veterinary Medicine, University of Ibadan were used for the study. The rats were acclimatized for 14 days and were fed with commercial rat cubes and water ad libitum. The study was approved by the institutional Ethics Committee on animal use in research.

Experimental design

The experimental animals were divided into four groups of seven rats per group. Group A or normal control received normal saline for the duration of the experiment. Group B animals were treated with single dose of CP (10 mg/kg i.p) on day 1 and 3 days after received normal saline for a period of 7 consecutive. Groups C and D animals also received 10 mg/kg dose of cisplatin on day 1 but after 3 days, they were treated respectively orally with 200 and 400 mg/

kg dose of the ethanol leaf extract of AP for 7 consecutive days. On day 10 the rats were kept in metabolic cages for 8 h and urine sample was collected for total protein analysis while on day 11 blood samples were collected for haematology.

Serum collection

About 5 ml of blood was collected from the retro-orbital venous plexus into sterile plain tubes and left for about 30 min to clot and the clotted blood was thereafter centrifuged at 4, 000 rpm for 10 min. The serum was decanted into eppendorf tubes and stored at −4 °C till the time of analysis.

Tissue preparation

After blood collection, the animals were sacrificed and the two kidneys from each rat were removed and rinsed in normal saline and immediately kept on ice to prevent denaturation of biomolecules. Subsequently, tissue samples were homogenized in ice cold phosphate buffer (0.1 M, pH = 7.4) with the Teflon homogenizer. The resulting homogenates were centrifuged at 10 000 g for 15 min at 4 °C so as to obtain the post mitochondrial fractions (PMFs). The supernatant was used for the biochemical analyses.

Chemicals

O-dianisidine, sodium potassium tartrate, ethanol, sodium azide, 2- dichloro-4-nitrobenzene (CDNB), thiobarbituric acid (TBA), hydrogen peroxide (H_2O_2), hydrochloric acid, sulphuric acid, xylenol orange, sodium hydroxide, potassium iodide, reduced glutathione (GSH), trichloroacetic acid, Ellman's reagent (DTNB), ammonium ferrous sulphate, sorbitol were purchased from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade and obtained from British Drug Houses (Poole, Dorset, UK).

Biochemical analyses

The activity of xanthine oxidase was determined according to methods of Akaike et al [29]. Presence of Advance oxidation protein products (AOPPs) was determined according to methods of Kayali et al [30]. Renal nitric oxide (NO) was measured as described by Crespo et al. [31]. Hydrogen peroxide generation was determined as described by Wolff [32]. Thiobarbituric acid reactive substance (TBARS) was quantified as malondialdehyde (MDA) in the renal PMF. The MDA was determined according to the method of Varshney and Kale [33]. Reduced glutathione content was determined using the method of Beutler et al. [34]. Glutathione peroxidase (GPX) activity was measured by the method of Rotruck et al. [35]. The glutathione-S-transferase (GST) estimation was by the method of Habig et al. [36] using 1-chloro-2, 4-dinitrobenzene as substrate. Superoxide dismutase (SOD) assay was carried out by the method of Misra and Fridovich [37] and Oyagbemi et al. [38]. Protein concentrations were determined by method described by Gornal et al. [39]. The serum myeloperoxidase (MPO) activity was determined according to the method of Xia and Zweier [40].

Measurement of urea and creatinine levels

The serum urea and creatinine levels were determined spectrophotometrically using commercial kits (Randox Laboratories, UK) that employed the principles of the Urease-Berthelot method (involving the formation of a green complex of 2-2-dicarboxylindophe-

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nol) and Jaffe alkaline picrate method (involving the reaction of serum creatinine with picrate in an alkaline medium to form a coloured complex), respectively [41].

Immunohistochemistry of renal Kim-1 and NrF-2 expressions

Immunohistochemistry of paraffin-embedded tissue of the kidney was performed after the tissues were obtained from buffered formalin as reported by Oyagbemi et al. [38]. Briefly, paraffin sections were melted at 60 °C in the oven. Dewaxing of the samples in xylene was followed by passage through ethanol of decreasing concentration (100–80 %). Peroxidase quenching in 3 % H_2O_2/methanol was carried out with subsequent antigen retrieval performed by microwave heating in 0.01 M citrate buffer (pH 6.0). All the sections were blocked in normal goat serum (10 %, HistoMark®, KPL, Gaithersburg, MD, USA) and probed with kidney injury molecule 1 (Kim-1) and nuclear erythroid factor 2 related (NrF2) antibodies (Abclonal®), 1:200 for 16 h at room temperature. Detection of bound antibody was carried out using biotinylated (goat anti-rabbit, 2.0 µg/mL) secondary antibody and subsequently, Horseradish Peroxidase streptavidin system was used according to manufacturer's protocol (HistoMark®, KPL, Gaithersburg MD, USA). Reaction product was enhanced with diaminobenzidine (DAB, Amresco®, USA) for 6–10 min and counterstained with high definition haematoxylin (Enzo®, NY, USA), with subsequent dehydration in ethanol. The slides were covered with coverslips and sealed with DBx mountant. The immunoreactive positive expression and intensive regions were viewed with ×400 magnifications using a photo microscope (Olympus) and a digital camera (Toupcam®, Touptek Photonics, Zhejiang, China).

Histopathology

Renal tissues were collected in 10 % formal saline buffer 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 [42].

Statistical analysis

All values are expressed as mean ± S.D. The test of significance between two groups was estimated by Student's t test. One-way ANOVA with Tukey's post-test was also performed using GraphPad Prism version 4.00. The level of statistical significance was considered as $p < 0.05$.

Results

Oxidative stress markers studied (AOPP, H_2O_2, MDA, MPO, XO) were significantly increased in CP only treated rats animals but reversed in group C and D. Antioxidant enzymes (SOD, GPx, GSH, GST) were increased significantly increased in C and D while these parameters were significantly decreased in group B animals (▶ **Figs. 1–14**). The serum creatinine and urea levels were significantly decreased for groups C and D, group B animals showed significant increase in the levels serum creatinine and urea (▶ **Table 1**). In histopathological study, there was severe infiltration of inflammatory cells into renal tissues of CP only treated animals whereas in rats post-treated with AP, only moderate glomerular degeneration was noted (▶ **Fig. 15**).
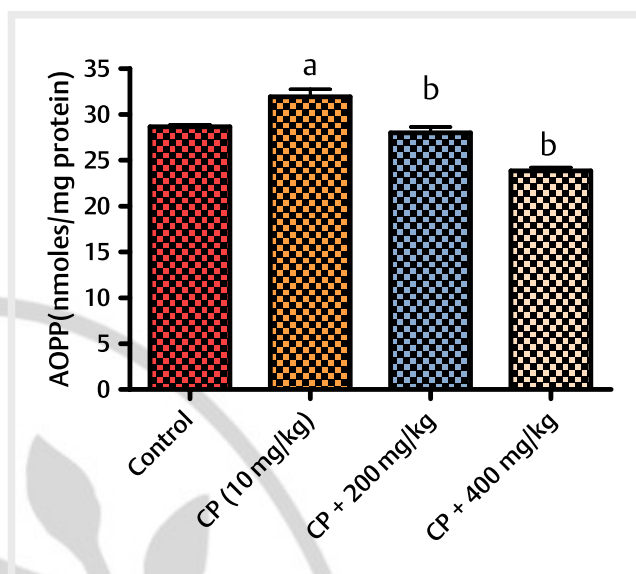
▶ **Fig. 1** Effect of Andrographis paniculata on advanced oxidative protein products in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - $p < 0.05$ compared with control, [b] - $p < 0.05$ compared with CP, [b] - $p < 0.05$.
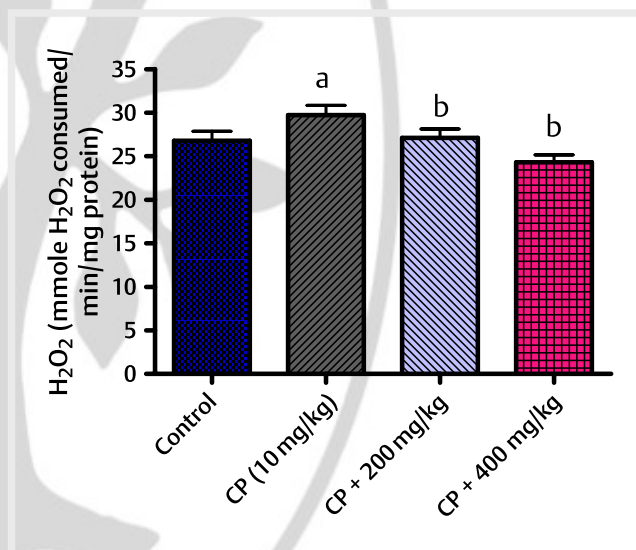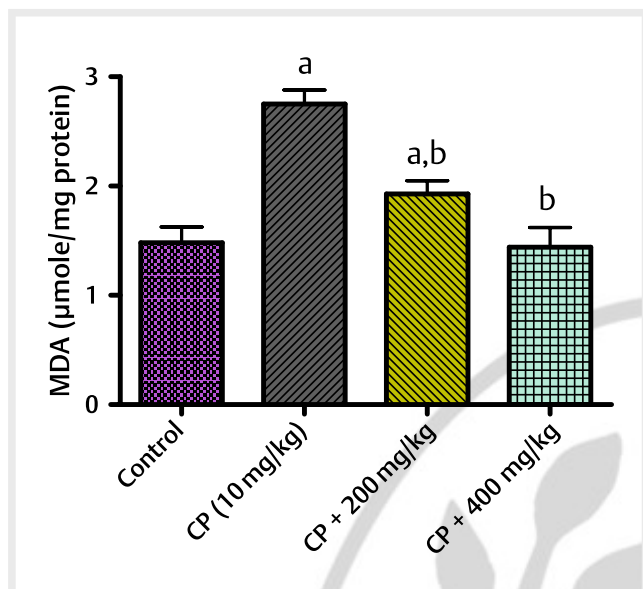

▶ **Fig. 2** Effect of Andrographis paniculata on hydrogen peroxide in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - $p < 0.05$ compared with control, [b] - $p < 0.05$ compared with CP.

In immunohistochemistry, there was higher expression of KIM-1 in CP only administered rats. However, there was a lower expression of KIM-1 for group C and D rats. Again, there was lower expression of Nrf2 for group B but higher expressions for groups C and D animals (▶ **Fig. 16** and ▶ **17**).

Discussion

In this study, the ability of the ethanol leaf extract of Andrographis paniculata to ameliorate renal injury caused by administration of
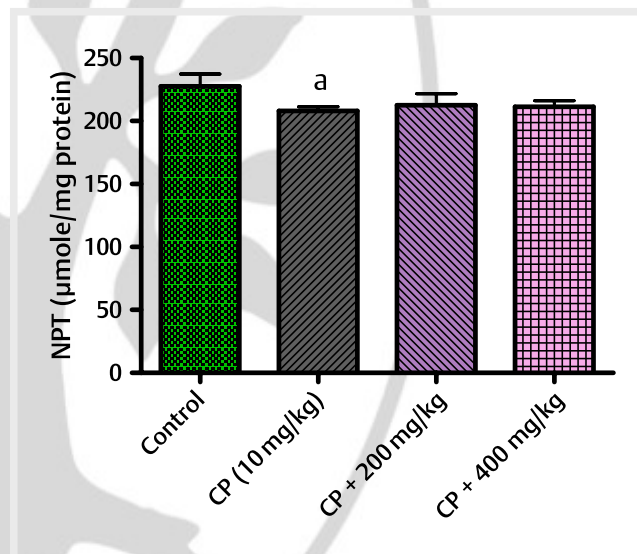
▶ **Fig. 3** Effect of Andrographis paniculata on lipid peroxidation (malondialdehyde) in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - $p < 0.05$ compared with control, [b] - $p < 0.05$ compared with CP.
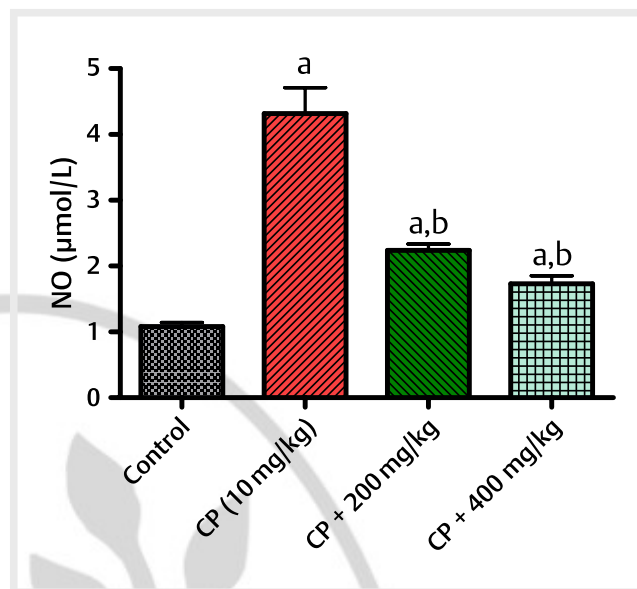
▶ **Fig. 5** Effect of Andrographis paniculata on nitric oxide in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - $p < 0.05$ compared with control, [b] - $p < 0.05$ compared with CP.
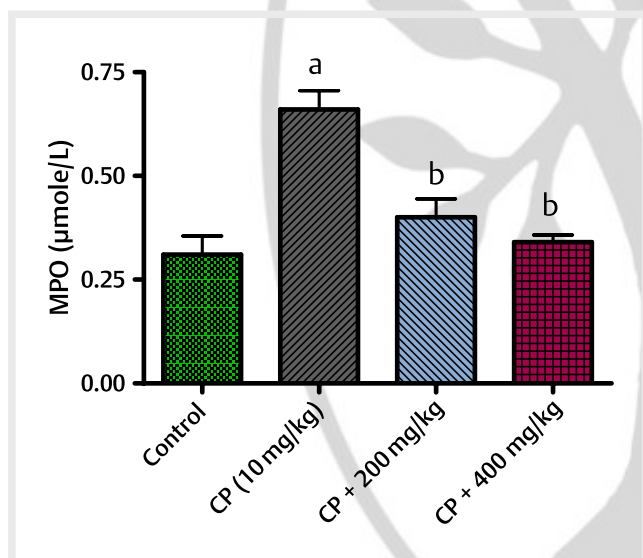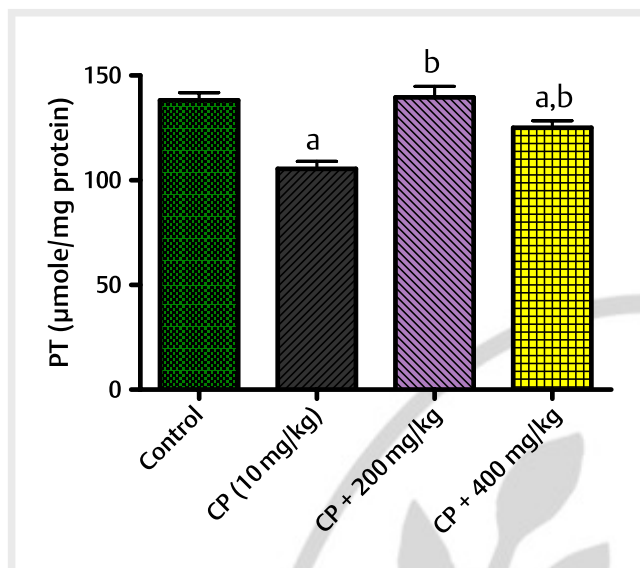

▶ **Fig. 4** Effect of Andrographis paniculata on myeloperoxidase in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - $p < 0.05$ compared with control, [b] - $p < 0.05$ compared with CP.
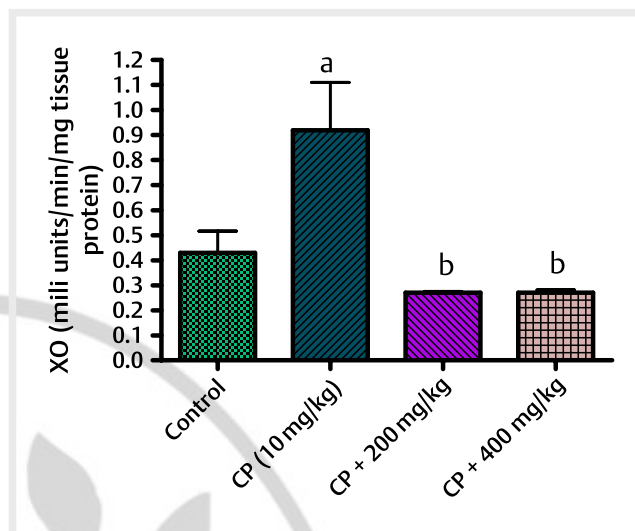

▶ **Fig. 6** Effect of Andrographis paniculata on non protein thiol in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - $p < 0.05$ compared with control, [b] - $p < 0.05$ compared with CP.

CP was assessed. All oxidative stress markers such as AOPP, H_2O_2, MDA, MPO, and XO carried out in the present study were significantly increased in group B rats but reverse was the case for rats in group C and D. On the other hand, the activity of SOD, GPx, GSH, GST significantly increased for group C and D rats, with a significant decrease in the activities of these antioxidant enzymes in group B rats (▶ **Figs. 1–14**). CP toxicity has been associated with oxidative stress that is known to generate ROS leading to decreased anti-oxidant defence system which is made up of anti-oxidant enzymes

and non enzymatic glutathione [43]. The result from this study is thus consistent with the fact that CP toxicity is associated with free radical generation. The ethanol leaf extract of AP at the two doses used caused a significant decrease in the oxidative markers and a significant increase in the anti-oxidant enzymes and non-enzymes carried out in this study. Qader et al [44] has shown that AP possessed anti-oxidant activity. Bardi et al [45] reported that increase in enzyme activities upon the use of ethanol leaf extract of AP in the treatment of thioacetamide-induced liver cirrhosis may stem from a decrease in ROS and free radicals, due to scavenging by an-

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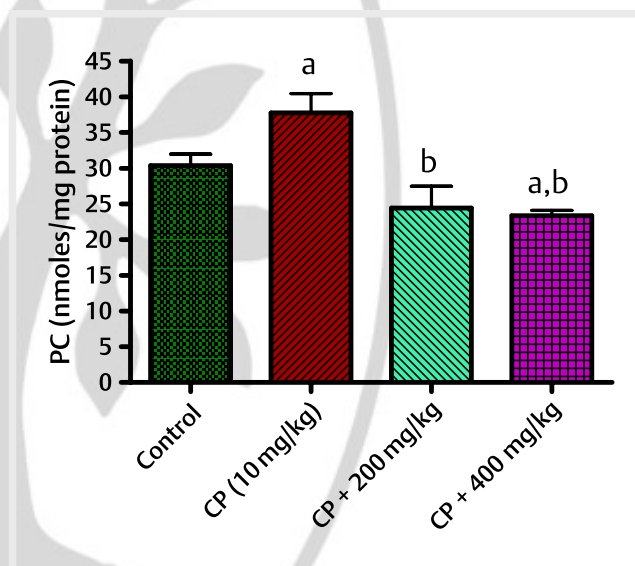► **Fig. 7** Effect of Andrographis paniculata on protein thiol in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - p < 0.05 compared with control, [b] - p < 0.05 compared with CP.

► **Fig. 9** Effect of Andrographis paniculata on xanthine oxidase in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - p < 0.05 compared with control, [b] - p < 0.05 compared with CP.
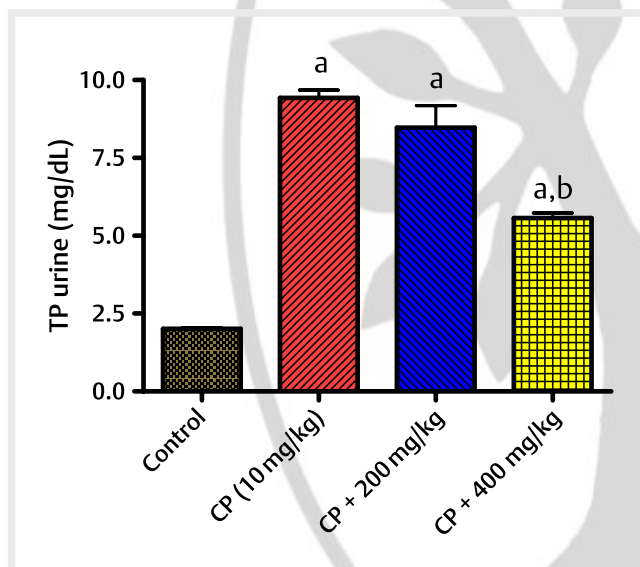
► **Fig. 8** Effect of Andrographis paniculata on total protein (urine) in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - p < 0.05 compared with control, [b] - p < 0.05 compared with CP.
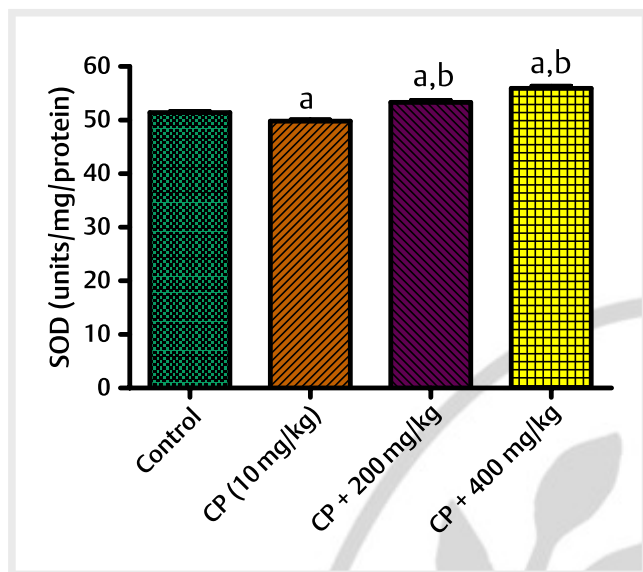

► **Fig. 10** Effect of Andrographis paniculata on protein carbonyl in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - p < 0.05 compared with control, [b] - p < 0.05 compared with CP.

drographolides contained in this plant. The report from this study is in agreement with similar reports on the effects of A. paniculata on liver damage [46]. These reports have thus corroborated the results from our study that AP has anti-oxidant property and may have been responsible for the ameliorative effect observed on the kidneys of rats used. It thus showed that AP having anti-oxidant property has the ability to protect the kidney against CP induced toxicity.

The serum creatinine and urea levels were significantly decreased in groups C and D animals however; group B animals experienced a significant increase in the levels of serum creatinine and urea (► **Table 1**). Serum urea and creatinine are biomarkers used to correlate with severity of kidney damage; hence, their measurement can assist in detection and prevention kidney disease at an early stage and thus, limit the progression to end stage renal disease [47] Elevation of these biomarkers of renal damage in this study thus confirms the nephrotoxic effect of CP. Since the leaf extract of the plant, A. paniculata caused a significant decrease in the levels of serum creatinine and urea; this therefore shows that the extract has protective effect against CP-induced kidney injury. Singh et al. [48] in a study on gentamicin-induced nephrotoxicity

▶ **Fig. 11** Effect of Andrographis paniculata on superoxide dismutase in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - p < 0.05 compared with control, [b] - p < 0.05 compared with CP.
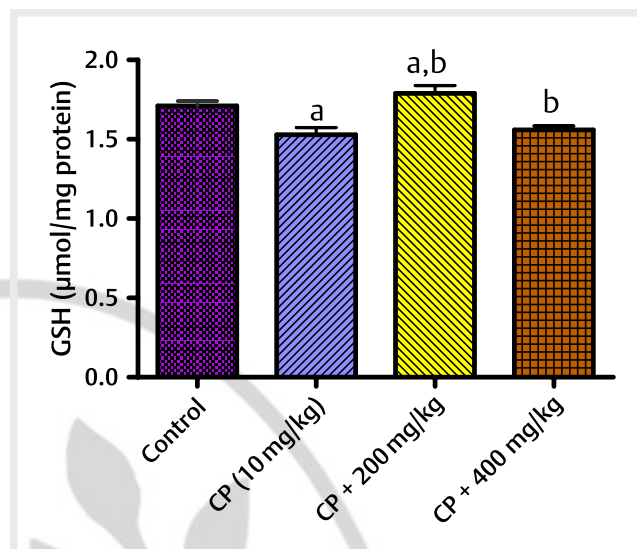
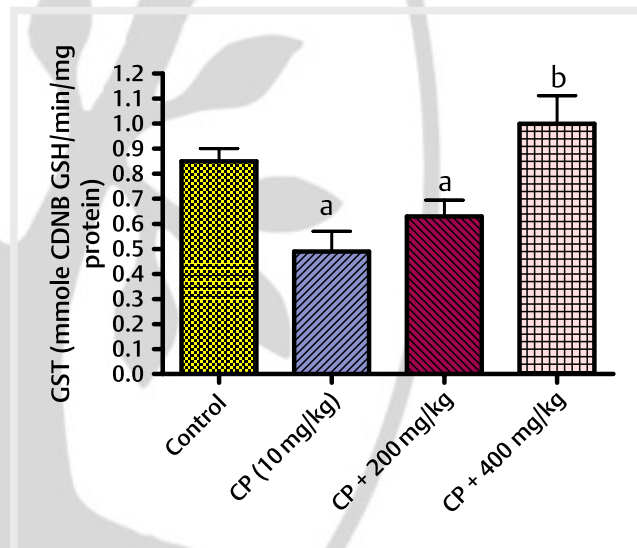▶ **Fig. 13** Effect of Andrographis paniculata on reduced glutathione in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - p < 0.05 compared with control, [b] - p < 0.05 compared with CP.
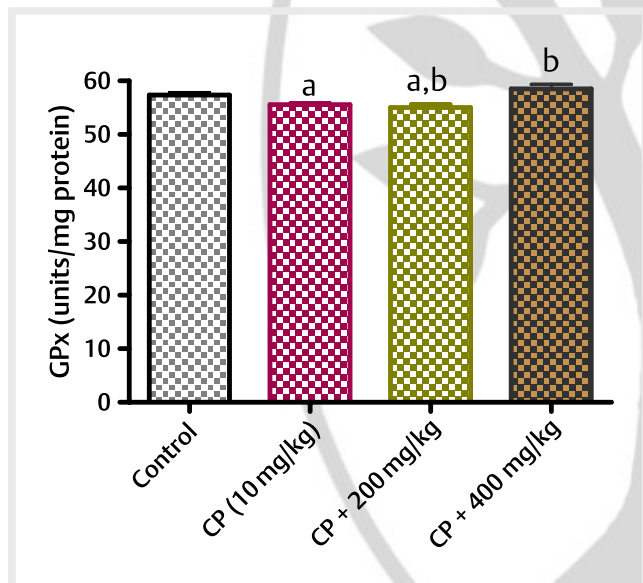
▶ **Fig. 12** Effect of Andrographis paniculata on glutathione peroxidase in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - p < 0.05 compared with control, [b] - p < 0.05 compared with CP.

▶ **Fig. 14** Effect of Andrographis paniculata on glutathione-S-transferase in cisplatin-induced acute kidney injury in rats as a model. n = 5. Values are mean ± SD, [a] - p < 0.05 compared with control, [b] - p < 0.05 compared with CP.

showed that the aqueous leaf extract of AP caused a significant reduction in the levels of serum creatinine and urea. Similar report was given by Singh et al. [49] indicating that serum creatinine and urea levels were always increased in toxicant induced kidney injury but that the extract of AP has proven to ameliorate the kidney injury by reducing the levels of these parameters. By the ameliorative ability of this plant, it shows that this plant has protective ability against CP-induced renal injury.

In histopathological study, there was severe infiltration of inflammatory cells into renal tissues of CP only treated animals whereas for rats that were post-treated with AP, only moderate glomerular degeneration was noted (▶ **Fig. 15**). In the pathogenesis of acute kidney injury, inflammatory process, potential tubular and vascular factors are involved [50]. Inflammation is particularly believed to play a major pathophysiological role in acute renal injury especially that the vascular endothelium plays a leading role in the recruitment and migration of circulating inflammatory cells into sites of inflammation [51, 52]. In the development of CP-induced

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|	Extract			
	Control	CP(10 mg/kg)	200 mg/kg	400 mg/kg
Creatinine(mg/dL)	2.21 ± 0.70	3.27 ± 0.46[a]	1.87 ± 0.35[b]	1.58 ± 0.43[b]
Urea (mg/dL)	28.83 ± 2.64	41.75 ± 6.36[a]	25.04 ± 7.35[b]	26.32 ± 1.68[b]
n = 5. Values are mean ± SD, [a] - p < 0.05 compared with control, [b] - p < 0.05 compared with CP				
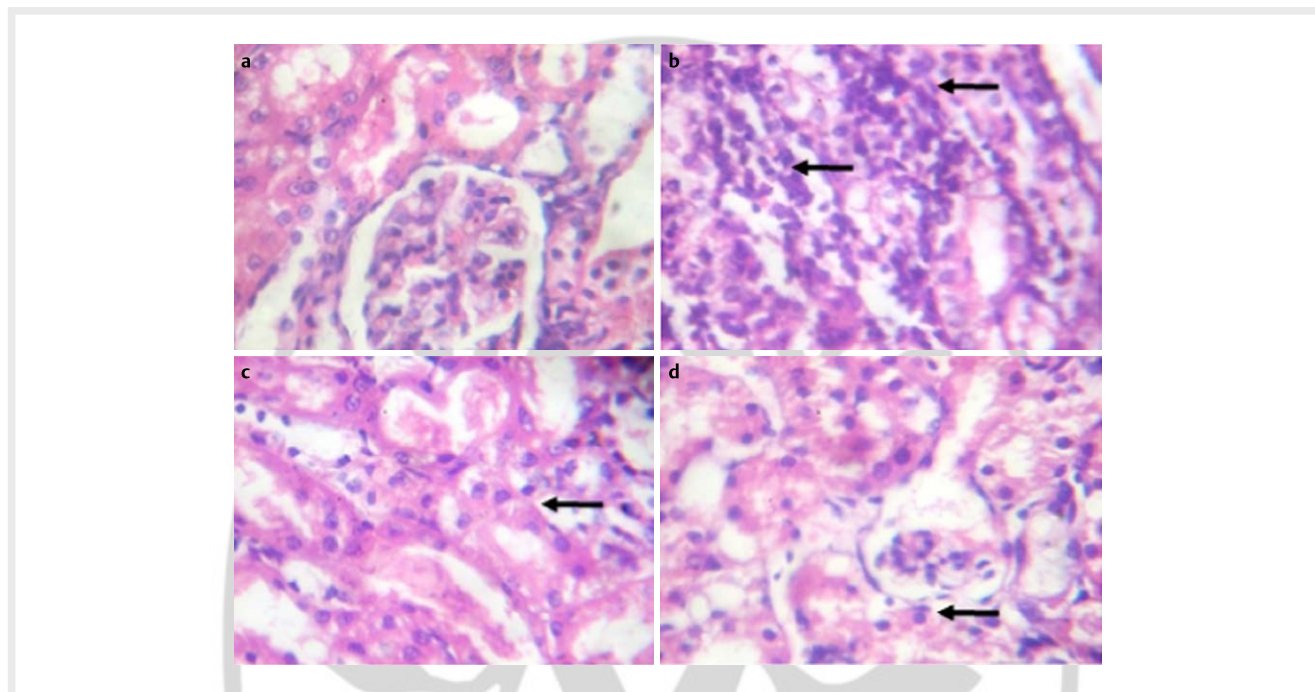
► **Fig. 15** The photomicrograph of rat kidney of cisplatin-induced acute kidney injury. **a** (Control): shows no visible lesion. b (CP 10 mg/kg): shows severe infiltration of inflammatory cells. c (CP + 200 mg/kg) & **d** (CP + 400 mg/kg): show moderate glomerular degeneration. Mag. × 400.

AKI, inflammatory cells of the immune system such as T cells, macrophages, neutrophils, and mast cells have a role to play in the infiltration of the kidney tissue thus a pathophysiological role for T lymphocytes, has been well established in CP-induced AKI [53]. Zou et al [54] reported that AP showed potent anti-inflammatory effect on pathogen-induced PID in rats, with a potential mechanism of inhibiting the NF-κB signal pathway. The moderate glomerular degeneration noted in this study may be attributable to the anti-inflammatory property of this extract, hence, the protective effect on the kidney of rats.

In immunohistochemistry, while there was higher expression of KIM-1 for group B rats, however, there was a lower expression for groups C and D. Again, there was lower expression of Nrf2 for group B but higher expressions KIM-1 for animals group C and D (► **Figs. 16**, **17**). In all forms of injury involving the kidney, kidney injury molecule 1 (KIM-1) is upregulated more than any other protein in the proximal tubule of the kidney [55]. It is a phosphatidylserine receptor that mediates phagocytosis of apoptotic bodies and oxidized lipids [56]. When it is expressed chronically, the result is progressive kidney fibrosis, hence, chronic kidney failure [57]. The resultant effect of chronic kidney failure may be related to its phagocytic function which enables KIM-1 to take up noxious compounds, including oxidized lipids. Apart from playing a role in phagocytosis,

KIM-1 can also activate signalling through the phosphoinositide 3 (PI3)-kinase pathway [58]. The upregulation of KIM-1 as a result of CP-induced kidney injury in this study is in agreement with the work of Dugbartey et al [59] who stated that KIM-1 is a novel biomarker for detection of subclinical nephrotoxicity in oncology patients treated with CP. The extract of Andrographis paniculata at both doses down regulated the expression of KIM-1 signifying its ability to protect the kidney from CP-induced injury. It is to be noted that acute kidney injury can progress to chronic kidney injury and end-stage renal failure if the underlying cause is not checked. KIM-1, an epithelial phosphatidylserine receptor that is expressed transiently after acute injury and chronically in fibrotic renal disease, is reported to promote kidney fibrosis [57]. The ability of AP extract to down regulate KIM-1 meant that it has the capacity to halt the progression of acute kidney injury to chronic acute injury and end-stage renal failure. The rats administered only CP showed lower expression of nrf2 while the opposite was obtained for the rats post-treated with the extract of AP. The erythroid transcription factor NF-E2-related factor 2 (Nrf2), is critically involved in cellular defence against oxidative stress [60]. Under ideal conditions, Nrf2 interacts with Kelch-like ECH-associated protein 1 (Keap1), leading to its degradation by the ubiquitin-proteasome pathway to maintain Nrf2 at a low level. But in response to electrophiles and oxidative stress,
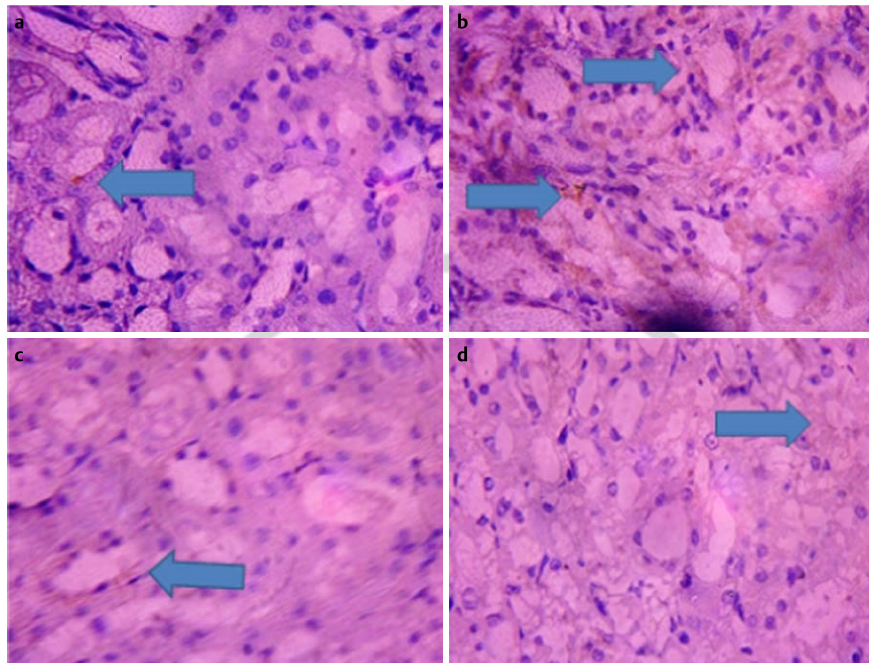
▶ **Fig. 16** Immunohistochemistry of kidney injury molecule 1 (KIM-1) in the kidney of cisplatin-induced acute kidney injury in rats. **a** (Control): shows positive and low expression of Kim-1. **b** (CP (10 mg/kg)) shows higher expression of Kim-1 than A (control). **c** (CP + 200 mg/kg) and **d** (CP + 400 mg/kg): show lower expression of Kim-1 than **b** (CP (10 mg/kg). The slides were counterstained with high definition of haematoxylin. Mag x100.
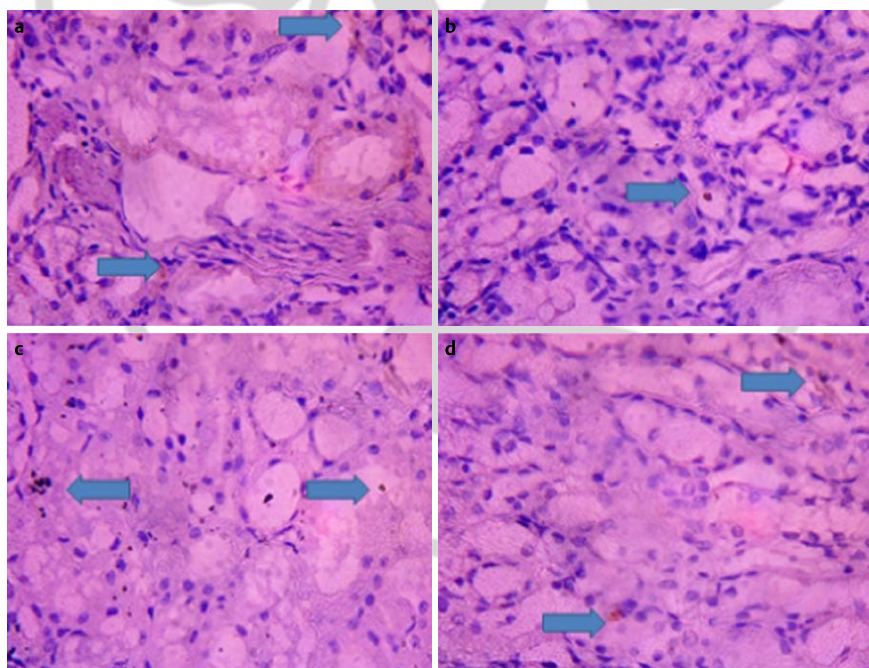
▶ **Fig. 17** Immunohistochemistry of nuclear factor erythroid-2- related factor 2 in the kidney of cisplatin-induced acute kidney injury in rats. **a** (Control): Shows higher expression of Nrf_2. **b** (CP 10 mg/kg) shows lower expression of Nrf2 than A (control). **c** (CP + 200 mg/kg) and **d** (CP + 400 mg/kg): show higher expression of Nrf2 than **b** (CP). The slides were counterstained with high definition of haematoxylin. Mag x100

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Nrf2 dissociates from Keap1, thus provoking its activation [61]. The activation of Nrf2 leads to its translocation to the nucleus. This in turn lead to the activation and induction of phase II detoxifying enzymes, such as NAD(P)H: quinone oxidoreductase (NQO1) and antioxidant proteins, like hemeoxygenase-1 (HO-1) [62]. It has been reported that the Nrf2-Keap1 pathway protects cells from many diseases including neurodegenerative disease and cancers [63]. It's down regulation by CP is a pointer to the fact that CP-induced kidney injury has to do with oxidative stress as already explained above. On the other hand, the up regulation of this protein by the extract of Andrographis p. has further confirmed its anti-oxidative potential. Wong et al. [64] showed that andrographolide, the major labdane diterpenoid originally isolated from Andrographis paniculata potently activated Nrf2 and also upregulated HO-1 expression in primary astrocytes. The report from this study is thus in agreement with our findings.

Conclusion

It could thus be concluded from this study that the ethanol leaf extract of Andrographis paniculata possesses protective properties against CP-induced kidney injury through its anti-inflammatory and anti-oxidant effects. Since the extract of AP is said to have anticancer property [65], concurrent use with cisplatin in cancer chemotherapy may be desirable.

Acknowledgement

Conflict of interest

The authors have no conflict of interest to declare.

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