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## **Cardioprotective activity of *Nelumbo nucifera* rhizomes in isoproterenol induced myocardial necrosis in male wistar rats**

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### **ABSTRACT**

The study was designed to scientifically evaluate the cardio protective effect of ethanolic extract of *Nelumbo nucifera* rhizomes, a traditional medicinal herb, on isoproterenol-induced myocardial necrosis in rat model. The albino male wistar rats were divided into five different groups with six animals in each group. Group I serves as a control group, group II were given isoproterenol (85 mg/kg s.c) for 2 days while Group III was treated with metoprolol (10 mg/kg) for 30 days and isoproterenol on last 2 days. This group serves as standard group and its effect was compared with that of test drug (*Nelumbo nucifera* extract). Group IV and V were given *Nelumbo nucifera* rhizomes extract (300 mg/kg and 150 mg/kg respectively) for 30 days and isoproterenol on 29<sup>th</sup> and 30<sup>th</sup> day. The level of enzymes Aspartate transferase (AST), Alanine transferase (ALT), Lactate dehydrogenase (LDH), Cretinine kinase (CK) were estimated in serum and the extent of necrosis was studied by grading. Isoproterenol significantly increased the activities of AST, ALT, CK, LDH in serum. Pretreatment with the ethanolic extract of *Nelumbo nucifera* had a significant decrease in the level of marker enzymes. The effect was compared with the effect on serum marker enzymes in the group treated with Metaprolol. The metaprolol treated group has shown reduced level of marker enzymes, nearer to the control group. It was found to be more protective at dose 300mg/kg as compared to 150 mg/kg dose.

**Keywords;** *Nelumbo nucifera*, Isoproterenol, cardioprotective effect, myocardial necrosis

### **INTRODUCTION**

Cardiovascular diseases are amongst the leading cause of death. Among these, the ischemic heart diseases, acute myocardial necrosis in particular, is one of the most alarming values. AMI give rise to a lot of unfavorable biochemical outcomes. The end results of which is the ultimate morbidity of patient or even death. [1]Cardiovascular disease (CVD) remains the principal cause of death in both developed and developing countries, accounting for roughly 20% of all worldwide deaths per year. In addition, hypertension, arrhythmia, coronary artery disease, myocardial infarction (MI) and cardiac failure represent the leading killer of males over the age of 45 and females over the age of 65 in the United States and accounts for 750,000 deaths annually. [2]

### **MYOCARDIAL INFARCTION**

Myocardial infarction or acute myocardial infarction (AMI), commonly known as a heart attack is the interruption of blood supply to a part of the heart, causing heart cells to die. This is most commonly due to occlusion of

a coronary artery following the rupture of a vulnerable atherosclerotic plaque, which is an unstable collection of lipids and white blood cells (especially macrophages) in the wall of an artery. The resulting ischemia (restriction in blood supply) and oxygen shortage, if left untreated for a sufficient period of time, can cause damage or death (infarction) of heart muscle tissue.

### THE HEART

The heart is a muscular organ that provides a continuous blood circulation through the cardiac cycle and is one of the most vital organs in the human body. The heart is divided into four main chambers: the two upper chambers are called the left and right atria and two lower chambers are called the right and left ventricles. There is a thick wall of muscle separating the right side and the left side of the heart called the septum. Normally with each beat the right ventricle pumps the same amount of blood into the lungs that the left ventricle pumps out into the body. Physicians commonly refer to the right atrium and right ventricle together as the right heart and to the left atrium and ventricle as the left heart.[3]

#### The Heart Wall

The heart is composed of cardiac muscle which enables the heart to contract and allow the synchronization of the heart beat. It is enclosed in a double-walled protective sac called the pericardium. The superficial part of this sac is called the fibrous pericardium. The fibrous pericardial sac is itself lined with the outer layer of the serous pericardium (known as the parietal pericardium). This composite (fibrous-parietal-pericardial) sac protects the heart, anchors its surrounding structures, and prevents overfilling of the heart with blood. The inner layer also provides a smooth lubricated sliding surface within which the heart organ can move in response to its own contractions and to movement of adjacent structures such as the diaphragm and lungs. [4]

The heart wall is divided into three layers:

**Epicardium** - outer protective layer of the heart.

**Myocardium** - muscular middle layer wall of the heart.

**Endocardium**-inner layer of the heart that is continuous with the inner lining of blood vessels. [3]

### EXPERIMENTAL SECTION

#### Material

##### Collection and authentication of plant material:

The rhizome of *Nelumbo nucifera* was purchased from the local market and authenticated from a botanist of National Botanical Research Institute Lucknow, Ref. no: NBRI/CIF/215/2011.(Appendix III)

##### Preparation of *Nelumbo nucifera* extract:

The shade dried and coarse powdered material of *Nelumbo nucifera* rhizome was extracted exhaustively with ethanol (80%)v/v and water in a Soxhlet apparatus. 5 gram of powdered drug was soaked in 50 ml of 80% ethanol in Soxhlet apparatus. The extract was concentrated at 40°C to obtain dark brownish syrupy residue. The yield obtained from the above process was found to be 8% w/w. The extracts were preserved in a refrigerator at 4°C.

##### Standard drug Metoprolol

Metoprolol is a relatively  $\beta_1$ -selective beta-blocker used extensively to treat hypertension and angina and as a prophylaxis after myocardial infarction. It is of similar potency to propranolol in blocking the cardiac  $\beta_1$  receptor. It is 1/50 times as potent as propranolol in inhibiting the vasodilator response to isoprenaline. However, it is much less active in blocking the  $\beta_2$  receptors in bronchii and blood vessels, hence causes less bronchial constriction. It is extensively metabolized in liver. [5]

**Drugs and Chemicals:****Table1; List of drugs and chemicals**

Normal saline 0.9%	Albert David Ltd, Ghaziabad
Isoprenaline Hydrochloride	Sigma Chemicals, USA (Gifted by Prof. S. K. Maulik. Dept. of pharmacology, AIIMS, New Delhi.)
Metaprolol Tartrate (METOLOR- 50)	Cipla Ltd, Sikkim
Ethanol	Manufactured by Fisher Scientific
Formaldehyde	Manufactured by Fisher Scientific
Serum ALT diagnostic kit	Span Diagnostics Ltd, Surat.
Serum AST diagnostic kit	Span Diagnostics Ltd, Surat.
Serum Creatinine kinase diagnostic kit	Span Diagnostics Ltd, Surat.
Serum LDH diagnostic kit	Accurex Biomedical Pvt.Ltd. Mumbai.
Diethyl Ether	Manufactured by Fisher Scientific

**INSTRUMENTS**

**Oral feeding needle:** It was used for oral administration of drugs.

**Animal cages:** Obtained from store of Faculty of Pharmacy, Integral University, Lucknow. It was taken to provide separate and clean housing for the test animals.

**Centrifuge machine:** from the pharmaceutics lab of Faculty of Pharmacy, Integral University, Lucknow. It was used to separate the serum from the blood collected from the heart of test animals.

**Ultraviolet Spectrophotometer:** SHIMADZU PHARMASPEC 1700 model, from the Instrumentation lab of Faculty of Pharmacy, Integral University, Lucknow. It was used to take the absorbance from the serum for the estimations of enzymes.

**Electronic Balance:** SHIMADZU AUX 220 model, from the Instrumentation lab of Faculty of Pharmacy, Integral University, Lucknow.

**Animals:**

Male albino wistar rats (150-200g) were used for the study. They were housed in sanitized polypropylene cages containing paddy husk as bedding under standard laboratory conditions at room temperature ( $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) with 12 h light / 12 h dark cycle. Six rats were taken in each cage. The animals were randomized into experimental and control groups. They were provided standard pellets as basal diet and water ad libitum. Ethical clearance was obtained from Institutional Animal Ethical Committee (IAEC) no. IU/pharm/m.pharm/cpcsea/10/33. (Appendix III) Faculty of pharmacy, Integral University, Dasauli, P.O. Bas-ha Kursi Road, Lucknow – 226026 (U.P).

**METHOD****ISOPRENALINE INDUCED MODEL FOR MYOCARDIAL NECROSIS**

The model of isoprenaline-induced myocardial necrosis is considered as one of the most widely used experimental model to study the beneficial effects of many drugs and cardiac function. The pathophysiological changes following ISO administration are comparable to those taking place in human myocardial ischemia/infarction. It is also well known to generate free radicals and to stimulate lipid peroxidation, which may be a causative factor for irreversible damage to the myocardial membrane. Increases in the formation of reactive oxygen species (ROS) during ischemia/reperfusion and the adverse effects of oxyradicals on myocardium have now been well established by both direct and indirect measurements. Thus, increased production of reactive oxygen species (ROS) may be a unifying mechanism in ischemic injury progression, and anti-oxidants may be the therapeutic value in this setting. Rats receiving subcutaneous injections of Isoproterenol on two consecutive days will develop myocardial necrosis. This depends on the size of the dose and the weight of the animal. The observation to develop a pharmacologic technique for producing myocardial necrosis of standard severity in animals. The myocardial necrosis was thought to result from a relative ischemia of the heart muscle. It was postulated that this was due to a combination of increased cardiac oxygen requirement as a result of the cardiac stimulant effect of the drug and a reduction of coronary blood flow secondary to a decrease in the systemic blood pressure.[6]

**Isoproterenol**

Isoproterenol (ISO) is a  $\beta$ -adrenergic agonist that causes severe stress in myocardium resulting in the infarct like necrosis of heart muscle [7]. Some of the mechanisms proposed to explain ISO induced damage in cardiac myocytes include hypoxia due to myocardial hyperactivity, coronary hypotension, calcium overload, depletion of energy reserves and excessive production of free radicals due to oxidative metabolism of catecholamines. ISO induced MN in rats is a widely used experimental model for evaluation of cardioprotective effect of various herbal drugs, because pathophysiological changes following ISO administration in rats are comparable to those taking place during MI in humans [8].

**Preparation of solution for oral dose of rats**

The ethanolic extract of *Nelumbo nucifera* was accurately weighed according to oral dose of 300mg / kg, and 150 mg / kg. The solution was prepared daily by dissolving it into 0.9% normal saline.

**Preparation of Isoprenaline Hydrochloride solution**

Isoprenaline Hydrochloride (85mg / kg) was dissolved in 0.9% normal saline and freshly prepare solution was used for inducing necrosis in rat heart.

**Table 2; Treatment protocol**

Groups	Number of animals	Treatment to be given
I	6	Normal control rats treated with normal saline
II	6	Rats treated with Isoprenaline (85mg/kg/day, subcutaneous)
III	6	Rats pretreated with standard drug metaprolol (metolor -50) (10 mg/kg day, oral ) And ISO (85 mg/kg/day for 2 days, subcutaneous.)
IV	6	Rats pretreated with <i>Nelumbo nucifera</i> extract (150 mg/ kg day, oral.) and ISO (85 mg/ kg□ day; for 2 days, subcutaneous.)
V	6	Rats pretreated with <i>Nelumbo nucifera</i> (300 mg/kg day oral) and ISO (85 mg kg day; for 2 days, subcutaneous.)

At the end of the 29 days of pretreatment with test drug rats were weighed and Isoprenaline Hydrochloride injection was administered at the dose of ISO (85 mg/ kg/day, s.c.) twice at an interval of 24 hours (i.e., on 29<sup>th</sup> and 30<sup>th</sup> day of extract treatment). The rats were sacrificed on day 31<sup>st</sup> blood was collected by cardiac puncture by using a 2ml syringe under light ether anesthesia and allowed to clot for 30minutes at room temperature. The serum was separated by centrifugation at 3000 rpm at 30°C for 15 minutes and used for the estimation of marker enzymes, including aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine phosphokinase (CPK). The hearts were dissected out immediately, chilled, and perfuse with Ice-cold saline. The animals were sacrificed 24 hr after the second dose of isoproterenol. The heart was dissected and immediately washed in ice cold saline. The hearts were stored in 10% formaline solution for histopathological analysis.

**Biological Screening:**

The serum was separated by centrifugation using the centrifuge machine and the different serum marker enzymes were assayed. The following enzymes were estimated

- (i) Aspartate Transaminase (AST) (Appendix II, method 1)
- (ii) Alanine Transaminase (ALT) (Appendix II, method 2)
- (iii) Lactate Dehydrogenase (LDH) (Appendix II, method 3)
- (iv) Creatinine (Appendix III, method 4)

**GROSS EXAMINATION OF RAT HEART****Table 3;**

<b>Grade-0</b>	No lesion.
<b>Grade-1</b>	Inflammation and redness, capillary dilatation.
<b>Grade-2</b>	Edema, capillary dilatation, ventricle portion yellowish.
<b>Grade-3</b>	Scar formation, yellowish colour of atrium and ventricle part of heart.
<b>Grade-4</b>	Diffuse heart, Scar formation, and yellowish color of atrium and ventricle part of heart.

### STATISTICAL ANALYSIS

The concentration of enzymes calculated in the serum was than subjected to statistical analysis. The statistical analysis was done by applying the student t-test. This involves, calculation of Standard Deviation & Standard Error and Determination of t- value.

$$S.D.(\sigma) = \sqrt{\frac{\sum (X - \bar{X})^2}{n - 1}}$$

Standard Deviation

Where, x = Independent values,  $\bar{x}$  = Mean value

= Variation from mean value, n = Total no. of values

$$S.E. = \frac{S.D.}{\sqrt{n}}$$

Standard Error

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(S.E._1)^2 + (S.E._2)^2}}$$

t-value

The t-value obtained was then compared with the values in the t-table to check the significance.

### RESULTS AND DISCUSSION

#### APPENDIX I

**Table 4;** Concentration of AST in serum of all groups

SN.	CONTROL	ISO.	STD.	HIGH	LOW
1	63.12	80.88	64.89	69.20	72.53
2	65.70	74.43	65.33	67.54	69.12
3	65.81	75.31	72.85	68.92	73.89
4	65.44	87.25	72.42	73.26	75.88
5	75.88	91.93	70.80	70.04	76.23
6	70.40	85.77	71.20	71.69	70.58
MEAN	67.73	82.96	69.59	70.11	73.04

**Table 5;** Concentration of ALT in serum of all groups

SN.	CONTROL	ISO.	STD.	HIGH	LOW
1	48.90	55.11	50.22	55.19	55.11
2	48.08	57.41	52.33	46.08	57.41
3	53.83	65.59	49.05	47.91	65.59
4	44.73	76.24	46.66	56.69	76.24
5	50.57	66.52	53.73	53.01	66.52
6	47.47	63.11	49.59	48.95	63.11
MEAN	48.93	64.00	50.26	51.31	64.00

**Table 6;** Concentration of LDH in serum of all groups

SN.	CONTROL	ISO.	STD.	HIGH	LOW
1	1160.10	1355.00	1209.87	1160	1230.39
2	1089.28	1245.54	1094.71	1213.32	1298.01
3	1131.46	1477.00	1070.05	1204.45	1307.89
4	1182.19	1475.00	1293.93	1326	1256.78
5	1190.86	1344.87	1282.30	1216.88	1215.33
6	1257.42	1295.96	1149.58	1257.01	1248.17
AVERAGE	1168.55	1365.56	1183.00	1229.61	1259

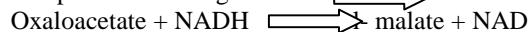
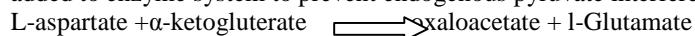
**Table 7;** Concentration of CK in serum of all groups

SN.	CONTROL	ISO.	STD.	HIGH	LOW
1	1.088	1.393	1.096	1.090	1.121
2	0.098	1.279	0.990	1.115	1.206
3	1.067	1.319	1.112	1.169	1.107
4	1.083	1.116	1.200	1.198	1.101
5	1.027	1.170	1.132	1.159	1.199
6	0.936	1.202	1.006	1.097	1.132
AVERAGE	1.031	1.247	1.089	1.138	1.144

### 1. APPENDIX II AST (GOT) Modified UV (IFCC), Kinetic Assay

#### Assay Principle

Aspartate aminotransferase (AST) catalyses the transamination of L-aspartate and  $\alpha$ -ketoglutarate to form oxaloacetate and L-glutamate. In subsequent reaction, Malate Dehydrogenase (MDH) reduces oxaloacetate to malate with simultaneous oxidation of nicotinamide adenine dinucleotide (reduced) (NADH) to nicotinamide adenine dinucleotide (NAD). The rate of oxidation of NADH is measured kinetically by monitoring the decrease in absorbance at 340 nm and is directly proportional to AST activity in the sample. Lactate dehydrogenase (LD) is added to enzyme system to prevent endogenous pyruvate interference, which is normally present in serum.

**Table 8;** Reagents Composition

S.no	Reagent	REF	Pack Size	Composition	Concentration
1	Buffer	77LS200-50	4X10 ml	Tris buffer (pH 7.8) MDH	80mmol/l
		77LS200-60	8X10 ml	LD	240mmol/L>600U/L
		77LS200-66	4X50 ml		
		77LS200-50	1X10 ml		
		77LS200-60	2X10 ml	$\alpha$ -ketoglutarate	12mmol/L
2	Substrate	77LS200-66	1X50 ml	NADH	0.18mmol/L

#### Working Reagent Preparation

Add reagent 2 to reagent 1 in 1:4 ratio i.e. 1 ml of reagent 2 + 4 ml of reagent 1

For kit code no. 77ls200-60/ 77ls200-50: 1 vial reagent + 2.5 ml of reagent 2

For kit code 77ls200-66: 1 vial reagent 1+ 12.5 ml reagent 2

#### Reagent Storage And Stability (Prior To Reconstitution)

Unopened reagents are stable 2-8°C until the expiry date mentioned on the container label.

After Reconstitution- working ALT reagent is stable at 2-8°C for 4 weeks. Do not freeze the working Alt reagent

#### ASSAY PARAMETERS

**Table 9;**

Mode	Kinetic
Reaction direction	Decreasing
Wavelength	340nm
Flow-cell temperature	37°C
Optical path length	1 cm
Banking	Purified water
Sample volume	100MI
Reagent volume	1000MI
Delay	60 seconds
Interval	30 seconds
Number of readings(s)	4
Permissible reagent blank absorbance	>1.0 AU
Kinetic factor	1768
Maximum ΔA/minute	0.26
Linearity	450 IU/l
Units	IU/L

**PROCEDURE****Table 10;**

Pipette Into Tube Marked	Test
Serum/plasma	100ML
Working ALT reagent	1000µL

Mix well and aspirate immediately for measurement.

Programme the analyser as per assay parameters.

Blank the analyser with Purified Water.

Read absorbance after 60 seconds. Repeat reading after every 30 seconds i.e. upto 120 seconds at 340 nm wavelength.

Determine the mean absorbance change per minute ( $\Delta A/\text{minute}$ )

**Calculations**

AST activity (IU/L) =  $\Delta A/\text{minute} \times \text{kinetic Factor}$

$\Delta A/\text{minute}$  = Change in absorbance per minute

Kinetic Factor (K) = 1768

Kinetic factor is calculated by following formula

$$K = \frac{1}{M} \times \frac{TV}{SV} \times \frac{1}{P} \times 10^6$$

M= Molar extinction coefficient of NADH and is equal to  $6.33 \times 10^3$  lit/mol/cm at 340 nm

TV= Sample Volume + Working Reagent volume

SV= Sample volume

P= Optical path length

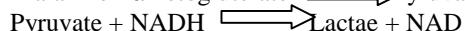
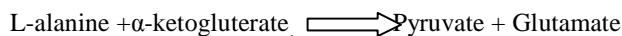
$10^6$ = constant

**Conversion Factor**

AST activity in nkat/L= AST activity in IU/L  $\times 16.67$

**2. ALT (GPT) Modified UV (IFCC), Kinetic Assay****Assay Principle-**

Alanine minotransferase(ALT) catalyses the transmission of L-alanine and  $\alpha$ -ketoglutarate to form pyruvate and L-glutamate. In subsequent reaction, Lactate Dehydrogenase (LD) reduces pyruvate to lactate with simultaneous oxidation of nicotinamide adenine dinucleotide (reduced) (NADH) to nicotinamide adenine dinucleotide (NAD). The rate of oxidation of NADH is measured kinetically by monitoring the decrease in absorbance at 340 nm . LD rapidly and completely reduces endogenous sample pyruvate during the initial incubation period , So it does not interfere with the assay.

**Table 11; Reagents Composition**

S.N	Reagent	REF	Pack Size	Composition	Concentration
1	Buffer	76LS200-50	4X10 ml	Tris buffer (pH 7.5) LD	100mmol/L
		76LS200-60	8X10 ml		500mmol/L
		76LS200-66	4X50 ml		>1200U/L
		76LS200-50	1X10 ml		
2	Substrate	76LS200-60	2X10 ml	$\alpha$ -ketoglutarate NADH	15mmol/L
		76LS200-66	1X50 ml		0.18mmol/L

**Working Reagent Preparation**

Add reagent 2 to reagent 1 in 1:4 ratio i.e. 1 ml of reagent 2 + 4 ml of reagent 1

For kit code no. 76ls200-60/ 76ls200-50: 1 vial reagent + 2.5 ml of reagent 2

For kit code 76ls200-66: 1 vial reagent 1+ 12.5 ml reagent 2  
 Reagent Storage And Stability ( Prior To Reconstitution)

Unopened reagents are stable 2-8°C until the expiry date mentioned on the container label.

After Reconstitution- working ALT reagent is stable at 2-8°C for 4 weeks. Do not freeze the working Alt reagent.

### ASSAY PARAMETERS

**Table 12**

Mode	Kinetic
Reaction direction	Decreasing
Wavelength	340nm
Flow-cell temperature	37°C
Optical path length	1 cm
Banking	Purified water
Sample volume	100Ml
Reagent volume	1000Ml
Delay	60 seconds
Interval	30 seconds
Number of readings(s)	4
Permissible reagent blank absorbance	>1.0 AU
Kinetic factor	1768
Maximum ΔA/minute	0.26
Linearity	450 IU/l
Units	IU/L

### PROCEDURE

**Table 13**

Pipette Into Tube Marked	Test
Serum/plasma	100µL
Working ALT reagent	1000µL

Mix well and aspirate immediately for measurement.

Programme the analyser as per assay parameters.

Blank the analyser with Purified Water.

Read absorbance after 60 seconds. Repeat reading after every 30 seconds i.e. upto 120 seconds at 340 nm wavelength.

Determine the mean absorbance change per minute (ΔA/minute)

### Calculations

ALT activity (IU/L) = ΔA/minute X kinetic Factor

ΔA/minute= Change in absorbance per minute

Kinetic Factor (K) = 1768

Kinetic factor is calculated by following formula

$$K = \frac{1}{M} \times \frac{TV}{SV} \times \frac{1}{P} \times 10^6$$

M= Molar extinction coefficient of NADH and is equal to  $6.33 \times 10^3$  lit/mol/cm at 340 nm

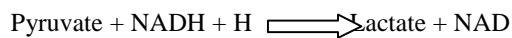
TV= Sample Volume + Working Reagent volume, SV= Sample volume, P= Optical path length

$10^6$ = constant.

### 3. LACTATE DEHYDROGENASE (LDH)

#### Principle:

Lactate dehydrogenase (LD or LDH) catalyzes the reduction of pyruvate by NADH to form Lactate and NAD. The catalytic concentration is determined from the rate of decrease of NADH measured at 340 nm.



Preparation of Working Solutions: working reagent was prepared by mixing Reagent R<sub>1</sub>& R<sub>2</sub> in the ratio 4:1 as per requirement.

#### Reagent Storage and Stability:

The Reagent Kit could be stored at 2-8°C till the expiry date mentioned. The reagent R<sub>1</sub>& R<sub>2</sub> were stable till expiry at 2-8°C. The working solution (4 R<sub>1</sub> + 1 R<sub>2</sub>) was stable for 30 days at 2-8°C.

**Table 14; Components & Concentration of Working Solution**

Component	Concentration
Tris buffer pH 6.8	1000mmol/l
EDTA	0.07gm/l
NADH	0.28mmol/l
Sodium Pyruvate	1.20mmol/l
Sodium Chloride	160mmol/l

**Table 15; Assay Parameters:**

Reaction Type	UV Kinetic
Reaction direction	Decreasing
Wavelength	340 nm
Flow cell Temperature	37°C
Zero Setting with	Distilled Water
Delay Time	60 seconds
No. of Readings	4
Interval	30 seconds
Blank Absorbance Limit	≥ 1.000 Abs.
Sample Volume	0.02ml (20 µl)
Working Solution Volume (4R1 : 1R2)	1.0 ml
Factor	8109
Linearity	2000 IU/l

#### Procedures:

Serum ---- 0.02 ml (20 µl)

Working solution ---- 1.0 ml (800 µl R<sub>1</sub> + 200 µl R<sub>2</sub>)

The following solutions were mixed thoroughly in the stated quantity and immediately transfer to the thermostated cuvette. The stop watch was started simultaneously and the first reading was recorded at 60 seconds while the subsequent three more readings with 30 seconds interval at 340 nm.

#### Calculations:

The average change in absorbance per minute = Δ Abs. / 30 seconds x 2.

Activity of LDH in IU/l = Δ Abs. / min x 8109.

### 4. CREATININE KINASE

**Principle:** Creatinine reacts with picric acid in an alkaline medium to form an orange colour complex. The rate of formation of complex is measured by reading the change in absorbance at 505 nm in a selected interval of time and is proportional to the concentration of picric acid and sodium hydroxide have been optimized to avoid interference from ketoacids.

**Table 16; Reagent Compositions**

Reagent no.	Reagent	REF	Pack Size	Composition	Concentration
1.	Picrate reagent	85LS200-66	1 x 25 ml	Picric Acid Preservatives	40mM/L Qs
2.	Sodium Hydroxide	85LS200-66	1 x 25 ml	Sodium Hydroxide	200mM/L
3.	Creatinine Standard	85LS200-66	1x2 ml	CreatinineStabiliser	2 mg/dl Qs

**Preparation of working Reagent:** The working reagent was prepared by mixing equal volumes of Reagent 1 (Picrate reagent) and Reagent 2 (NaOH) to make up the desired volume and mixed gently for 2 minutes. Reagent 3 was ready to use.

**Reagent storage and stability:** the unopened reagent 1 & 2 were stable at room temperature (15-30°C) and reagent 3 was stable at 2-8°C until the date of expiry.

After Reconstitution, the working reagent was stable for 7 days at 2-8°C.

**Table 17; Assay Parameters**

Mode	Initial rate
Flow-cell Temperature	37°C
Wavelength	505 nm
Reaction Directions	Increasing
Optical Path Length	1 cm
Blanking	Purified water
Sample Volume	100 µl
Working Reagent Volume	1000 µl
Concentration of Standards	2 mg/dl
Delay	30 seconds
Interval	120 seconds
Number of Readings	1
Permissible Reagent Blank Absorbance	< 0.35 AU
Linearity	Upto 20 mg/dl
Units	Mg/dl

### Procedure

The standard and test solution was prepared with following composition.

**Table18;**

Pipette into tubes marked as	Standard	Test
Serum	-----	100 µl
Reagent 3	100 µl	-----
Working Creatinine Reagent	1000 µl	1000 µl

Both the solutions was mixed well.

The analyser was programmed as per the assay parameters.

Analyser was blanked with purified water.

Initial absorbance of Standard (AS1) is measured after 30 seconds and the final absorbance (AS2) after an interval of another 120 seconds.

After taking the standard absorbance, the absorbance of Test sample i.e AT1 & AT2 was taken accordingly in the same procedure.

### Calculations:

Serum Creatinine (mg/dl) = AT2 – AT1 / AS2 – AS1 x 2

Conversion Factor: Creatinine concentration in µmol/L = Creatinine concentration in mg/dl x 88.4.

## RESULTS

Effect of pretreatment of administration of ethanolic extract of *Nelumbo nucifera* (150/300 mg/kg, p.o., once daily) for 28 days and Isoproterenol on day 29<sup>th</sup>& 30<sup>th</sup> (2 days s.c. 85 mg/kg/day) on serum enzymes levels in rats was observed as follows:

### **1. Effect on serum AST**

Rats treated with Iso(85mg/kg/day, s.c.,) for 2 days had serum AST level of (82.95±2.83 IU/L) when measured after experiment. This was very significantly higher ( $P<0.01$ ) when compared to serum AST levels in normal control rats (67.72±1.89 IU/L).

Iso Rats treated with Ethanolic extract of N.N. (150 & 300mg/kg/day, p.o, once daily) for 28 days had serum AST level of (73.04±1.16 and 70.11±0.84 IU/L) when measured after experiment. This was significantly lower ( $P<0.05$ ) when compared to AST levels in Iso control rats (82.95±2.83 IU/L).

### **2. Effect on serum ALT**

Rats treated with Iso(85mg/kg/day, s.c.,) for 2 days had serum ALT level of (63.99±3.06 IU/L) when measured after experiment. This was very significantly higher ( $P<0.01$ ) when compared to serum ALT levels in normal control rats (48.93±1.25 IU/L).

Iso Rats treated with Ethanolic extract of N.N. (150 & 300mg/kg/day, p.o, once daily) for 28 days had serum ALT level of (55.50±1.60 and 51.31±1.74 IU/L) when measured after experiment. This was significantly lower ( $P<0.05$ ) when compared to AST levels in Iso control rats (63.99±3.06 IU/L).

### **3. Effect on serum C.K.**

Rats treated with Iso (85mg/kg/day, s.c.,) for 2 days had serum C.K. level of (1.24±0.045 mg/dl) when measured after experiment. This was very significantly higher ( $P<0.01$ ) when compared to serum C.K. levels in normal control rats (1.03±0.02 mg/dl).

Iso Rats treated with Ethanolic extract of N.N. (150 & 300mg/kg/day, p.o, once daily) for 28 days had serum C.K. level of (1.144±0.018 and 1.138±0.017 mg/dl) when measured after experiment. This was significantly lower ( $P<0.05$ ) when compared to C.K. levels in Iso control rats (1.24±0.045 dl/mg).

### **4. Effect on serum LDH**

Rats treated with Iso(85mg/kg,s.c.,) for 2 days had serum LDH level of (1365.56±38.37 IU/L) when measured on day 66.This was very significantly higher ( $P<0.01$ ) when compared to serum C.K. levels in normal control rats (1168.55±23.31IU/L).

Iso Rats treated with Ethanolic extract of N.N. (150 & 300mg/kg/day, p.o, once daily) for 28 days had serum LDH level of (1259±15.01 and 1229.61±23.10 IU/L) when measured after experiment. This was significantly lower ( $P<0.05$ ) when compared to AST levels in Iso control rats (1365.56±38.37 IU/L).

**Table 19;**Effect of *Nelumbo nucifera* (300 mg/kg & 150 mg/kg) on serum enzymes in Iso. treated rats

ANIMAL GROUPS	AST (IU/L)	ALT (IU/L)	LDH (IU/L)	CK (mg/dl)
<b>CONTROL</b>	67.73±1.90	48.93±1.26	1168.55±23.32	1.031±0.025
<b>ISO.</b>	82.96±2.83 <sup>*</sup>	64.00±3.06 <sup>*</sup>	1365.56±38.38 <sup>*</sup>	1.247±0.045 <sup>*</sup>
<b>STD.</b>	69.59±1.45	50.26±1.02	1183.00±38.54	1.089±0.324
<b>HIGH</b>	71.09±1.21 <sup>**</sup>	51.46±1.40 <sup>**</sup>	1229.00±21.41 <sup>**</sup>	1.120±0.017 <sup>**</sup>
<b>LOW</b>	74.66±1.51 <sup>**</sup>	55.39±1.18 <sup>**</sup>	1254.00±18.40 <sup>**</sup>	1.129±0.031 <sup>**</sup>

Values expressed as mean ±SEM for six animals

\* $p<0.01$  considered statistically very significant as compared to normal control group

\*\*  $P<0.05$  considered statistically significant as compared to ISO group.

t - test was done with the help of Prism 3.0.01 version, available on ggraphpad.com.

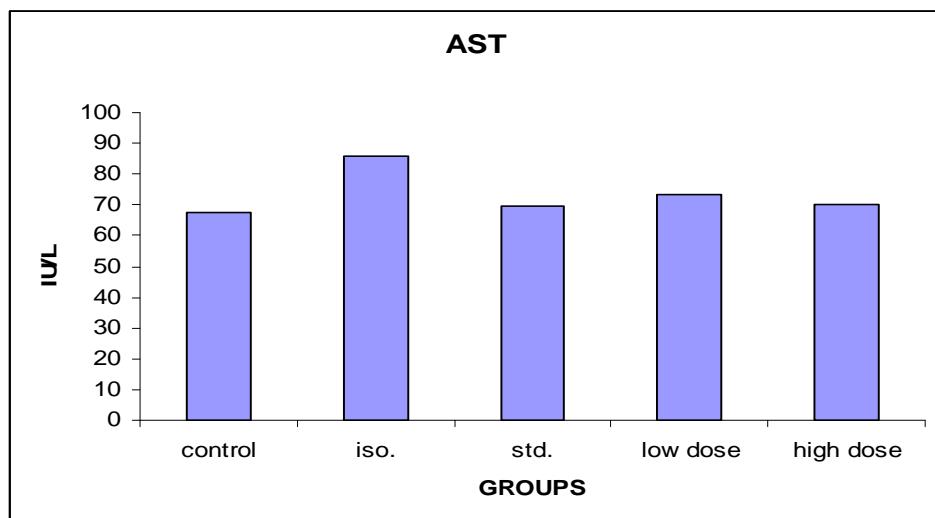
**Graphical Representation of Enzyme Concentrations**

Fig.1 Effect of N.N.E. (150mg / kg &amp; 300 mg/kg, p.o.) on serum AST levels

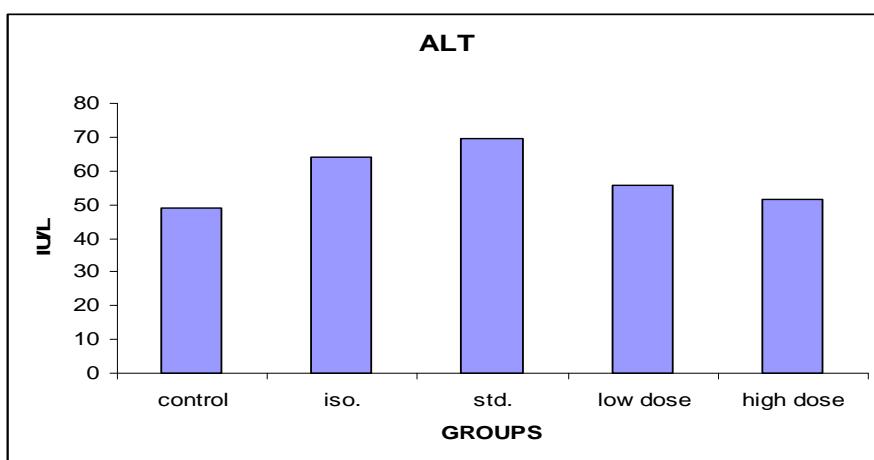


Fig 2: Effect of N.N.E. (150mg / kg &amp; 300 mg/kg, p.o.) on serum ALT levels

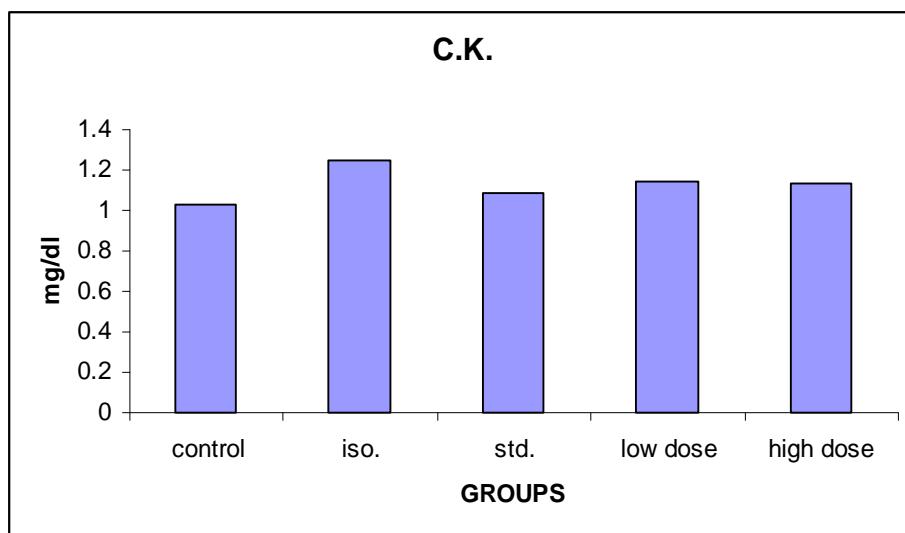


Fig 3; Effect of N.N.E. (150mg / kg &amp; 300 mg/kg, p.o.) on serum CK levels

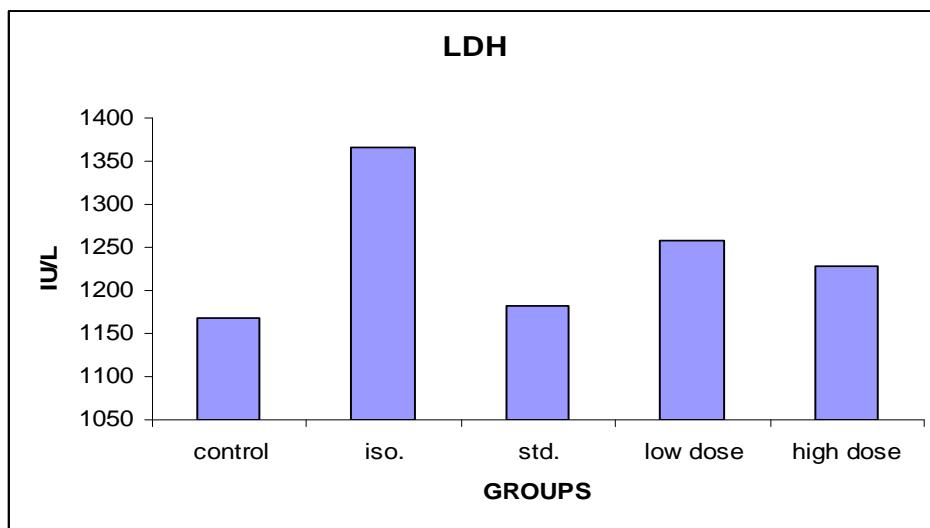
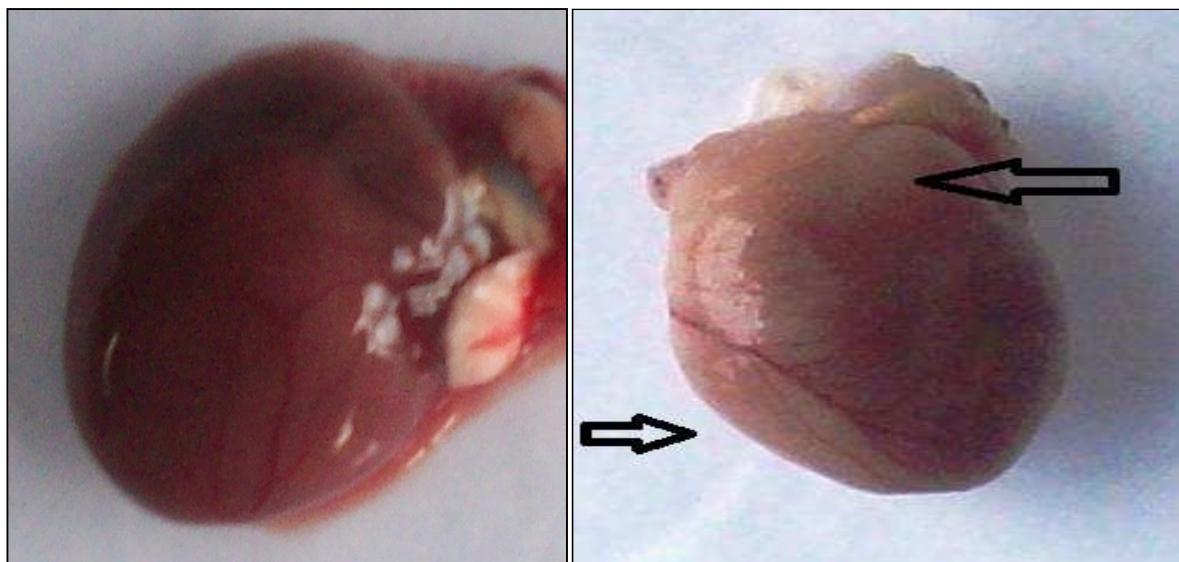


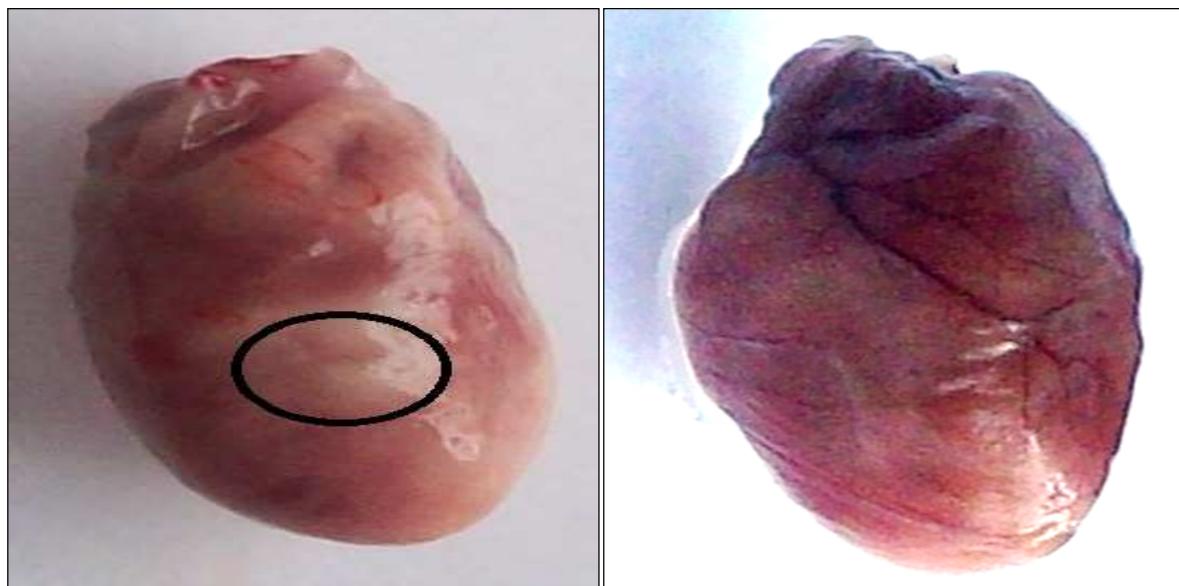
Fig 4; Effect of N.N.E. (150mg / kg &amp; 300 mg/kg, p.o.) on serum LDH levels

Assessment and Grading of Heart



a) Normal saline (0.9 % w/v)  
(fig. H 1)

b) Iso 85 mg/kg  
(fig. H 2)



c) Standard drug  
(metoprolol tartrate) 10mg/kg  
(fig. H 3)

d) *Nelumbo nucifera* 150 mg/kg  
(fig. H 4)

Fig.5; Showing heart of various groups and low dose

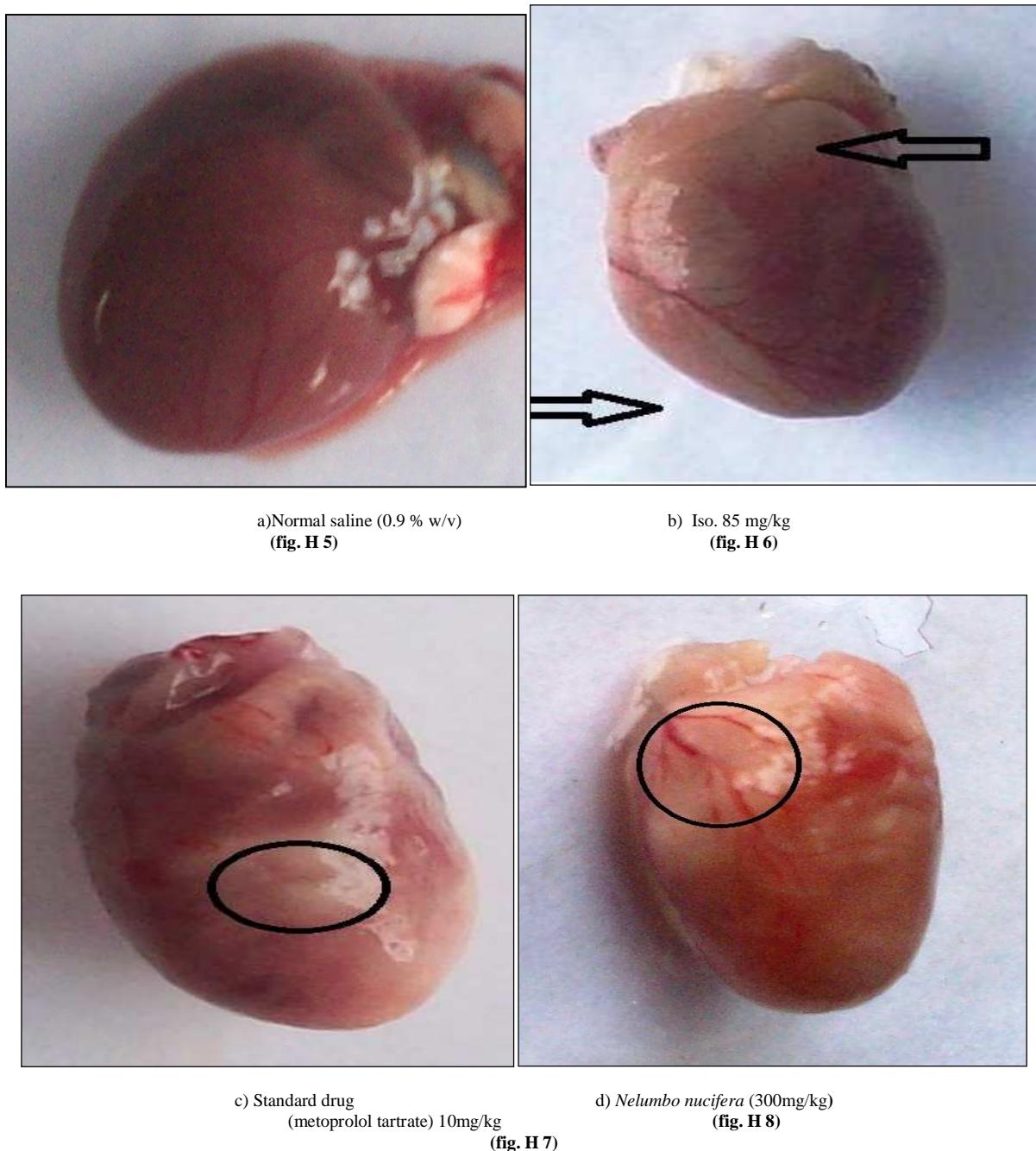


Fig.6; Showing heart of various groups and high dose

The grading was done on the visual basis of heart. The grade helps in comparing the condition of heart of different groups. Grading was done by numbers i.e., 0,1,2,3 & 4. The grade was decided after seeing the hearts very carefully as the necrosis increases the grade also increases, grade 0 shows no necrosis while grade 4 shows maximum necrosis.

#### **Grading Parameters**

Grade 0 = No Lesion

Grade 1 = Inflammation, redness of heart, capillary dilations.

Grade 2 = Yellowish ventricle portion, scar formation.

Grade 3 = Atrium & ventricle turns yellow,

Grade 4 = Diffuse Heart, absolute scar formation, increased necrosis portion.

Observation 1 (150mg/kg)

Figure H 1: No lesions. Grade 0

Figure H 2: Diffuse heart, formation of scar, yellowness in maximum part. Grade 4

Figure H 3: Redness present, no scar formation, some yellowish portion. Grade 2

Figure H 4: Redness present, little scar formation capillary dilation and small yellowish portion. Grade 3

Observation 2 (300mg/kg)

Figure H 5: No lesions. Grade 0

Figure H 6: Diffuse heart, formation of scar, complete yellowness. Grade 4

Figure H 7: Redness present, no scar formation, some yellowish portion. Grade 2

Figure H 8: Redness present, smaller yellowish portion. Grade 1

## DISCUSSION

Myocardium contains an abundant concentration of enzymes like creatinine kinase, lactate dehydrogenase, Alanine Transaminase, Aspartate Transaminase. These Enzymes serves as sensitive index to assess the severity of myocardial infarction. The increase in activities of these enzymes in serum could be due to leakage of enzymes from heart. This is because of lipid peroxidation of membrane bound polyunsaturated fatty acids leading to impairment of structural and functional integrity of cardiac cell membrane as result of sequence of biochemical alteration such as increased calcium overload, activation of ATPase, phospholipase, degradation of phospholipids, by free radicals generated by isoproterinol. This increase in the serum enzyme level of creatinine kinase, lactate dehydrogenase, Alanine Transaminase, Aspartate Transaminase strongly suggest about the myocardial necrosis. Ethanolic extract of *Nelumbo nucifera* (150mg/kg & 300 mg/kg p.o once daily) pretreated-ISO administered rats maintains the levels of AST, ALT, CK, and LDH near to normal. This data suggested that N. N. is effective cardioprotective effect at the dose of 150mg/kg & 300mg/kg p.o. once daily

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