



PHYTOCHEMICAL ANALYSIS AND CARDIOPROTECTIVE ACTIVITY OF *PLUMBAGO ZEYLANICA* LINN IN ISOPROTERENOL PROVOKED OXIDATIVE MYOCARDIAL INJURY IN ALBINO WISTAR RATS

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ABSTRACT

The phytochemical investigation of *Plumbago zeylanica* is done by using chemical tests and also by using analytical instruments like Fourier transform-Infrared spectroscopy and confirmed the presence of flavonoids. Isoproterenol a β -adrenergic agonist is used to induce the myocardial injury as it causes the generation of reactive oxygen free radicals and results in the necrosis of cardiac myocytes. The Myocardial infarction is confirmed by serum analysis for Lipid profile, Cardiac biomarkers and the oxidative markers are also estimated in the tissue homogenate, the cardio protective effect of *Plumbago zeylanica* is confirmed by analysis of the above parameters and concluded that the hydro alcoholic extract of *Plumbago zeylanica* has the cardio protective effect.

Keywords: Isoproterenol, *Plumbago zeylanica*, Cardiac biomarkers, Flavonoids, Myocardial infarction.

INTRODUCTION

Myocardial infarction, also known as "heart attack," is the death of cardiac muscle resulting from ischemia. It is by far the most important form of IHD and alone is the leading cause of death in the United States and industrialized nations.

A new look at the cardiovascular crisis in India

Cardiovascular diseases in India have quadrupled in the last 40 years and WHO estimates that by 2020 close to 60% of cardiac patients worldwide would be Indians.

The field of environmental cardiology is relatively new. A lot of research is under way at the moment mainly focusing on environmental factors and possible correlation with cardiovascular diseases. Tobacco smoke was one of the earliest environmental factors to be linked with cardiovascular diseases and the moving on to second hand smoke as well. These factors will cause the generation of reactive free radicals thus causing the damage to the myocytes of cardiac muscle resulting in tissue necrosis and lipid peroxidation that results in increase in lipid profile.

In the present study Isoproterenol is used to induce myocardial necrosis which has the same mechanism of generating free radicals causing lipid peroxidation damage to proteins by ROS produces carbonyls and other amino acid modifications. Carbonyl derivatives are the result of direct free radical damage to various amino groups such as lysine, arginine, proline and threonine. This can also lead to dysfunction of membrane channels and pumps, inactivation of enzymes and reduction in the ability of hemoglobin to carry oxygen¹. Elevation of lipid profile in the laboratory animals at a dose of 85mg/kg.

AIM OF WORK

To identify a plant with potential cardioprotective activity. In the present study phytochemical analysis and cardioprotective activity of *Plumbago zeylanica* Linn in isoproterenol provoked oxidative myocardial injury in albino wistar rats is identified and its efficiency is compared with the standard marketed product Propranolol.

COLLECTION OF PLANT MATERIAL

Fresh plant of *Plumbago zeylanica* LINN was collected from Alagar Hills, Madurai, Tamil Nadu. The plant specimen was authenticated by Dr.D.Stephen, Lecturer, Dept of Botany, The American College, Madurai, Tamil Nadu.

MATERIALS AND METHODS

Soxhlet apparatus, Pet. Ether, Chloroform, Ethanol, Distilled water, Shade dried plant of *Plumbago zeylanica*, Albino wistar rats (180-220gm), Isoproterenol and Propranolol.

PREPERATION OF PLANT EXTRACT

The whole plant of *Plumbago zeylanica* was dried in the shade. Then the shade dried plants were powdered to get coarse powder and about 500 gms of dried powder of *Plumbago zeylanica* was soaked in extractor and macerated for 30 hr with petroleum ether, followed by chloroform. Then it is extracted with water: ethanol by continuous hot percolation technique using Soxhlet apparatus for 72hrs. Crude extract was distilled under vaccum condition. After concentration, the hydro alcoholic extract gives brownish residue which weigh about 7.2gms. The extract was used for experimentation.

SELECTION AND ACCLIMITIZATION OF ANIMALS

Albino rats of wistar strains weighing between 180-220gm were selected and were fed with standard pellet diet and water ad libitum. They were housed in well ventilated cages (3-4 per cage), maintained at room temperature under 12hr light dark cycle. Animals were acclimatized for a week to the laboratory conditions.

TREATMENT PROTOCOL

The acclimatized animals were divided into 5 groups of each 6 animals, designated as

- Group 1: Served as normal control and receive normal diet and water.
- Group 2: Served as treatment control given normal saline for 28 days and at 29th & 30th day Isoproterenol (85mg/kg I.P)² was administered at 24 hr interval.
- Group 3: The HAEPZ (200mg/kg)³ given orally for 28 days and at 29th and 30th day Isoproterenol (85mg/kg I.P) was administered at 24 hr interval.
- Group 4: The HAEPZ (400mg/kg)³ is given orally for 28 days and at 29th and 30th day Isoproterenol (85mg/kg I.P) was administered at 24 hr interval.
- Group 5 : Served as standard control the animals are fed with normal diet and water for 15 days and from 16th day it was given Propranolol (10mg/kg I.P)⁴ and on 29th & 30th day Isoproterenol (85mg/kg I.P) is administered at 24 hr interval.

After acclimatization of animals and completing the drug administration as per the treatment protocol, the electro cardio gram was recorded by using the BIO PAC system and the waves are recorded. The blood was collected through retro orbital plexes bleeding and the hearts were isolated.

The collected blood was centrifuged and the serum was separated immediately and then the bio chemical parameters like cardiac bio markers, lipid profile were estimated. The hearts were homogenized by triturating with Krebs solution and immediately centrifuged in a cooling centrifuge at 2500 rpm for 10 minutes and the supernant liquid was subjected for the evaluation of anti-oxidant parameters.

To confirm the incidence of myocardial necrosis, the hearts were subjected to histopathological evaluation.

PHARMACOLOGICAL EVALUATION

Serum cardiac marker enzyme assay

The marker enzymes AST, LDH, CK-MB, C-TROPONIN were assayed in the serum by following the standard procedure using the auto analyser COBAS MIRA PLUS-S from ROCHE-SWITZERLAND. The results were expressed in terms of mg/dl.

Lipid peroxidation

The quantitative estimation of lipid peroxidation was conducted by determining the concentration of thiobarbituric acid reactive substances in heart using the method of Ohkawa and Yagi⁵. The amount of malondialdehyde (MDA) formed was quantified by reaction with TBA and used as an index of lipid peroxidation. The results were expressed as nmol of MDA/g of wet tissue using molar extinction coefficient of the chromophore (1.56 × 10⁻⁵/M/cm) and 1, 1, 3, 3-tetraethoxypropane as standard.

Glutathione estimation

GSH was estimated in the heart homogenate using DTNB by the method of Ellman⁶. The absorbance was read at 412 nm and the results were expressed as μmol of GSH/g of wet tissue.

Antioxidant enzyme assays in heart homogenate

SOD was assayed by the method of Sun et al, in which the activity of SOD is inversely proportional to the concentration of its oxidation product adrenochrome, which is measured spectrophotometrically at 320 nm⁷. CAT was estimated by the method of Clairborne et al, which is a quantitative spectroscopic method developed for following the breakdown of H₂O₂ at 240 nm in unit time for routine studies of catalase kinetics⁸.

Estimation of serum lipid profile

The serum was analyzed for total cholesterol, triglycerides, high density lipoproteins, low density lipo proteins, very low density lipoproteins using standard protocol methods. (COBAS MIRA PLUS-S from ROCHE-SWITZERLAND).

Histoarchitectural studies

The parts of the hearts stored in 10-percent (w/v) buffered formalin were embedded in paraffin, sections cut at 5 μm, and stained with hematoxylin and eosin. These sections were examined under a light microscope for histoarchitectural changes.

Statistical analysis

The results of cardioprotective and antioxidant activities are expressed as mean ± SEM from six animals in each group. Results were statistically analyzed using one-way ANOVA followed by Newman Kelus Multiple range test. P<0.05 was considered significant.

Graph Pad In Stat version 3.00 of Graph Pad Software, Inc. (San Diego, CA), was used for statistical analysis.

TABLE 1: RESULTS FOR SERUM CARDIAC BIO- MARKERS

GROUPS	LDH (IU/L)	CK-MB (IU/L)	AST (IU/L)	ALT (IU/L)	TROPONIN (mg/dl)
G 1	119.83±2.41	104.76±2.29	40.22±0.76	26.19±1.06	3.95±0.18
G 2	206±5.95***(a)	257.87±6.71***(a)	58.41±1.89***(a)	44.68±1.71***(a)	6.85±0.83***(a)
G 3	191.66±4.10**(b)	242.66±5.40**(b)	52.92±1.47**(b)	38.92±1.65**(b)	5.03±0.37**(b)
G 4	146.5±3.55***(a)	134.78±3.79***(a)	43.47±1.11***(a)	32.08±1.47***(a)	4.0±0.24***(a)
G 5	130.33±2.92***(a)	115.18±2.27***(a)	41.52±1.00***(a)	29.18±1.28***(a)	4.08±0.22***(a)

(Iu/l-International units/litre, Mg/dl- Milligram/desiletre), Values are expressed as Mean± SEM, Values were find out by using ONE WAY ANOVA followed by Newman Keuls multiple range test, ***(a) values were significantly different from normal control P<0.001., ***(b) values were significantly different from normal control P<0.05.

TABLE 2: RESULTS FOR SERUM LIPID PROFILE

GROUPS	TC (mg/dl)	TGL (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	CHO/HDL (mg/dl)
G 1	91.26±1.76	96.43±1.70	40.73±1.00	22.75±0.87	13.46±0.56	1.08±0.04
G 2	164.16±3.06***(a)	156.18±2.92***(a)	28.14±0.66***(a)	52.63±1.73***(a)	29.08±1.17***(a)	1.97±0.10***(a)
G 3	154.16±2.79**(b)	145.0±2.63**(b)	32.16±0.70**(b)	45.5±1.62**(b)	24.52±1.11**(b)	1.64±0.07**(b)
G 4	122.55±2.15***(a)	113.66±2.42***(a)	35.5±0.85***(a)	29.38±1.28***(a)	16.53±0.87***(a)	1.36±0.06***(a)
G 5	110.16±1.95***(a)	105.03±2.06***(a)	38.9±0.87***(a)	25.43±1.26***(a)	14.95±0.79***(a)	1.23±0.05***(a)

(Mg/dl-milligram/desilitre), Values are expressed as Mean± SEM, Values were find out by using ONE WAY ANOVA followed by Newman Keuls multiple range test, ***(a) values were significantly different from normal control P<0.001., ***(b) values were significantly different from normal control P<0.05.

TABLE 3: RESULTS FOR ANTI-OXIDANT PARAMETERS IN HEART HOMOGENATE

GROUPS	MDA nmol/g wet tissue	GSH μ mol/g wet tissue	SOD unit/mg protein	CAT unit/mg protein
G 1	38.83±1.37	2.10±0.10	11.72±0.74	22.25±1.01
G 2	86.44±2.40***(a)	1.21±0.01***(a)	5.78±0.17***(a)	11.77±0.66***(a)
G 3	78.41±2.05**(b)	1.58±0.04**(b)	7.26±0.31**(b)	15.5±0.67**(b)
G 4	55.95±1.80***(a)	1.85±0.05***(a)	8.90±0.32***(a)	10.83±0.60***(a)
G 5	43.30±1.68***(a)	1.98±0.09***(a)	10.83±0.60***(a)	20.60±1.01***(a)

Values are expressed as Mean± SEM, Values were find out by using ONE WAY ANOVA followed by Newman Keuls multiple range test, ***(a) values were significantly different from normal control P<0.001., ***(b) values were significantly different from normal control P<0.05.

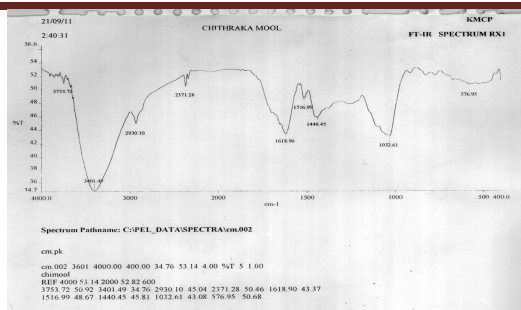


Table no 4

FREQUENCY	GROUPS ASSIGNED
3753.72	O-H aromatic phenolic group.
2930.10	C-H stretching
1618.90	C= C stretching (aromatic)
1032.61	O-H bending
760.90	C-H bending

TABLE 5 PHYTOCHEMICAL INVESTIGATION OF EXTRACT OF *Plumbago zeylanica*

CONSTITUENT	NAME OF THE TEST	RESULT
CARDIAC GLYCOSIDES	LEGAL'S TEST	+ve
	KEDDYS TEST	+ve
ALKALOIDS	Dragendoff's test	+ve
	Mayers test	+ve
	Wagners test	+ve
TANNINS	Ferric chloride test	+ve
STERIODS	Lieberman-Burchard's test	+ve
	Salkowski's test	+ve
FLAVONOIDS	Ferric chloride test	+ve
	Lead acetate test	+ve
	Sodium hydroxide test	+ve
SAPONINS	Frothing test	+ve
ANTHRAQUINONES	Brontragers test	+ve

ELECTRO CARDIO GRAM

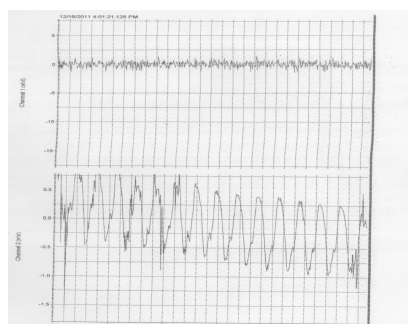


Fig no: 1 Toxic control (iso-85mg/kg)

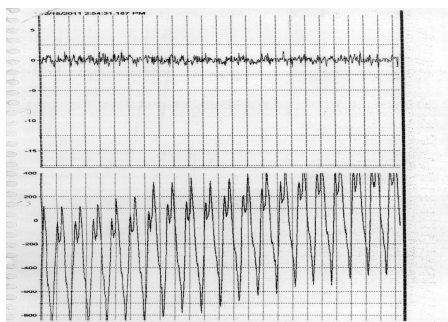


Fig no: 2 HAEZP 400 Mg/Kg

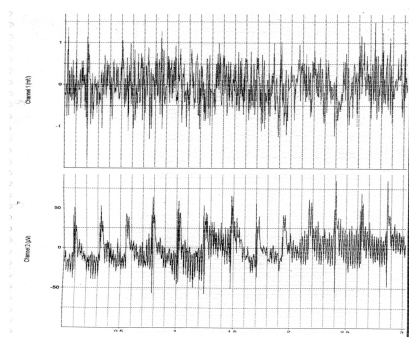


Fig no: 3 PROPRANOLOL 10mg/kg

HISTOARCHITECTURAL STUDIES

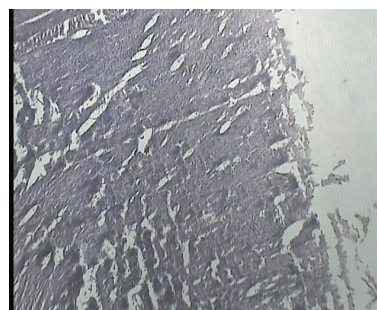


Fig no: 4 Toxic control (iso-85mg/kg)

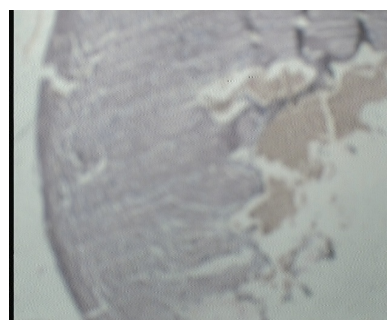


Fig no: 5 HAEZP 400 Mg/Kg



Fig no: 6 PROPRANOLOL 10mg/kg

RESULTS

Electro cardiogram

ISO treated group Showed significant changes in repolarization phase of the ECG. Significant prolongation of QT interval and elevation of ST segment, with no significant effect on QRS complex as compared to normal group. Pre treatment with HAEPZ 200mg/kg, 400mg/kg and propranolol 10mg/kg significantly reduced the ECG alterations when compared to ISO induced myocardial infarcted rats.

Histoarchitectural studies

Normal control- Section shows normal cardiac muscle.

Toxic control- Myocardial wall is thinned. Heart muscles show oedema between the fibers. Cytoplasm shows coagulation necrosis as shown in the fig 4.

HAEPZ 200mg/kg + ISO- Myocardial wall is thinned. Heart muscles show oedema between the fibers. No coagulation necrosis in the cytoplasm.

HAEPZ400mg/kg + ISO- Myocardial wall is thickened. Heart muscles show no oedema between the fibers. No coagulation necrosis in the cytoplasm as shown in the fig 5.

Propranolol10mg/kg+ ISO- Myocardial wall is thickened. Heart muscles show no oedema between the fibers. No coagulation necrosis in the cytoplasm as shown in the fig 6.

Serum cardiac biomarkers

In the present study administration of Isoproterenol 85mg/kg I.P resulted in cardio toxicity. The serum concentrations of various cardiac biomarkers investigated varied drastically following ISO treatment. The LDH, CK-MB, AST, ALT and TROPONIN values for G1 are 119.83±2.41, 104.76±2.29, 40.22±0.76, 26.19±1.06 & 3.95±0.18 respectively. It increased significantly to 206±5.95, 257.87±6.71, 58.41±1.89, 44.68±1.71 & 6.85±0.83 respectively (P<0.001) in G2. Treatment with HAEPZ 200mg/kg and 400mg/kg in G3 & G4 reduced the values to 191.66±4.10, 242.66±5.40, 52.92±1.47, 38.92±1.65 & 5.03±0.37 in G3 (P<0.05) and 146.5±3.55, 134.78±3.79, 43.47±1.11, 32.08±1.47 & 4.0±0.24 respectively in G4 (P<0.01) as shown in the table 1. The G5 treated with Propranolol 10mg/kg has regained the values to 130.33±2.29, 115.18±2.27, 41.52±1.00, 29.18±1.28 & 4.08±0.22 respectively which is near to the normal values as shown in the table 1.

Serum lipid profile

The serum lipid profile parameters such as TC, TGL, LDL, VLDL, CHO/HDL has increased significantly in G2 (ISO control) when compared with the G1 (normal control) indicating the lipogenesis. Upon treatment with 200mg/kg of HAEPZ, 400mg/kg of HAEPZ and propranolol 10mg/kg the values are regained to near normal values as shown in the table 2. The HDL values in the G2 has been decreased to 28.14±0.66 when compared with the values in G1 40.73±1.00. The G3, G4, & G5 which are treated with 200mg/kg HAEPZ, 400mg/kg HAEPZ and Propranolol10mg/kg the values obtained for HDL are 32.16±0.70, 35.5±0.85 and 38.9±0.87 respectively. The values are regained near to the normal values as shown in the table 2.

Anti oxidant parameters in heart homogenate

The MDA antioxidant parameters of the tissue homogenate in G1 (Normal control) is 35.83±1.37 which is increased drastically in G2 (ISO control) to 86.44±2.40. In the G3, G4 & G5 which are treated with HAEPZ 200mg/kg, 400mg/kg and Propranolol 10mg/kg the values are 78.41±2.05, 55.95±1.80 & 43.30±1.68 respectively which are near to the normal values as shown in the table 3. The GSH, SOD, CAT

antioxidant parameters of the tissue homogenate in G1 are 2.10±0.10, 11.72±0.74 & 22.25±1.01, which decreased significantly to 1.21±0.01, 5.78±0.17 & 11.77±0.66 in G2 which is served as ISO control. In the G3, G4 & G5 the values are increased to the near normal values as shown in the table 3.

DISCUSSION

Isoproterenol, a synthetic β -adrenergic agonist by its positive inotropic and chronotropic actions, increases the myocardial oxygen demand that leads to ischemic necrosis of myocardium in rats. Free radical-mediated lipid peroxidation and consequent changes in membrane permeability are the primary factors for cardiotoxicity induced by ISO. In addition, ISO administration reduces blood pressure that triggers reflex tachycardia, there by increases myocardial oxygen demand⁹.

Oxidative stress increases cAMP levels by exhausting ATP and decreases sarcolemmal Ca^{+2} transport, resulting in intracellular calcium overload, which leads to ventricular dysfunction and contractile failure in rat heart¹⁰. An elevation of ST segment observed in Group II Isoproterenol. Lipid peroxidation in vivo has been identified as one of the basic deteriorative reactions in cellular mechanisms of myocardial ischemia¹¹.

Hypercholesterolemia and hyper triglyceridemia were seen in ISO-treated rats which might be due to increased mobilization of lipids from adipose tissue. Lipid metabolism plays an important role in myocardial necrosis produced by ischemia. The significant increase observed in the lipid profiles except phospholipids in the rat treated with ISO alone could be due to enhanced lipid biosynthesis by cardiac cyclic adenosine mono phosphate (cAMP)¹².

The diagnostic marker enzymes AST, ALT, LDH, CK-MB and TROPONIN serve as a sensitive index to assess the degree of myocardial necrosis. Rats treated with ISO exhibited increased activities of serum marker enzymes accompanied by concomitant reduction in the heart, indicative of the onset of myocardial necrosis. ISO-generated free radicals are known to initiate peroxidation of membrane-bound polyunsaturated fatty acids, leading to damage of the structural and functional integrity of the myocardium. Metabolically impaired myocardium releases its marker enzymes into the bloodstream. Hence, the activities of AST, ALT, LDH, CK-MB and TROPONIN were found to be decreased in the heart tissue of ISO-treated animals compared with normal animals. Increased levels of MDA in animals treated with ISO reflect excessive formation of free radicals by auto-oxidation of ISO and greater formation of lipid peroxides, resulting in severe damage to the myocardium. The ISO-elevated MDA levels were significantly decreased probably by preventing formation of lipid peroxides from fatty acids of the myocardium. Reduced glutathione is one of the most abundant non-enzymatic antioxidant bio-molecules present in tissues. Decreased GSH levels in ISO treated rats may be due to its increased utilization to augment the activities of GPx and GST. The GSH levels depleted by ISO were significantly restored by HAEPZ oral administration. Free-radical scavenging enzymes such as SOD, CAT, and GSH are known to be the first line cellular defense against oxidative damage, disposing of O_2 and H_2O_2 prior to the interaction to form the more harmful hydroxyl ($OH\cdot$) radical¹³. In the present study SOD activity decreased significantly in the ISO-treated group, which might be due to an excessive formation of superoxide anions. These excessive superoxide anions might inactivate SOD and decrease its

activity. Administration of HAEPZ to ISO-challenged rats effectively prevented the depletion of SOD, CAT, and GSH activities, which can be correlated to the scavenging of free radicals by HAEPZ.

CONCLUSION

Summarizing the effect of HAEPZ treatments on marker enzymes and endogenous antioxidants, showed significant cardio protective and antioxidant activities. HAEPZ 400mg/kg elicited better cardiac protection than HAEPZ 200mg/kg when compared with the standard propranolol 10mg/kg. The biochemical results are in agreement with the histological findings. It is possible that HAEPZ exerts its cardioprotective effect by stabilizing the myocardial membrane. Membrane stabilization might be due to an augmentation of basal endogenous antioxidants, leading to increased myocardial antioxidant reserve and strengthened myocardial defense mechanisms.

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