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In-vitro Antioxidant and Cardio-Protective Activity of Hydro-Alcoholic Bark extract of *Terminalia paniculata* Roxb on Isoproterenol Induced Myocardial Infarction in Rat's

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ABSTRACT

The present study was designed to evaluate the In vitro anti-oxidant cardio-protective activity of Hydro alcoholic bark extract of *Terminalia paniculata* Roxb on Isoproterenol induced Myocardial Infarction in Rat. Wistar albino rats used in this experiment were pretreated with vehicle, HAETP (200,400 and 600 mg/kg, p.o), Vit-C (20 mg/kg, p.o) for 14 days, on 14th and 15th day, Isoproterenol (85 mg/kg, s.c) was injected. After 24 h of last dose of ISO administration, ECG was recorded and serum biomarkers CK-MB, LDH, SGOT and SGPT were estimated. Further antioxidant levels were estimated from the tissue homogenates. Finally histopathological examination of heart was performed. HAETP have shown significant antioxidant activity in DPPH and reducing power methods. Moreover pretreatment with HAETP and Vit C improved ECG pattern and histopathology of heart.
Keywords: Antioxidant; Cardiprotective; Isoproterenol; *Terminlia paniculata* Roxb.

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INTRODUCTION

Myocardial infarction (MI), commonly known as heart attack is a disease that occurs when the blood supply to a part of the heart is interrupted, causing death of heart tissue. It means necrosis of a region of myocardium caused by an interruption in the supply of blood to the heart usually as a result of occlusion of a coronary artery also called as cardiac infarction [1]. Isoproterenol (ISO) induced MI serves as a well-standardized model because the pathophysiological changes following isoproterenol administration are comparable to those taking place in human MI [2]. ISO a synthetic sympathomimetic amine that is structurally related to epinephrine but acts almost exclusively on beta receptors that is a β1, β2 non selective agonist with very low affinity for alpha-adrenergic receptors. The isopropyl amine group in ISO makes it selective for β receptors. ISO hydrochloride is a racemic compound [3] of changes in cAMP concentration. Under certain circumstances, β2 receptors may couple to Gq proteins. These receptors have been demonstrated to activate additional kinases, such as MAP kinases, by forming multi-subunit complexes within cells. MI induced by ISO in rats has been shown to be accompanied by hyperglycemia, hyperlipidemia and increase in serum creatine phosphokinase, alanine aminotransferace, aspertate aminotransferase and lactate dehydrogenase activities [4,5].

Terminalia paniculata Roxb belonging to Combretaceae distributed in semi evergreen forests of Western Ghats**,** Karnataka, Andhra Pradesh, and Tamil Nadu. The plant is used as *"*Kashaya*"* "Tikta*" "*Lakhu" "Rooksha*" and"* Seeta*"* in Ayurvedic medicine. Bark is the useful part.

Terminalia paniculata Roxb is used as diabetic complication, cardiac effects, cough, bronchitis, wounds, and skin diseases. Cardio-Protective, Hypoglycemic, Hypolipidemic, Anti-Inflammatory were reported [6-8].

The present study was design to investigate the modulation of HAETP in ISO-induced electrocardiographic, biochemical and histopathological changes.

MATERIALS AND METHODS

Isoproterenol purchased from Bangalore Fine Chemicals, Auto analyzer from Awareness Technology INC, Langendorff and Digital physiograph from INCO, UV spectrophotometer from shimadzu.

Plant Material

The *Terminalia paniculata* Roxb bark of plant was collected from the Tirupathi, Chittoor District (Andhra Pradesh), India identified and authenticated by Dr. K. Madhava Chetty, Asst. Professor, Dept. of Botany. Sri Venkateswara University, Tirupathi.

Preparation of Plant Extract (HAETP)

The shade dried Bark was powdered; the coarse powder was subjected to extraction with Hydro-Alcohol (70% v/v) extraction in Soxhlet's extractor for 18 h and the extract thus obtained was evaporated to obtain the solid or semisolid mass and stored in air tight container.

Animals

Male Wistar rats weighing about 150-200 gms were used,The animals were kept in climatized environment (25 ± 2°C), with light/dark control each 12 hours. The animals were placed in cages.Food and water was ad libitum. These were acclimatized to laboratory condition for one week prior to start of dosing. All the studies conducted were approved by the Institutional Animal Ethical Committee (IAEC) of Gautham College of Pharmacy, Bangalore (REF-IAEC/003/5/2010) according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg No: 491/01/c/CPCSEA), Govt. of India. We selected male rats for our studies, since females are shown to be protected from cardiovascular complication.

In-vitro Antioxidant Activity

1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging capacity [9]

DPPH scavenging activity was measured by the slightly modified spectrophotometric method. A solution of DPPH in methanol (6 × 10-5 M) was prepared freshly. 3 ml aliquot of this solution was mixed with l mL of the HAETP at varying concentrations (50–250 µg/ml). The solutions in the test tubes were shaken well and incubated in the dark for 15 min at room temperature. The decrease in absorbance was measured at 517 nm. The percentage inhibition of the radicals due to the antioxidant property of the HAETP was calculated using the formula.
Calculation: Scavenging activity of DPPH free radical in percent was calculated according to the equation:

$$Percent\ inhibition = (A0-A1/A0) \times 100$$

Where, A0 is the absorbance of control reaction and A1 is the absorbance of test compounds.

Reducing Power Method [9]

Different concentrations of the HAETP (50-250 µg) extract in 1 ml of distilled water were mixed in to the mixture of 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50o C for 20 min. Following incubation, 2.5 ml of 10% trichloro acetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1 %) and the absorbance was measured at 700 nm. Vit C was used as

the reference material. All the tests were performed in triplicate and the results averaged. Increased absorbance of the reaction mixture indicated the increased reducing power [10].
The % reducing power was calculated by using the formula:

$$Percent\ inhibition = (A0-A1/A0) \times 100$$

Where, A0 is the absorbance of control reaction and A1 is the absorbance of test compounds.

Acute Toxicity Studies (LD_{50}) [11]

Female Albino rats of weighing 180-200 g were used for the study. They were nulliparous and non-pregnant. These were acclimatized to laboratory condition for one week prior to start of dosing.

Preparation of Dose

HAETP was dissolved in tween 80, were dissolved in saline/water, to prepare a dose of 2000mg/kg. The doses were selected according to the OECD guidelines 425.

Preparation Vit C solution

500 mg of Vit C was weighed and dissolved in 100 mL of distilled water to obtain 5 mg/mL.

Treatment protocol

Animals were subdivided into six groups containing six animals in each group.

Group-I: Normal control group.
Group II: ISO-induced myocardial damage.
Group-III: Standard drug on ISO induced myocardial damage.
Group-IV: HAETP 200 mg/kg (p.o) on ISO-induced myocardial damage.
Group-V: HAETP 400 mg/kg (p.o) on ISO-induced myocardial damage.
Group-VI: HAETP 600 mg/kg (p.o) on ISO-induced myocardial damage.

The I group, treated with normal Saline (Normal control group), the second group received ISO (85 mg/kg, s.c) from IIIrd group to VIth group recieves Vit C (20mg/kg, p.o), 200,400,600 mg/kg (p.o) of HAETP respectively for 14th days. From IInd group to VIth group receives ISO on 14th and 15th days .After 24 hr of second ISO injection the animals were anaesthtised with light anaesthetic ether [12] and ECGs were recorded. After recording ECG, blood was collected from retro-orbital plexus; serum was separated and used for estimation of marker enzymes like AST, ALT, CK-MB and LDH. At the end of study, all rats sacrificed by cervical decapitation and the hearts were dissected out, washed in ice cold saline and histopathological studies were carried out.

Statistical evaluation

The data were expressed as Mean ± S.E.M. Statistical comparisons were performed by one-way ANOVA followed by Tukey's post-test using Graph Pad Prism version 5.0, USA.

RESULTS

Acute Toxicity Studies (LD_{50})

In both phase I and II procedures, none of the animals did not show any toxicity upon the single administration of HAETP (2000 mg/kg,). Thus, a low dose 200 mg/kg, a medium dose 400 mg/kg, and a high dose 600 mg/kg, were selected for the present study.

In-vitro Antioxidant Activity

It is observed that the HAETP have been demonstrated there was a dose dependent significant increase in DPPH radicals and reducing power activity (Table and Figure No 1 and 2).

***In-vitro* Antioxidant Activity**

Table No 1: DPPH Assay

Groups	Absorbance (Mean ± SEM)	% Decrease
Control	1.204±0.021	-----
Vit C 50 µg	0.196±0.004	83.72
HAETP 50 µg	1.086±0.021	9.80
HAETP 100 µg	0.986±0.013	18.10
HAETP 150 µg	0.893±0.010	25.83
HAETP 200 µg	0.746±0.010	38.03
HAETP 250 µg	0.529±0.009	56.06

Values are the mean ± SEM., n=3, HAETP: Hydro alcoholic extract of *Terminalia paniculata*. Std: Vit C, ***Significant at $p<0.05$ compared to control.

Figure No 1: DPPH Radical Scavenger Activity of HAETP

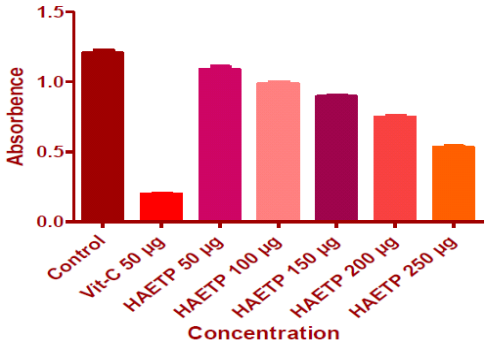

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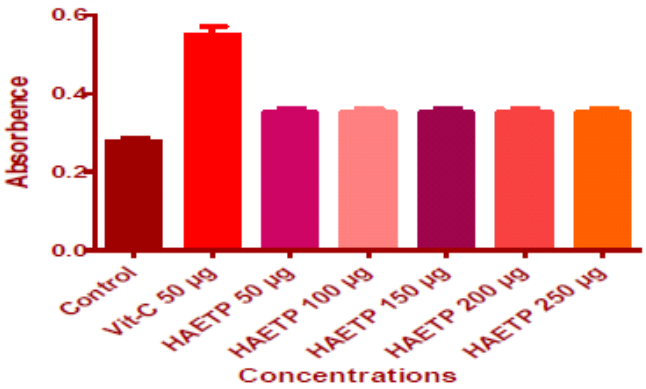
Table No 2: Reducing Power Assay

Groups	Absorbance Mean ± SEM	Increase
Control	0.277 ± 0.007	------
Vit C 50 µg	0.548 ± 0.020	97.83
HAETP 50 µg	0.350 ± 0.010	26.35
HAETP 100 µg	0.402 ± 0.004	45.12
HAETP 150 µg	0.448 ± 0.009	61.73
HAETP 200 µg	0.476 ± 0.004	71.84
HAETP 250 µg	0.491 ± 0.004	77.25

Values are the mean ± SEM., n=3, Significance *** P<0.05 compared to control. Std: Vit C, HAETP: Hydro alcoholic extract of *Terminalia paniculata.*

Figure No 2: Effect of HAETP on Reducing Power



Effect of HAETP on Different ECG Parameters

Fig No: 5 Show the electrocardiographic pattern of control and experimental animals. Normal control and Vit C (20mg/kg, p.o) treated rats showed a normal ECG pattern, whereas animals treated with ISO (85 mg/kg, s.c) alone showed significant elevation in ST segment, reduction in P wave, QRS complex, R-R interval and QT interval . In addition there was an increase in heart rate and cardiac cycles compared to normal control animals. Pretreatment of HAETP (200 and 400mg/kg, p.o) administered rats exhibited normal ECG pattern with a slight elevation in ST segment resulted in significant ($p<0.001$) increase in P wave, QRS complex and R-R interval, as compared to the ISO alone treated group. Whereas heart rate and cardiac cycle were maintained near to normal values. Whereas pretreatment with HAETP (600 mg/kg, p.o) is not shown the significant changes as compared to the ISO alone treated group. The data of the experimental animals such as P wave, QRS complex, QT interval, R-R interval, heart rate and cardiac cycle are shown in Table No: 5.

Effect of HAETP on Serum Enzyme Markers (Table No: 3, Fig No: 3):

There was significant ($p<0.001$ and $p<0.01$) increase in CK-MB, LDH, ALT and AST in ISO alone treated groups. When the animals are pretreated with Vit C (20 mg/kg), HAETP (200 and 400 mg/kg) followed by two doses ISO (85 mg/kg) has shown the significant ($p<0.001$ and $p<0.01$) decrease in the CK-MB, LDH, AST and ALT when compared to the ISO alone treated groups. When the animals are treated with HAETP (600 mg/kg) has not shown the significant decrease in the serum enzyme markers. Pretreated groups have shown the good cardio-protective activity when compared to the ISO alone treated group.

Effect of HAETP on Biochemical Parameter in HTH (Table No: 4, Fig No: 4).

The animal are pretreated with ISO (85 mg/kg) alone there was significant ($p<0.001$) reduction CAT, GSH, LPO and SOD when compared to the normal control group. When the animal are pretreated with Vit C (20 mg/kg) and HAETP (200 and 400 mg/kg) followed by two doses of ISO has shown the significant ($p<0.001$ and $p<0.01$) increase in the biochemical parameters when compared to the ISO alone treated group. There is no significant increase in the biochemical parameters in HAETP (600 mg/kg) when compared to ISO alone treated group.

Histopathological Changes (Fig No: 6)

Histopathological examination of myocardial tissue obtained from normal control animals exhibited clear integrity of myocardial membrane. Control rats showed normal cardiac fibers without any infarction. The heart sections obtained from ISO treated animals showed abundant areas of necrosis and aggregation of acute inflammatory cells and damaged vascular spaces. Animals pretreated with HAETP (200 and 400 mg/kg, p.o) showed a better protection against ISO toxicity by improvement in the cell integrity evidenced by decreased necrotic area and reduction in infiltration of inflammatory cells. The HAETP (600 mg/kg, p.o) pretreatment is not shown the better protection ISO induced toxicity. The animals treated with Vit C (20 mg/kg, p.o) showed marked reversal of ISO induced histolopathological changes.

Pharmacological Evaluation

The general appearance of all groups of animals was recorded throughout the study. In ISO treated group, the animal fur became scruffy and developed a pink tinge. Loss of body weight, edema and necrosis observed at the site of ISO injection. These observations were markedly reduced in HAETP (200, 400 and 600 mg/kg, p.o) and Vit-C (20 mg/kg, p.o) treated groups.

Estimation of Serum Enzyme Markers

Table No 3: Effect of HAETP on CK-MB, LDH, ALT and AST in ISO Induced Myocardial Infarction in rats

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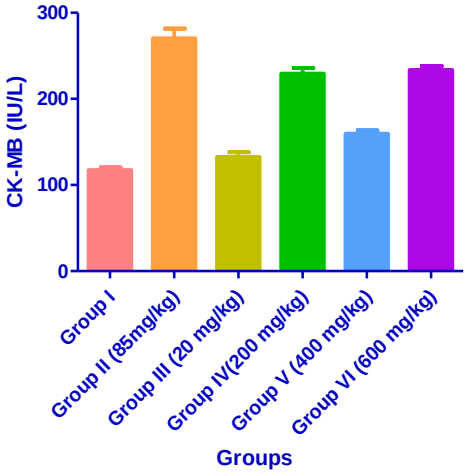
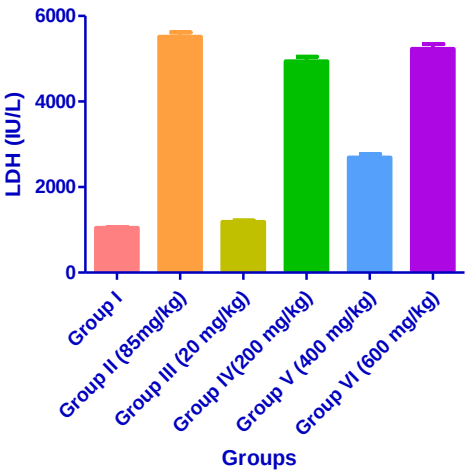
Treatment	CK-MB (IU/L)	LDH (IU/L)	ALT (IU/L)	AST (IU/L)
Group I (Control)	117.3±3.084	1050±19.31	95.96±3.250	305±7.445
Group II (ISO 85 mg/kg, s.c)	270.4±11.23$^{###}$	5518±97.64	135.6±4.929$^{###}$	435.1±9.236
Group III (Vit C 20 mg/kg, p.o + ISO)	132.6±5.540***	1189±26.37***	86.25±1.863***	265.7±11.07***
Group IV (HAETP 200mg/kg, p.o + ISO)	229.3±6.614**	4942±107.1**	111.6±4.975**	377.1±9.022**
Group V (HAETP 400mg/kg, p.o+ ISO)	159.5±3.840***	2691±80.51***	67.12±4.015***	224.8±12.21***
Group VI (HAETP 600mg/kg, p.o+ ISO)	233.6±4.437**	5232±110.6	115.6±2.354**	389.1±13.91

Each bar represent the Mean ± SEM (n = 6). **p<0.01; ***p<0.001 compared with ISO alone rats. $^{###}$p<0.001 compared with normal control. One-way ANOVA followed by Tukeys post-test.

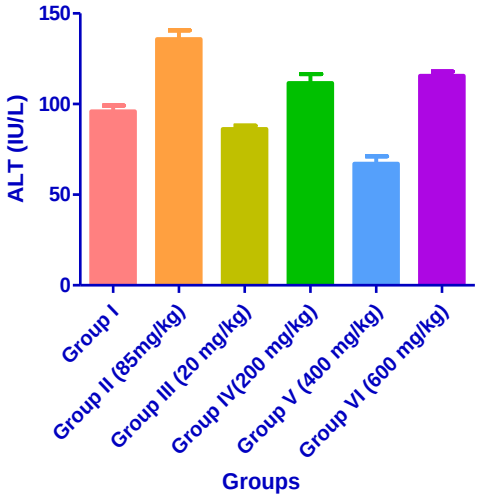
Figure No 3: Effect of HAETP on CK-MB, LDH, ALT and AST in ISO Induced Myocardial Infarction in rats:



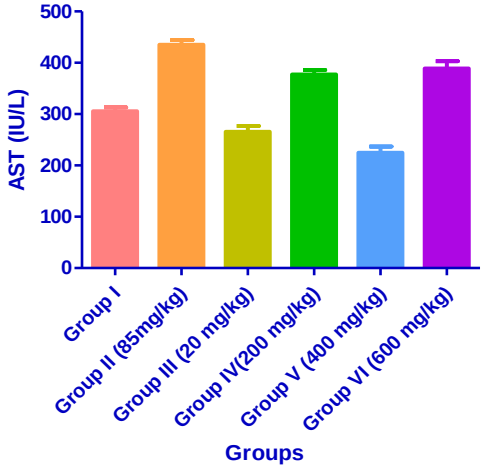
CK-MB



LDH



ALT



AST

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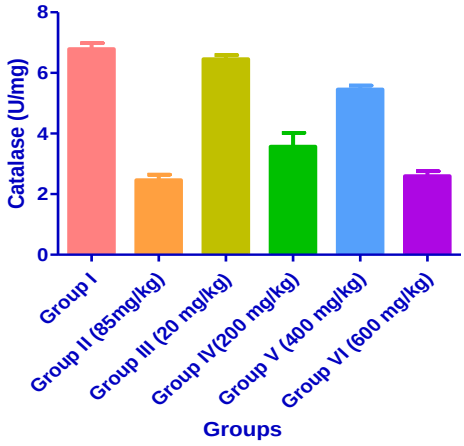
Table No 4: Effect of HAETP on CAT, GSH, LPO, and SOD in Heart Tissue Homogenate of ISO Induced Myocardial Infarction

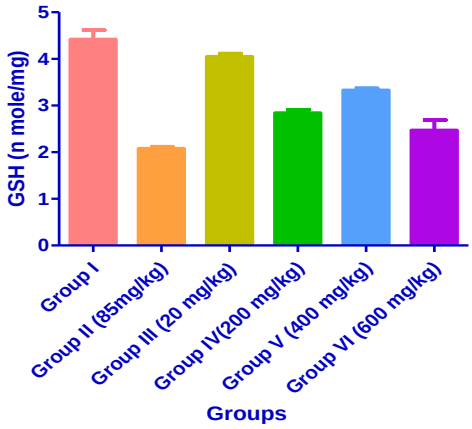
Treatment	CAT (U/mg)	GSH (nmole/mg)	LPO(µmole MDA/mg protein)	SOD(µmole MDA/mg protein)
Group I (Control)	6.793±0.192	4.420±0.197	2.938±0.037	10.82±0.168
Group II (ISO 85 mg/kg, s.c)	2.465±0.177$^{###}$	2.078±0.041$^{###}$	1.155±0.074$^{###}$	5.213±0.193$^{###}$
Group III (Vit C 20 mg/kg, p.o + ISO)	6.458±0.134***	4.048±0.065***	2.908±0.050***	10.18±0.260***
Group IV (HAETP 200mg/kg, p.o + ISO)	3.573±0.450*	2.838±0.071**	1.903±0.024***	7.433±0.228***
Group V (HAETP 400mg/kg, p.o+ ISO)	5.455±0.127***	3.325±0.041***	2.233±0.034***	9.618±0.082***
Group VI (HAETP 600mg/kg, p.o+ ISO)	2.595±0.160	2.470±0.219	1.145±0.037	5.900±0.329

Each bar represent the Mean± SEM (n=6). *p<0.05; **p<0.01; ***p<0.001 compared with ISO alone rats. $^{###}$p<0.001 compared with normal control. One-way ANOVA followed by Tukeys post-test.

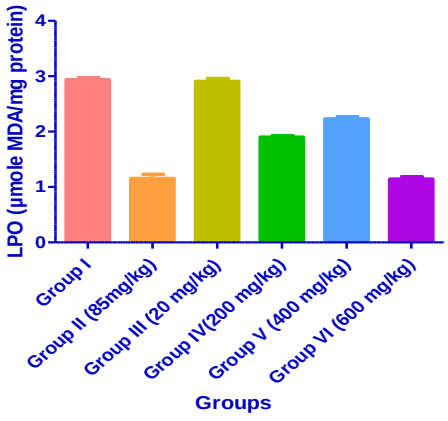
Figure No 4: Effect of HAETP on CAT, GSH, LPO, and SOD in Heart Tissue Homogenate of ISO Induced Myocardial Infarction:



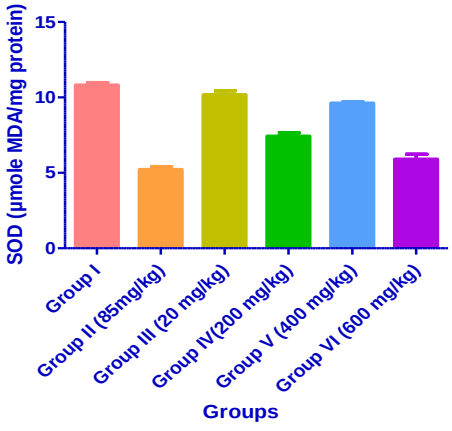
CAT



GSH



LPO



SOD

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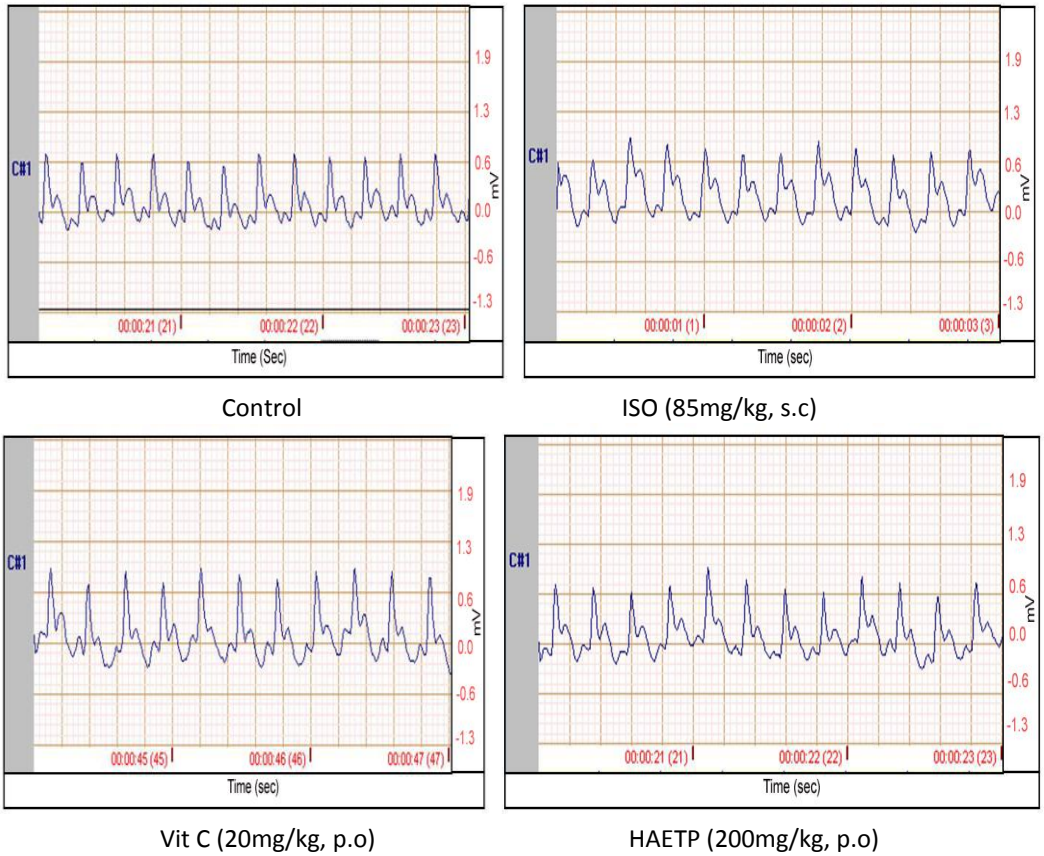
Table No 5: Effect of HAETP on Different ECG Parameters in ISO - Induced Cardiotoxicity in Rats

Treatment	P Wave	QRS Complex	QT Interval	R-R Interval	Heart Rate	Cardiac Cycle
Group I (Control)	0.05623±0.0001	0.07513±0.0002	0.1088±0.0003	0.2756±0.002	226.7±4.84	0.1900±0.001
Group II (ISO 85 mg/kg, s.c)	0.04358±0.0011###	0.06379±0.0006###	0.1373±0.0007###	0.2435±0.001###	267.2±2.40###	0.2062±0.001###
Group III (Vit C 20 mg/kg, p.o + ISO)	0.05601±0.0003***	0.06858±0.0009***	0.1186±0.0004***	0.2670±0.002***	219.0±2.55***	0.1821±0.001***
Group IV (HAETP 200mg/kg, p.o + ISO)	0.05081±0.0001***	0.06872±0.0001***	0.1300±0.0003***	0.2618±0.0006***	245.0±2.75***	0.1903±0.001***
Group V (HAETP 400mg/kg, p.o+ ISO)	0.05528±0.0002***	0.07217±0.0002***	0.1277±0.0004**	0.2717±0.0005***	230.3±3.26**	0.1867±0.001**
Group VI (HAETP 600mg/kg, p.o+ ISO)	0.04722±0.0001*	0.06631±0.0001*	0.1329±0.0001**	0.2577±0.0004**	265.7±2.82	0.1993±0.009

The ECG parameters are expressed in seconds (sec) and the heart rate as beats per min (BPM). Each Values are expressed as mean ± SEM (n= 6) animals in each group, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ as compared to ISO alone group.$^{###}p<0.001$ as compared to control group. One-way ANOVA followed by Tukeys post-test.

Figure No: 5 Effect of HAETP on Different ECG Parameters in ISO - Induced Cardiotoxicity in Rats



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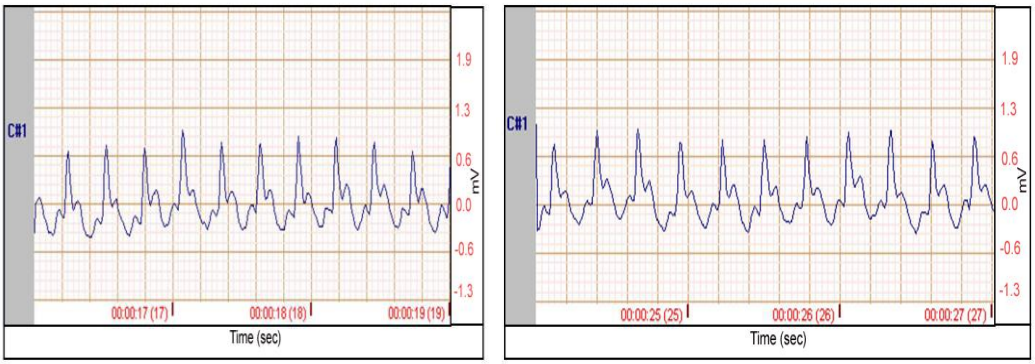
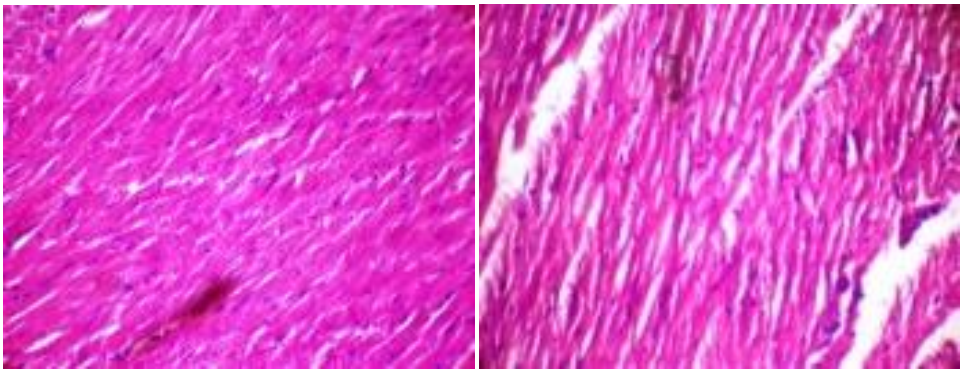
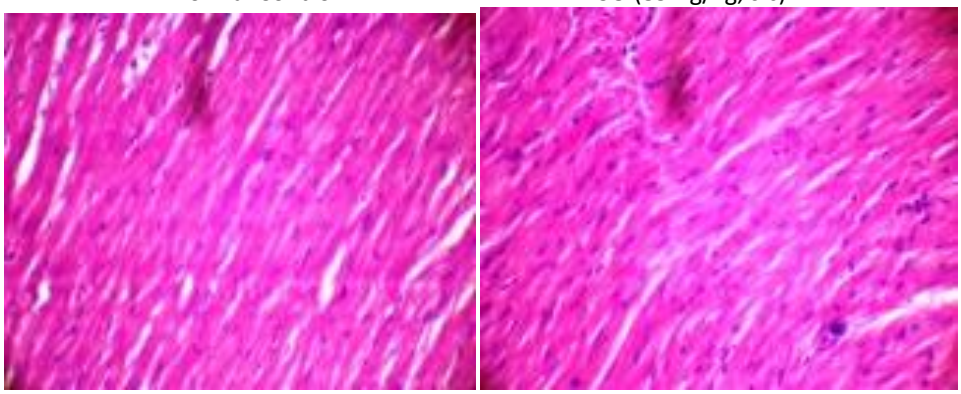


HAETP (400mg/kg, p.o) HAETP (600mg/kg, p.o)

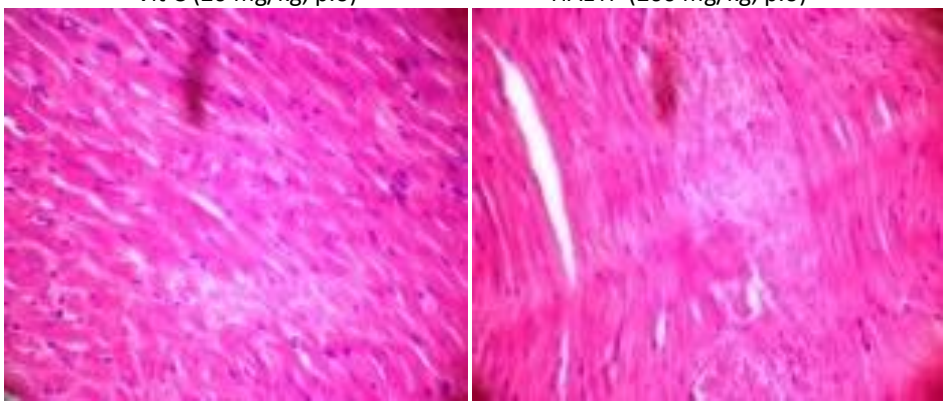
Figure No: 6 Histopathology



Normal Control ISO (85mg/kg, s.c)



Vit C (20 mg/kg, p.o) HAETP (200 mg/kg, p.o)



HAETP (400 mg/kg, p.o) HAETP (600 mg/kg, p.o)

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DISCUSSION

Myocardium contains an abundant amount of diagnostic marker enzymes for MI and once metabolically damaged, it releases its intracellular contents into the extracellular fluid[13]. Hence the serum levels of these marker enzymes reflect the alterations in membrane integrity and/or permeability. Cytosolic enzymes CK-MB, LDH, SGOT and SGPT which serve as the diagnostic markers, leak out from the damaged tissue to blood stream when cell membrane becomes permeable or rupture [14,15]. Assay of the activity of CK-MB in serum is an important diagnostic, because of the marked abundance of this enzyme in myocardial tissue and virtual absence from most of other tissues and its consequent sensitivity. CK-MB isoenzyme activity is useful not only as an index of early diagnosis of MI, but also any type of myocardial injury. ISO administration to rats showed elevated levels of serum CK-MB, which was found to be significantly low in the HAETP pretreated rats. Lactate dehydrogenase (LDH) is an intracellular enzyme, which catalyses the readily reversible reaction involving the oxidation of lactate to pyruvate with nicotinamide adenine dinucleotide (NAD) serving as coenzyme. This clinically significant enzyme rises within 24-48 hours after a heart attack and peaks in two to three days in the serum. Consistent with the above clinical observations, in the present study we observed a significant rise in the LDH levels of rats treated with ISO after 48 or 72 h of the respective treatment. HAETP pretreatment significantly reduced the elevated levels of LDH indicating the reduction in the severity of MI.

It has been reported that infarction of as little as 10% of the total myocardium produces a significant rise in the serum levels of SGOT and is linear with the amount of infarction. SGPT is an important cytosolic enzyme, which also rises to a significant concentration in the serum after acute MI. In accordance to the above reports, ISO treatment elevated these enzyme levels in serum to a significant extent. Pretreatment with HAETP (200, 400 and 600 mg/kg, p.o) or Vit-C (20 mg/kg, p.o) significantly lowered the ISO -induced elevation of serum levels of these diagnostic marker enzymes. It demonstrates that HAETP could maintain membrane integrity thereby restricting the leakage of these enzymes as that of Vit-C.

Administration of supra maximal doses of ISO had been reported to induce severe oxidative stress and impair the myocardial function. Overproduction of ROS can cause severe impairment of cellular functions and necrotic lesions in the myocardium of rats. On the other hand CAT, TT, GSH constitute a mutually supportive defense team against oxidative injury.

The ECG is considered the single most important initial clinical test for diagnosis of myocardial ischemia and infarction. Its correct interpretation is usually the basis for immediate therapeutic interventions and/or subsequent diagnosis tests. ISO administration in rats showed pathological ECG wave. The appearance of pathological Q waves is the most characteristic ECG finding of transmural myocardial infarction of the left ventricle. The Q wave appears when the infarcted muscle is electrically inert and the loss of forces normally generated by the infarcted area leaves unbalanced forces of variable magnitude in the opposite direction from the remote region. ISO administration in rats also showed a decrease in P wave intensity, QRS complex, R–R interval and increase in the heart rate. These changes could be due to the consecutive loss of

cell membrane in injured myocardium. It has been demonstrated that an increase in heart rate is responsible for increased oxygen consumption leading to accelerated myocardial necrosis. ISO administration also resulted in increased 'ST-segment' and decreased 'R-amplitude'. This is in consistent with the observations of the earlier reports. ST segment elevation reflects the potential difference in the boundary between ischemic and non-ischemic zones and consequent loss of cell membrane function, whereas decreased R-amplitude might be due to the onset of myocardial edema following ISO administration [16]. HAETP extract pretreatment in ISO treated rats prevented the pathological alterations in the ECG suggestive of its cell membrane protective effect as that of Vit-C (20 mg/kg, p.o).

The results of the serum, tissue biomarkers and ECG are well correlated with the histopathological changes in the heart of disease and treatment groups. The myocardial tissue in control rats illustrated clear integrity of the myocardial cell membrane and absence of inflammatory cell infiltration. ISO injected rats showed coagulative necrosis, separation of cardiac muscle fibers and infiltration of inflammatory cells. The reduced inflammatory cell infiltration and normal cardiac muscle fiber architecture in HAETP treated rats further confirmed the cardioprotective effect of the extract.

CONCLUSION

The present study provides experimental evidence that HAETP have shown significant *In-vitro, In-vivo* anti-oxidant activities. HAETP has shown the good cardioprotective effect against ISO induced Myocardial injury. These findings might be helpful to understand the beneficial effects of HAETP extract against myocardial injury although further study is needed to confirm its mechanism.

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