International Journal of Medicine and Pharmaceutical Research

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R E S E A R C H A R T I C L E

Investigation of Cardiac Protective Activity of Methanolic Extracts of *Garcinia Indica* in Rats

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A B S T R A C T

Atherosclerotic heart disease, heart attack and heart stroke, but atherosclerosis is primary cause of death. Developing countries are reliant on medicinal plants as their main source of treatment for diseases. As *Garcinia indica* have the native habitat the production is more so it is locally available cost effective with no side effects. As *Garcinia indica* is cost effective and beneficiary in metabolism of cholesterol, so it has been taken in to consideration in order "To evaluate cardioprotective activity of Methanolic Extracts of *Garcinia indica* in isoproterenol induced cardiac toxicity in rats. And the final results showed that this plant shows the cardiac protective activity and also shows minimal side effects towards the cardiac and cardiac muscle tissues.

Keywords: *Garcinia indica*, cardiac muscle, cardioprotective.

A R T I C L E I N F O

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PAPER-QRCODE

A R T I C L E H I S T O R Y: Received 07 Sept 2019, **Accepted** 07 October 2019, **Available Online**10 December 2019

Citation: *Vijaya Kumar Gampa, et al.* Investigation of Cardiac Protective Activity of Methanolic Extracts of Garcinia Indica in Rats.*Int. J. Med. Pharm. Res., 2019, 7(6):184-190.*

CONTENTS

1. Introduction

The medicinal plants are potential sources of drugs as they are rich in secondary metabolites and essential oils of therapeutic importance. Uses of medicinal plants in various ailments are due to being economical, effective, their ease availability and due to their safety. Because of these advantages the use of medicinal plants has been widely increased by the traditional medical practitioners in their day to day practice. Foods are used commonly to meet our

nutritional needs. However, foods obtained by plants contain a wide range of non nutrient phytochemicals that are synthesized by plants for their own defense and for other biological functions. When we ingest these plant foods to meet our nutritional needs, we also ingest a wide variety of these non-nutrient phytochemicals. These phytochemicals have the potential for preventing chronic diseases and also non-toxic.

Cardiovascular disease is the number one cause of death globally and is projected to remain the leading cause of death. As many as 1.4 million children are suffering from heart related diseases in Pakistan and some 8,000 need heart surgeries annually, but out of them only 1,200 are operated upon. (Sixth "Biennial International Conference," organized by the Pakistan Society of Cardiovascular and Thoracic Surgeries). Free radicals play deleterious role to body stablished ischemia. Presence of various antioxidant compounds in fruits and vegetables, for example, vitamins C and E, b-carotene and polyphenolics have been associated with decreased risks of several chronic diseases, such as coronary heart disease and some cancers. Antioxidants scavenging the free radicals and protect the body.

There is inverse relationship between intake of polyphenols and heart diseases. There is a large and increasing global burden of cardiovascular disease. Approximately 14 million individuals died of cardiovascular disease in 1990, and this is projected to rise to about 25 million by 2020. The global burden of disease due to cardiovascular diseases (CVDs) is escalating, principally due to a sharp rise in the developing countries which are experiencing rapid health transition. The continuous increase in incidences of cardiovascular disease is a manifestation of chronic poor diet the backbone of Indian traditional system of medication is herbal source, Ayurveda the oldest traditional medicinal system that is based on plant medicines and treatments. Garcinia indica, commonly known as Kokum belonging to the Clusiaceae family, is a tropical fruit native to India. It does not require irrigation, spraying or fertilizers.

The Garcinia indica tree's major health benefits are derived from the fruit which is rich in polyisoprenylated benzophenone derivatives such as Garcinol a yellow, fat soluble pigment and isogarcinol its colourless isomer. Garcinol possess anti-oxidative, chelating, free radical scavenging, anticancer, anti-inflammatory, and antiulcer activities. Hydroxycitric acid (HCA), a water soluble constituent that possess apetite suppressant effect and antihyperlipidemic activity. The fruit also contains other compounds including hydroxycitric acid lactones, citric acid and oxalic acid. Malic acid, polyphenols, carbohydrates, anthocyanin, pigments and ascorbic acid. Kokum seed butter has nongreasy moisterising properties that are being usedin many cosmetics, creams, conditioners, and soaps. Kokum tel is used as foot massage. It is an Indian spice used in many parts of the country for making several vegetarian and non- vegetarian 'curry' preparations like chutneys, pickles and the popular 'solkadhi'. The fruits are steeped in sugar syrup to make 'Amrutkokum', a healthy soft drink to relieve sunstroke, which is popular during summer. Kokum is loaded with B complex, vitamins and minerals which help to control heart rate and blood pressure. This is versatile golden fruit has long been used to combat digestive problems such as indigestion, flatulence, acidity and constipation. The detailed plant information and its uses could help to lead the discovery of various new plant based drugs and treatment of various disorders and diseases.

To evaluate the cardioprotective effect of *Garcinia indica* Methanolic Extracts on isoproterenol induced cardiac toxicity in rats. *Garcinia indica* is a plant species in the genus Dracocephalum, endemic to China. The specific epithet, "rupestre", is derived from Latin, and pertains to the plant growing among rocks. *Garcinia indica* is a rhizomatous herb having numerous purplish, upwards-rising and unbranching stems (15–42 cm) scantily covered in backward-pointing hairs. Triangular-ovate, sparsely villous leaves (1.4–5.5 × 1.2–4.5 cm) are numerous. Inflorescences are verticillastrate with bluish-purple petalled flowers. Flowering period is from July–September

2. Materials and Method

Materials:

Reduced nicotinamide adenine dinucleotide (NADH), Glutathione reduced are bought from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Hydrogen peroxide, Ethanol, 2,4-dinitro phenylhydrazine (DNPH), Dipotassium hydrogen phosphate, Potassium dihydrogen phosphate are bought from Merck, Mumbai, India.Azathioprine is bought from RPG Life sciences Pvt, Ltd, Hyd. Ascorbic acid is bought from Finar chemicals, Ahmedabad, India. Normal saline is bought from Claris life sciences. Ltd., Ahmedabad, India.

Methods

Collection and Authentification of Plant Material:

The whole plant of *Garcinia indica* for the study were procured and authenticated

Extraction of Plant Material:

The plant flowers are grinded in to a coarse powder with the help of suitable grinder.

Cold Extraction (Methanol Extraction):

In this work the cold extraction process was done with the help of methanol. About 200gms of powdered material was taken in a clean, flat-bottomed glass container and soaked in 750 ml of methanol. The container with its contents were sealed and kept for period of 7 days accompanied by continuous shaking with the shaker. The whole mixture then went under a coarse filtration by a piece of a clean, white cotton wool.

Evaporation of Solvent:

The filtrates (methanol extract) obtained were evaporated using Rotary evaporator in a porcelain dish. They rendered a gummy concentrate of greenish black. The extract was kept in vacuum desiccator for 7 days.

Preliminary Phytochemical Screening:

Preliminary phytochemical screening of the *Garcinia indica* extract was carried out for the analysis of Alkaloids,

Carbohydrates, Tannins, Saponins, Steroids, Phenols, Flavonoids as per the standard methods.

Animals:

Healthy Adult Male rats of 5 weeks old with Average weight in the range of 100-150gms were selected. Animals are housed 4 per cage in temperature controlled (27 ^{0}C ±3 ^{0}c) room with light/dark cycle in a ratio of 12:12 hrs is to be maintained. The Animals are allowed to acclimatize to the environment for seven days and are supplied with a standard diet and water *ad libitum*. The guidelines of committee for the purpose of control and supervision of experiments on Animals (CPCSEA), Govt of India were followed and prior permission was sought from the Institutional Animal Ethics Committee (IAEC) for conducting the study.

Acute toxicity studies:

The Acute Toxicity Studies was performed using female rats as per OECD Guideline No.423 (Short term toxicity). Male rats were selected of weight around 100-150 gm for main test. Single animals are dosed in sequence usually at 48 h intervals. A Dose Progression Factor of 3.2 is used. Using the default dose progression factor, doses would be selected from the sequence (1.75, 5.5, 17.5, 55, 175, 550, 1750, and 5000). However, the time intervals between dosing are determined by the onset, duration, and severity of toxic signs. Treatment of an animal at the next dose should be delayed until one is confident of survival of the previously dosed animal. If the animal survives, the second animal receives a higher dose. If the first animal dies or appears moribund, the second animal receives a lower dose. The toxicological effects were observed in terms of mortality expressed as LD50. The number of animals dying or surviving during a period was noted.

Disease induction:

20 mg/kg of isoproterenol is given to the animals in all groups on the 17th day of the experiment except for the normal control group.

Experimental design for cardioprotective activity:

The rats will be divided into six groups (6 rats in each group). All animals were deprived of food and water 2 hours before, and 2 hours after administration of different doses of the methanolic extract of *Garcinia indica* leaves for a period of 14 days.

Group I : Control rats.

Group II : Isoproterenol (20 mg/kg. b.wt. Sigma Chemical Co, USA) was given by s.c injection for 1 day (17 th day).

Group III : methanolic extract of *Garcinia indica* treated rats (250 mg/kg b.wt was given orally for 30 days). Isoproterenol was given by s.c injection on 17 th day.

Group IV : methanolic extract of *Garcinia indica* treated rats (500 mg/kg b.wt was given orally for 30 days). Isoproterenol was given by s.c injection on 17 th day.

Group V : Atorvastatin was given orally (10 mg / kg b.wt) for 30 days.

Isoproterenol was given by s.c injection on 17th day. Atorvastatin was used as a positive control.

After the experimental regimen, all the animals were sacrificed by cervical dislocation under mild, chloroform anesthesia. Blood was collected into clean centrifuge tubes by carotid bleeding and allowed for clotting. Then the serum was separated by centrifugation at 3000 rpm for 15 minutes and was kept at 4°C'to assess the activities of serum enzymes. The heart was excised immediately, rinsed in ice- cold saline and used for biochemical assays. A portion of heart tissue was fixed in 10% buffered neutral formalin solution for histopathological studies.

Assay of hematological parameters:

Hemoglobin content, red blood cells (RBC), white blood cells (WBC), differential count, total count, and platelet count were assayed in the blood as per the standard methods

Estimation of Hemoglobin (Drabkin and Austin, 1932):

Pipetted out 0.02 ml of serum and 5.0 ml of Drabkin's solution into a test tube. Simultaneously, a blank was set up with Drabkin's solution and distilled water. Mixed well the above tubes and allowed to stand at room temperature for 5 minutes. Measured the absorbance of test at 546 nm. Take the absorbance of cyanmethemoglobin standard was taken directly without adding working reagent against blank at 546 nm. The results were expressed as g/dl in serum.

Estimation of Red Blood Cells (Chesbrough and McArthur, 1972):

Procedure:

The whole blood was taken into the RBC pipette exactly up to the 0.5 mark (Thoina pipette mark 101) and the diluting fluid (formal citrate solution) was immediately drawn up to the mark 101. The pipette was rotated between the thumb and the forefinger. This gave a dilution of 1:200. The cover glass was placed in position over the ruled area using gentle pressure. The suspension was mixed thoroughly by rotating the pipette for about a minute, holding it in horizontal position, and finally shook it sidewise. The fluid was expelled from the stem of the pipette and filled the chamber immediately by holding the pipette at an angle of 45° and slightly touching the tip against the edge of the cover glass. There should not be any bubbles under the cover glass. Then the red corpuscles were allowed to settle for 2 to 3 minutes. The number of RBCs was counted in 180 small squares (4 squares of 16 at each four corners and one of 16 at centre).The cells touching the lower and right hand lines were not counted, but the cells touching the upper and left hand lines were counted. The cells counted are expressed as million cells /rnmj blood.

Estimation of White Blood Cells (Chesbrough and McArthur, 1972):

Procedure:

The whole blood was taken up to the mark 0.5 in WBC pipette and diluted up to the mark 11 with WBC fluid as described in RBC counting and filled the counting chamber in the same manner. Then the cells area was allowed to settle for 3 minutes. The neubaur counting chamber was used to count the cells in the four comers and each of these 4 sq mm. areas is subdivided into 16 squares by using the low power objective and a medium ocular. While counting, the cells included were those touching the lines on the left and bottom. The difference between the two squares millimeter area as thousand cells /mm blood.

Estimation of Lactate Dehydrogenase (LDH) (King, 1965b):

Procedure:

Placed 1.0ml buffered substrate and O.lml sample into each of two tubes. Added 0.2ml water to the blank. Then to the test added 0.2ml of NAD. Mixed and incubated at 37°C for 15 mins. Exactly after 15 mins, 1.0ml of dinitrophenyl hydrazine was added to each (test and control). Left for further 15 mins. Then added 10ml of 0.4N Sodium hydroxide and the color developed was read immediately at 440 nm. A standard curve with sodium pyruvate solution with the concentration range 0.1 -l.O umole was taken. LDH activity in serum was expressed as umoles of pymvate liberated / L and in cardiac homogenate as nmoles of pyruvate liberated / minute / rng protein.

Estimation of Creatine Kinase (Okinaka, 1961):

The incubation mixture containing 0.75ml of double distilled water, 0.05 ml of serum, 0.1 ml of ATP solution, O.lml of magnesium - cysteine reagent and 0.1 ml of creatine was incubated at 37°C for 20 mins. The tubes were centrifuged and the supernatant was used for the estimation of phosphorus. The enzyme activity is expressed as IU/L.

Estimation of Creatine Kinase- Mb Activity (Neumeir, 1981):

To the test tubes added lOOOul of the reagent and 50fil of the sample. The mixture was mixed and incubated at 37 C. The absorbance was measured after 300 seconds. Two additional absorbance was taken at 1 minute interval. The mean absorbance change/minutes (AA/min) was calculated. The change in absorbance/ minute was multiplied by factor 3376 that is equal to CK-MB.

Measurement of blood pressure: Before the systolic blood pressure measured by the tailcuff method using PowerLab data acquisition systems (ADInstruments, Bella Vista, NSW, Australia), the rats were warmed for 10 min. Five readings were obtained from each rat and averaged.

DPPH Free Radical-Scavenging Activity:

The methanolic solution of DPPH (0.1 mM, 1 ml) was incubated with 3 ml of different concentrations of the root extract ranging from 10-100 µg/ml. Incubation was carried out at room temperature (25^0C) for 30 min. For each concentration, the assay was run in triplicate. At the end of the incubation period, the optical density of each sample was determined at 517 *nm*. Ascorbic acid solution was used as a standard. EC_{50} values (concentration required to scavenge 50% of the free radicals) for both ascorbic acid and the root extract were determined. The radical scavenging activity of the tested sample was expressed as an inhibition percentage (IP) [102].

$$\textbf{DPPH Scavenged (\%)} = (A_{DPPH} - A_{test} / A_{DPPH}) \times 100$$

Where,

A_{DPPH} is the absorbance of the 0.1 mM of DPPH solution

A_{test} is the absorbance in the presence of the extract or ascorbic acid.

IC_{50} value was determined from the graph obtained using standard ascorbic acid by using the "y = mx + c" formula from the slope of the graph.

Histopathological Studies:

Whole hearts were serially sectioned (at 10 µm thickness) using a Leica SM2400 heavy duty sledge-type microtome. The tissue was allowed to relax in a water bath (Leica Microsystems, HI 1210) at 39°C for 2–15 min, depending on the size of the tissue section, and then carefully (aiming for minimal distortion and avoidance of tissue folds) mounted to positively charged slides (SuperFrost, VWR). Slides were air-dried in a laminar airflow hood (overnight), followed by de-waxing and trichrome staining using an automated stainer (Leica AutoStainer XL, ST50-10). Trichrome stain allows one to identify collagen (bluish green), myocytes (pink), cytoplasm (orange, highlighting non-myocytes), and nuclei (blue-black). The stained and mounted sections were imaged at high resolution with Leica Application Suite, operating the LAS Power Mosaic Module, integrated with an automated Leica DM4000B light microscope and a Märzhäuser inverted microscope scanning stage.

Statistical Analysis:

Results are expressed as Mean ± S.D .all the results were compared with control subject one-way analysis of variance (ANOVA), followed by the dunnet t-test using Graph Pad Prism Software 6 version. P Values < 0.05 were as considered statistically significant.

3. Results and Discussion

%Yield of Methanolic Extract from Aerial Parts of *Garcinia indica* was found to be **34.75.**

Preliminary Phytochemical Screening:

Investigation revealed the presence of Alkaloid, Tannin, Saponin, Phenol in Methanolic Extract of *Garcinia indica* while only Phenol were present in Methanolic Extract of *Garcinia indica*

Acute toxicity studies:

As per (OECD) draft guidelines 423 adopted, Female albino rats were administered with *Garcinia indica* and doses was be selected in the sequence (1.75- 5000) using the default dose progression factor, for the purpose of toxicity study. Animals are observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours and daily thereafter, for a total of 14 days,. In all the cases, no death was observed within 14 days. Additional observations like behavioral changes in skin, fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems and somato motor activity and behavior pattern were also found to be normal. Attention was also given to observation of tremors and convulsions, salivation, diarrhoea, lethargy, sleep and coma. Overall results suggested the LD_{50} value as 5000 mg/kg. Hence therapeutic dose was calculated (i.e. 400mg/kg and 500 mg/kg) of the lethal dose for the purpose of cardiac protective investigations.

Haematological Parameters:

Alterations in blood parameters may be due to changes in cellular integrity and membrane permeability of cells or even due to exposure to toxic chemicals (Hoffbrand and Petrit 1997).Results of the haematological studies data showed that all the haematological parameters for the control rats were not significantly different ($P<0.05$) from those treated with the methanolic extract of *Garcinia indica* As far as the haematological and biochemical parameters are concerned, no index of significant alteration in relation to control appeared, after 14 days of treatment. All values

found for these parameters are comparable to those existing in specialized literature (Mitruka and Rawnstley 1977; Rodrigues *et al* 1998).

Certain medicinal herbal preparations or convectional drugs or chemicals adversely affect various blood components (Synder *et al.,* 1977; King and Kelton 1984). Decrease or increase in cell counts and depletion of plasma constituents or their elevation beyond reference range could equally demonstrate haematotoxicity (Dioka *et al.,* 2002). The methanolic extracts did not affect the haematograms of the rats in a manner that would suggest adverse effects on their bone marrow, which is a source of reticulocytes.Blood is an important index of physiological and pathological status in man and animals and the parameters usually measured are hemoglobin, packed cell volume, white blood cell count and platelets count (Schalm *et* a/., 1975).

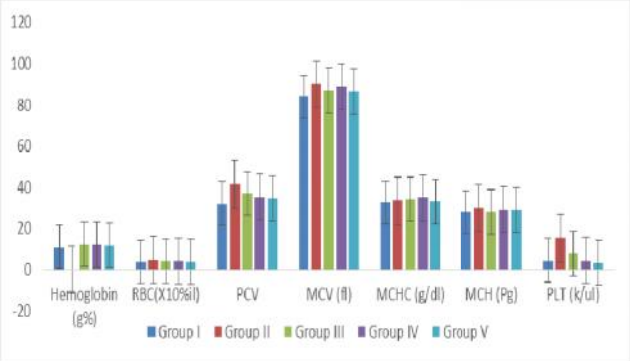

Fig 1: Effect of methanolic extracts on haematological profile in blood of control and experimental rats

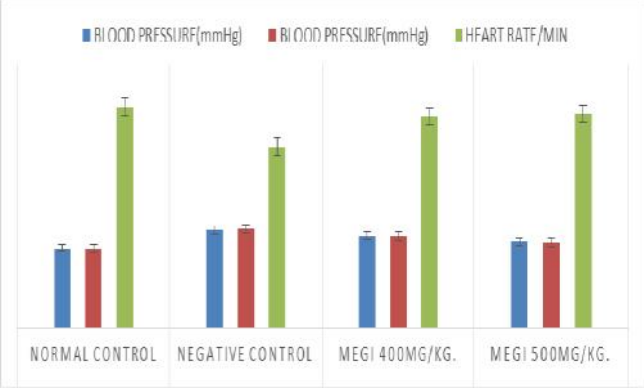

Fig 2: Estimation of blood pressure and heart rate

Estimation of Lactate Dehydrogenase (LDH) (King, 1965b):

Marker enzymes namely (LDH) were determined after administration of MEGIdid not produce any deleterious alteration in the levels of LDH in both serum and heart of extract treated rats compared to control rats. It was found that most of the parameters were slightly changed with respect to control group rats but remain within the normal range. This indicates insignificant adverse effect of MEGIon cardiac function. The cardiac function tests involve mainly the determination of LDH (Tilkian,1979) and any marked necrosis of the cardiac cells lead to a significant rise of these enzymes in the serum.

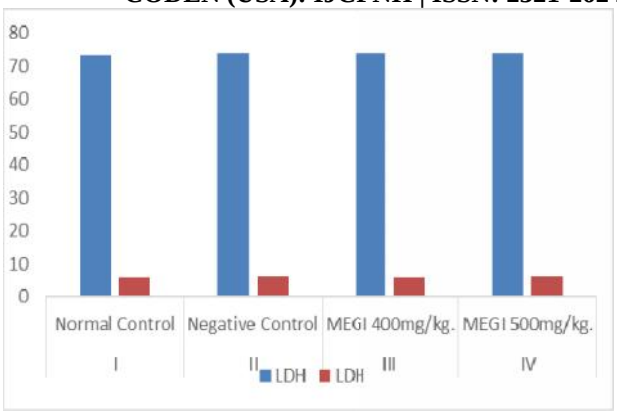

Fig 3: Activities of cardiac marker enzymes in serum and cardiac of control and experimental rats

Estimation of CK and CK-MB

The significant (P<0.05) rise observed in the activities of diagnostic marker enzyme namely CK and CK-MB in the serum of Isoproterenol administered rats as compared to that of control rats is an indication of the severity of the necrotic damage to the myocardial membrane. MEGI pretreatment at all the three doses significantly reduced (PO.05) the activities of the marker enzymes as compared to the rats treated with DOX alone. This reduction in enzyme activities could be due to its action on maintaining membrane integrity thereby restricting the leakage of this enzyme.

The activities of both CK and CK-MB in heart tissues were decreased on DOX treatment whereas it reverted back towards normalcy on treatment with methanolic extracts of MEGI at all the selected concentrations. Creatine kinase is a muscle specific enzyme mainly for heart and brain; therefore, its increase in serum is the result of myocarditis, cardiac insufficiency, arrhythmias and myocardial infarction (Okinaka et al., 1961). DOX is a well-known cardiotoxic agent; due to its ability, it will destruct myocardial cells. As a result of this, cytosolic enzyme creatine kinase was released into blood stream and serve as the diagnostic marker of myocardial tissue damage. The amount of this cellular enzyme present in blood reflects the alterations in plasma membrane integrity and / or permeability.

CK-MB is the diagnostic marker of MI. This enzyme is released from the heart into the blood during myocardial damage due to DOX induced necrosis in the myocardium. When myocardial cells containing CK-MB are damaged or destroyed, the cell membrane becomes permeable or may rupture, which results in the leakage of these enzymes. This accounts for the increased activities of serum CK-MB in DOX treated rats. Pretreatment with methanolic extracts of MEGI decreased the activity of serum CK-MB in DOX-induced rats.

Punithavathi and Prince, 2009 who worked on Preventive effect of naringin on cardiac markers, electrocardiographic patterns and lysosomal hydrolases in normal and

Isoproterenol-induced myocardial infarction in Wistar rats, reported similar results.

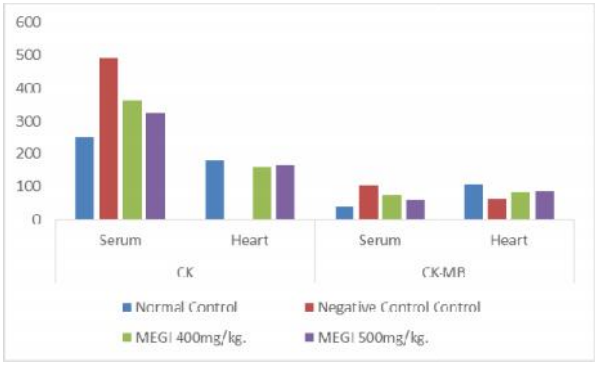

Fig 3: Activities of creatine kinase (CK) and creatine kinase MB (CK-MB) in serum and heart of control and experimental rats

***In Vitro* Anti-Oxidant Activity**

DPPH free radical scavenging activity:

DPPH is a relatively stable free radical which when encounters proton donors' such as antioxidants, the radicals get quenched and absorbance gets reduced. Results indicated definite scavenging activity of the extract towards DPPH radicals when compared with standard ascorbic acid. IC50 value for standard Ascorbic acid was found to be 43.137µg/ml., whereas the IC50 value for methanolic extract of MEGI was found to be 41.024µg/ml.

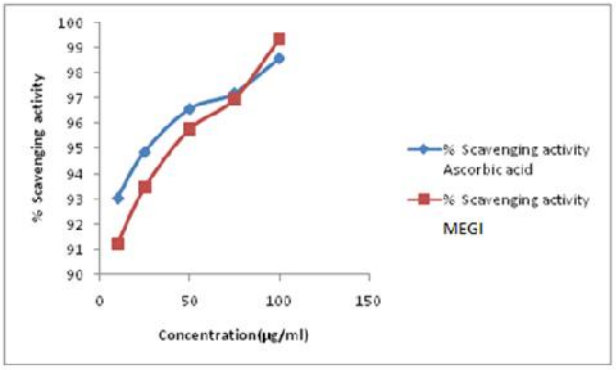

Fig 4: Anti-oxidant activity of MEGI & ascorbic acid

Histopathological studies:

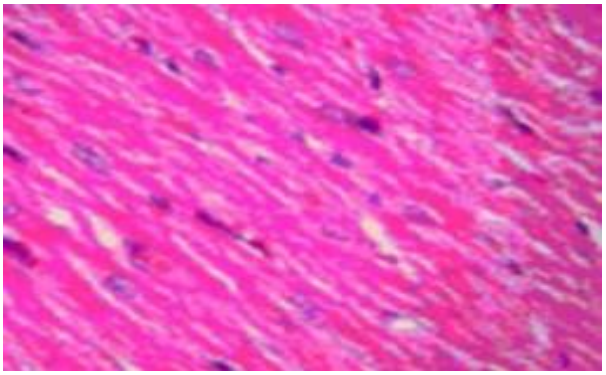

Fig 5: Normal control rats showing healthy mayocardial tissue

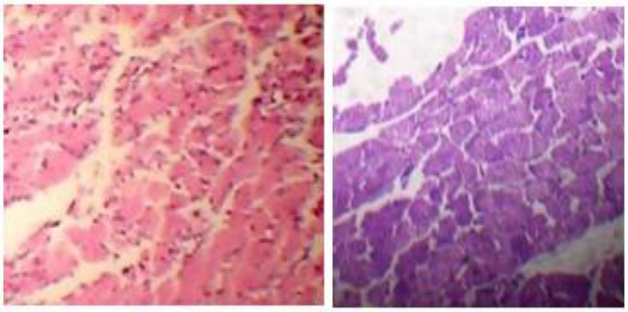

Fig 6: Isoproterenol induced cardiac toxic rats showing cell damage

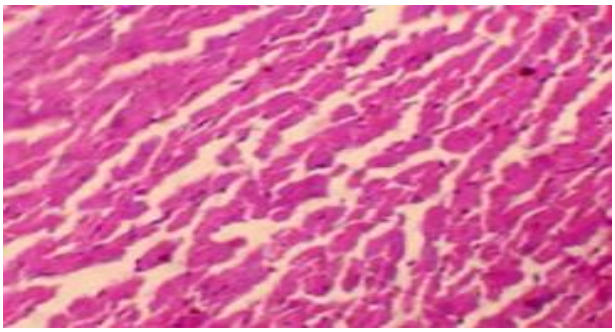

Fig 7: Myocardium of Rats treated with plant extract 400mg/kg

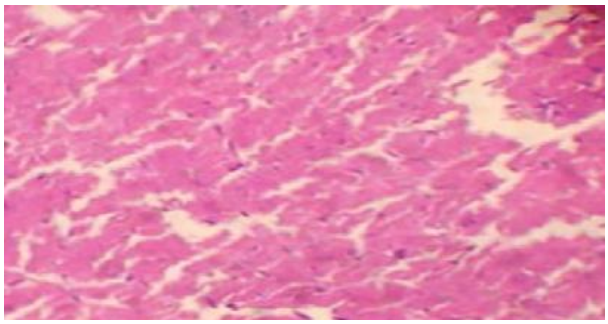

Fig 8: Myocardium of Rats treated with plant extract 500mg/kg

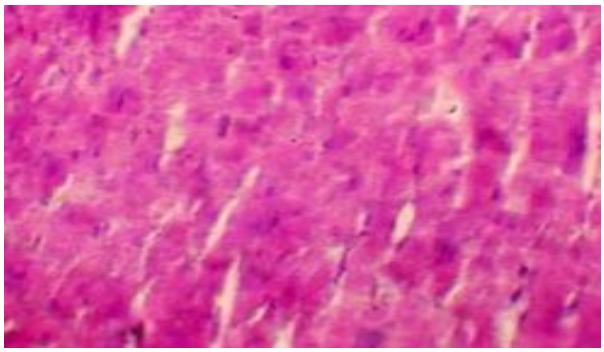

Fig 9: Myocardium of Rats treated with standard drug atorvastatin

4. Conclusion

Natural products extracts of therapeutic relevance are of paramount importance as reservoirs of structural and chemical diversity. A recent review on national pharmacopoeias from several countries reveals at least 120 distinct chemical substances from different plants that have

utility as life saving drugs. This has been achieved through chemical and pharmacological screening of only 6% of the total plant species. Untapped, hidden wealth in the flora needs to be unearthed and explored to cure diseases like heart disease, cancer, diabetes, AIDS etc. Most countries face high and increasing rates of cardiovascular disease. Combating these cardiovascular diseases is of a paramount importance today. Ischemic heart disease leading to myocardial infarction (MI) is a major clinical concern and remains as a clinical challenge and a problem of great importance despite considerable advances in therapy and management that have been made over the past three decades. MI continues to be a major public health problem, not only in western countries but also increasingly in developing countries and makes significant contribution to the mortality statistics. The results concluded that MEGI (500 mg/kg) have definite cardioprotective activity in the respective concentrations Further studies on this extract may lead to identify the possible mechanism of action.

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