

Protective Effect of *Microcosmus exasperatus* Against Isoproterenol Induced Myocardial Ischemia- A Biochemical and Histopathological Approach

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ABSTRACT

Ascidians, commonly called as sea – squirts are marine sedentary organisms found in abundance in the coastal regions of Tuticorin. Among these, *Microcosmus exasperatus* is a simple ascidian belonging to the class Ascidiacea and family Pyuridae. Many biologically active compounds with pharmacological properties have been reported from this group. The present study was undertaken to evaluate the protective effect of *Microcosmus exasperatus* against isoproterenol induced myocardial ischemia in rats. Wistar male albino rats were divided into 3 main groups - control (saline only), ISO control (saline+isoproterenol) and *Microcosmus exasperatus* treated (ME+ISO). The extract of *Microcosmus exasperatus* was administered at a dose of 50, 100 and 150 mgkg⁻¹ i.g.c for 28 days. On 29th and 30th day of the experiment, the animals of ISO control and ME pretreated groups were given isoproterenol (150 mgkg⁻¹) at an interval of 24 hours. A significant increase in the level of LDH, AST, ALT in the serum and marker enzyme - MDA in serum and heart tissue and decrease in the enzymatic and non-enzymatic antioxidant enzymes - SOD, CAT, GPx in heart tissue and GSH in both serum and tissue were noted in ISO treated groups. In the pretreated groups all the parameters were normal. Restoration of enzyme level observed in the co-treated groups indicate that the extract of *Microcosmus exasperatus* has protective role against ISO induced myocardial ischemia. Histopathological architecture also confirmed the cardioprotective effect of the extract.

Keywords: *Microcosmus exasperatus*, myocardial ischemia, cardiotoxicity, lipid peroxidation, antioxidant.

INTRODUCTION

Number of diseases, play a vital role in disturbing human health. Among these, cardio vascular disease (CV) causes sudden death. Epidemiological studies reveal that ischemic heart diseases (IHD) especially, myocardial infarction (MI) will constitute the major threatening disease causing death worldwide in 2020¹. Myocardial infarction occurs, when there is interruption in supply of blood to any part of the heart, causing cell injury known as myocardial ischemia which is one of the most lethal manifestations of cardio vascular diseases. Isoproterenol (L-β-3,4, dihydroxy phenyl- α iso propylaminoethanol hydrochloride), a sympathomimetic β – adrenergic receptor agonist, causes severe stress to the myocardium resulting in an infarct like necrosis of heart muscles². Research suggests that isoproterenol induced cell death to myocardial cells include hypoxia, calcium overload, depletion of energy reserves and excessive production of free radicals obtained during oxidative metabolism of catecholamines³. Several studies indicate that ISO induced MI serves as a well standardized model in assessing the beneficial effects of many drugs and heart function. Review of literature has shown that a number of plant extracts have been screened for their cardio protective effects against isoproterenol induced toxicity⁴⁻⁷. Studies on chemical

screening^{8,9}, anti-bacterial activity¹⁰, acute and subchronic oral toxicity¹¹, anti diabetic activity¹² and hepato protective activity¹³ with the extract of *Microcosmus exasperatus* have been carried out so far. *Microcosmus exasperatus* is a simple ascidian belonging to the family pyuridae. It is a continuous breeder occurring in the coastal regions of Tuticorin. The present study aims to investigate the protective effect of *Microcosmus exasperatus* against isoproterenol induced cardiotoxicity.

MATERIALS AND METHODS

Animal material

Microcosmus exasperatus was collected from Tuticorin harbour area with the help of SCUBA diver. It was identified and authenticated using key to identification of ascidians¹⁴. A voucher specimen AS 2240 has been deposited in the museum, Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin (Plate 1).

Plate I: *Microcosmus exasperatus*



Systematic position

Phylum: Chordata, Subphylum: Urochordata, Class: Ascidiacea, Order: Pleurogona, Suborder: Stolidobranchia, Family: Pyuridae, Genus: *Microcosmus*, Species: *exasperatus*.

Preparation of powder and extract

The animals were cleaned with sea water, dried at 45° C and homogenized to get a coarse powder which was stored in an air tight container. The extract was prepared from 100 gm powder with ethanol using soxhlet apparatus, cooled to room temperature and evaporated in a rotary evaporator to get a residue which was used for further investigations.

Experimental animal

Mature adult male albino rats weighing about 180-200 gm were selected for the study. The animals were maintained in a well ventilated animal house with constant 12 hours of darkness and 12 hours of light schedule, room temperature (24±2° C) and humidity (60-70 %). Clean water and standard pellet diet “ad Libitum” (Hindustan Lever Ltd., India) were given to them. Before commencement of the experiment, the animals were kept under fasting for about 16 hours.

Acute toxicity studies

To determine the minimum lethal dose, acute oral toxicity studies were performed as per OECD guidelines 2002¹⁵. Male albino rats weighing about 180-200 gm were used. An oral dose of 2000 mgkg⁻¹ body weight of the ethanolic extract of *Microcosmus exasperatus* was given orally using intra gastric catheter to overnight fasted animal. They were observed continuously for any gross behavioral changes and toxic manifestations like hypersensitivity, grooming, convulsions, sedation, hypothermia and mortality during the first 3 hours. The number of dead and surviving animals after 24 hours was recorded. The experiment was repeated with the same dose of the extract for 7 more days. Thereafter the animals were continuously monitored at regular intervals for 14 days.

Induction of myocardial ischemia

Myocardial ischemia was induced by subcutaneous injection of isoproterenol hydrochloride (ISO) 150 mgkg⁻¹ dissolved in saline, once a day for 2 days.

Experimental protocols

Forty eight rats were randomly selected, divided into eight experimental groups, each containing six animals. They were acclimatized for an hour, pre and co-treated orally with saline/*Microcosmus*

exasperatus along with isoproterenol subcutaneously on the scheduled days as per the groups in table I. Twenty four hours after the second dose of isoproterenol (31st day), rats were euthanized.

Table I. Experimental protocol

Groups	Treatment Days 1-28 (mgkg ⁻¹ i.g.c)	Treatment Days 29 &30
I - Saline	Saline	Saline i.g.c. + saline s.c
II - ISO control	Saline	Saline i.g.c. + ISO150 mgkg ⁻¹ s.c
III - ME 50	<i>Microcosmus exasperatus</i> - 50	<i>Microcosmus exasperatus</i> 50 mgkg ⁻¹ i.g.c. + saline s.c
IV - ME 100	<i>Microcosmus exasperatus</i> - 100	<i>Microcosmus exasperatus</i> 100 mgkg ⁻¹ i.g.c. + saline s.c
V - ME 150	<i>Microcosmus exasperatus</i> - 150	<i>Microcosmus exasperatus</i> 150 mgkg ⁻¹ i.g.c. + saline s.c
VI – ME 50 + ISO 150	<i>Microcosmus exasperatus</i> - 50	<i>Microcosmus exasperatus</i> 50 mgkg ⁻¹ i.g.c. + ISO 150 mgkg ⁻¹ s.c
VII – ME 100 + ISO 150	<i>Microcosmus exasperatus</i> - 100.	<i>Microcosmus exasperatus</i> 100 mgkg ⁻¹ i.g.c. + ISO 150 mgkg ⁻¹ s.c
VIII – ME 150 + ISO150	<i>Microcosmus exasperatus</i> - 150	<i>Microcosmus exasperatus</i> 150 mgkg ⁻¹ i.g.c. + ISO 150 mgkg ⁻¹ s.c

ME – *Microcosmus exasperatus*; ISO – Isoproterenol; i.g.c. – Intra gastric catheter; s.c. – sub cutaneously

Preparation of serum and tissue for biochemical studies

Samples of blood were collected by cardiac puncture. The serum obtained was used for the analysis of lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT), reduced glutathione (GSH) and lipid per oxidation marker enzyme-malondialdehyde (MDA). The heart of the animals were dissected out, weighed, washed in ice-chilled physiological saline, frozen and homogenized in a Teflon homogenizer with 10 times (w/v) ice-cold phosphate buffer saline of pH 7.8. The homogenate was centrifuged at 1000 rpm at 4° C and the supernatant was used for the estimation of enzymatic and non-enzymatic myocardial antioxidant enzymes. LDH, AST, ALT, extent of lipid per oxidation, SOD, CAT, GPx and GSH was assayed by standard procedures¹⁶⁻²³.

Assay of Lactate Dehydrogenase (LDH)

1ml of the buffered substrate was pipette out and 0.1 ml of serum was added. The tubes were incubated for 15 minutes at 37° C. The incubation was continued for another 15 minutes by adding 0.2 ml of NAD⁺. 1ml of dinitrophenyl hydrazine reagent was added to arrest the reaction and then the tubes were incubated for a further period of 15 minutes at 37° C. After the incubation period 7.0 ml of 0.4N sodium hydroxide solution was added and the colour developed was measured at 420 nm using a spectrophotometer. Following the same procedure, aliquots of the standards were analyzed. The enzyme activity is expressed as IUmg⁻¹.

Assay of AST and ALT

To 1ml of the buffered substrate, 0.1 ml of serum was added and incubated for 1hr at 37° C. At the end of the incubation, 0.07 ml of aniline citrate reagent was added and the incubation further continued for 20 minutes. Then 1ml of dinitrophenyl hydrazine (DNPH) reagent was added and left for 20 minutes. 10 ml of 0.4N Sodium hydroxide was added to arrest the reaction and the colour developed was read at 540 nm using spectrophotometer. Similarly the standards were also analyzed. The enzyme activity in serum is expressed as IUmg⁻¹.

Lipid peroxidation assay

Malondialdehyde, a stable product of lipid per oxidation in heart tissues, react with thio barbituric acid resulting in a coloured complex- chromogen. To 0.2 ml of tissue homogenate, added 1.5 ml of 20% acetic acid, 0.2 ml SDS and 1.5 ml of TBA. By the addition of distilled water, the mixture was made up to 4 ml and then heated for 30 minutes at 95° C. 4 ml of n-butanol-pyridine mixture was added and shaken well. The organic layer was separated after centrifugation at 4000 rpm for 10 minutes and absorbance was read spectrophotometrically at excitation wavelength 532 nm. The lipid peroxide concentration was expressed in n.mol. MDA ml⁻¹ in serum and mg⁻¹ wet weight for heart tissue.

Assay of super oxide dismutase (SOD)

The method is based on the principle of the inhibitory effect of SOD on the reduction of NBT (nitro blue tetrazolium) dye by superoxide radicals, which are generated by the auto oxidation of hydroxylamine hydrochloride. The reaction mixture contained 2.7 ml of 0.067M phosphate buffer, pH7.8, 0.05 ml of 0.12mM riboflavin, 0.1 ml of 1.5mM NBT, 0.05 ml of 0.01M methionine and 0.1 ml of enzyme samples. For uniform illumination, the tubes were kept in air aluminium foil in a box with a 15W fluorescent lamp for 10 minutes. Blank without samples were also run simultaneously. The reduction of this dye is followed by an absorbance increase at 540 nm. One unit is defined as the amount of enzyme, which caused half- maximal inhibition of NBT reduction and expressed as U mg^{-1} protein.

Assay of catalase (CAT)

This enzyme catalyzes decomposition of hydrogen peroxide (H_2O_2) which can be measured spectrophotometrically. Samples were diluted in 3 ml of 0.66M phosphate buffer (pH 7.0), incubated and centrifuged. Supernatants were combined and 0.04 ml was added to 30% w/v H_2O_2 mixed well and read on a spectrophotometer. Blanks in reference cuvette containing H_2O_2 free phosphate buffer was also run simultaneously. Decrease in absorbance at 240 nm is followed until the optical density reaches 0.05 and it was recorded. By applying the formula $(0.1175/t)$ the catalase activity was calculated and expressed in U mg^{-1} protein.

Assay of glutathione peroxidase activity (GPx):

The assay mixture contained 0.2 ml of tissue homogenate, 0.2 ml of EDTA (0.8mM), 0.1 ml of sodium azide, 0.1 ml of GSH (4mM), 0.1 ml of H_2O_2 , 0.4 ml of phosphate buffer (0.4M) pH7.0. After incubation for 10 minutes at 37°C , the reaction was terminated by the addition of 0.5 ml of 10 % TCA, centrifuged at 2000 rpm for 10 minutes. The remaining GSH in the supernatant was quantified as the non – protein thiol (-SH) groups by reacting with 0.1 ml of 0.04 % DTNB solution (Ellman's reagent). The optical density of the chromophoric product formed was read at 412 nm against a blank (without homogenate). The results are expressed as U mg^{-1} protein.

Assay of reduced glutathione activity (GSH):

0.1 ml of tissue homogenate was added to each tube containing 0.1 ml of TCA (10%). The tubes were centrifuged at 5000 rpm for 10 minutes. Accurately 0.1 ml of resulting clear supernatant, 2 ml of DTNB (0.6mM), 1.9 ml of 0.2 M phosphate buffer (pH 9.0) were mixed in separate test tubes. Then the optical density was measured at 412 nm against a reagent blank (containing TCA only). A series of standard was made for each sample to determine the glutathione content. The data is expressed as n.mol. protein ml^{-1} in serum and mg^{-1} in tissue.

Histopathological studies of cardiac muscle

The heart tissues were excised and immediately fixed in 10% buffered neutral formalin. Sample tissues were dehydrated and embedded in molten paraffin wax. Tissue sections were cut at 5 μm thickness, stained with haematoxylin and eosin. The myocardial architecture was examined under light microscope and photomicrographs were taken.

Statistical analysis

Values are expressed as mean \pm SEM. The statistical analysis was done by one- way analysis of variance (ANOVA) followed by Dunnet's test. P values less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

A study undertaken to ascertain the cardioprotective effect of the extract of *Microcosmus exasperatus* against isoproterenol induced myocardial ischemia showed the following results (Table II &III).

Table II. Effect of the extract of *Microcosmus exasperatus* on serum LDH, AST, ALT and lipid peroxidation marker enzyme – MDA

Groups & Treatment	LDH IU mg ⁻¹	AST IU mg ⁻¹	ALT IU mg ⁻¹	MDA	
				Serum n mol. ml ⁻¹ protein	Heart Tissue n mol. mg ⁻¹ protein
I - Control	152.42±1.25	12.61±0.89	10.23±0.75	8.31±0.58	5.32±0.39
II - ISO 150 mg	234.59±2.83 ^{aa}	21.93±0.15 ^{aa}	20.35±0.13 ^{aa}	19.65±0.34 ^{aa}	14.36±0.11 ^{aa}
III - ME 50 mg	143.64±1.51	11.82±0.48	9.86±0.48	7.95±0.38	4.31±0.41
IV - ME 100 mg	149.15±1.62	12.93±0.68	11.36±0.69	8.49±0.41	4.91±0.31
V - ME 150 mg	155.36±1.72	13.21±0.39	12.84±0.59	8.93±0.11	5.41±0.24
VI - ME 50 mg + ISO	184.14±1.12 ^{ab}	18.23±0.59 ^a	19.86±0.84 ^{aa}	13.86±0.58 ^{ab}	13.15±0.48 ^{aa}
VII - ME 100 mg + ISO	178.19±1.73 ^{ab}	13.65±0.68 ^b	16.43±0.55 ^{ab}	12.61±0.29 ^{ab}	10.63±0.52 ^{ab}
VIII - ME 150 mg + ISO	163.86±1.59 ^{abb}	11.15±0.38 ^{bb}	12.15±0.62 ^{bb}	11.29±0.41 ^{abb}	6.56±0.89 ^{bb}

Data represented as mean± S.E.M (n=6). Significance between Group I and Group II to VIII ^a < 0.05; ^{aa} < 0.01. Significance between Group II and Groups VI to VIII ^b < 0.05; ^{bb} < 0.01.

Table III. Effect of the extract of *Microcosmus exasperatus* on enzymatic and non- enzymatic myocardial antioxidant enzymes

Groups & Treatment	SOD U mg ⁻¹ protein	Catalase U mg ⁻¹ protein	Gpx U mg ⁻¹ protein	GSH	
				Serum n mol. ml ⁻¹	Heart Tissue n mol. mg ⁻¹ protein
I - Control	8.36±0.93	3.22±1.84	4.09±0.68	4.56±0.94	2.13±0.14
II - ISO 150 mg	4.12±0.63 ^{aa}	1.98±0.62 ^{aa}	3.26±0.84 ^a	2.04±0.11 ^{aa}	1.05±0.16 ^a
III - ME 50 mg	6.84±1.38	2.08±0.84	3.93±0.64	4.16±0.14	2.08±0.34
IV - ME 100 mg	6.86±1.38	2.19±0.85	4.16±0.26	4.84±0.19	2.13±0.84
V - ME 150 mg	8.16±1.14	2.84±0.68	4.64±0.15	4.89±0.06	2.98±0.14
VI - ME 50 mg + ISO	6.29±1.62	2.26±1.14	3.40±0.65	2.88±0.052 ^a	1.84±0.48
VII - ME 100 mg + ISO	7.31±2.16 ^b	2.84±0.54	4.29±0.88 ^b	3.09±0.014 ^b	1.98±0.93
VIII - ME 150 mg + ISO	8.61±3.38 ^{bb}	3.04±1.12 ^b	4.96±0.16 ^{bb}	4.19±0.046 ^{bb}	2.96±0.84 ^b

Data represented as mean± S.E.M (n=6). Significance between Group I and Group II to VIII ^a < 0.05; ^{aa} < 0.01. Significance between Group II and Groups VI to VIII ^b < 0.05; ^{bb} < 0.01.

Acute oral toxicity

The animals administered with 2000 mg/kg body weight did not show any mortality during the twenty four hours experimental duration. This single dose of 2000 mg/kg body weight induced irritability, tremor, laboured breathing, staggering and convulsion but not mortality indicating its safety. Based on the results, sub lethal dose of 50,100 and 150 mg/kg body weight were selected for the present study.

ISO administration at a dose of 150 mg/kg induced myocardial ischemia which is evidenced by a significant increase in the levels of serum LDH, AST, ALT and lipid per oxidation marker enzyme MDA in serum and heart tissue. The elevated level of serum enzymes can be accepted as a reliable index of damage to myocardium caused by ISO which is anticipated as a cardio toxic mediator because of its ability to cause necrotic damage of the myocardial cells and membrane. Due to deficiency of oxygen supply, the myocardial membrane becomes permeable. This leads to a leakage of enzymes into blood stream thus increasing their concentration in the serum which is a diagnostic marker of myocardial infarction. The present observations on treatment with ISO are in agreement with the earlier reports using plant extracts^{24,25}. In the groups pre treated with the extract, an increase was observed in the enzyme levels which were brought back to normal in the group which received the highest dose. This could be due to potential of extract in repair and maintenance of membranes of the myocardium as observed on treatment with *Terminalia arjuna* extracts⁴. A dose dependent significant reduction in the marker enzymes was noted in the groups co treated with extract and ISO, indicating the protective effect of the extract on the myocardial tissues by restoring the level to that of the control. The lower levels of enzyme was recorded during co treatment could be attributed to the reduction in the damage to the myocardium

by the bioactive compounds present in the extract which would have prevented the release of the marker enzymes from the heart tissues.

Subsequent to ISO administration, an increased formation of lipid peroxidation product, MDA was observed. This is an important pathogenic event causing cardiac damage²⁶. The increase in the levels of lipid peroxides in ISO induced rats might be the result of free radical mediated membrane stress. On the pre treated groups, no significant change in the level of MDA in the serum and heart tissue was noted indicating the non toxic nature of the extract. During co treatment, the group which received the lowest dose showed a higher value of MDA with a dose dependent decrease. The decreased level of MDA in serum and heart might be due to augmentation of endogenous antioxidants by the active components of the extract of *Microcosmus exasperatus*. The results are in line with previous reports using plant extracts²⁷.

The activities of SOD and CAT decreased significantly in ISO induced rats when compared to control group. SOD protects the cells from oxidative stress caused by free radicals. During myocardial infarction, SOD and catalase are structurally and functionally impaired by free radicals causing myocardial necrosis²⁸. Hence, reduction in SOD and catalase activity may be due to the involvement of superoxide and free radicals in the myocardial cells causing damage. A gradation in the level of SOD and CAT was observed in the pre treated groups. The antioxidant properties exhibited may be by the presence of compounds such as Tetradecanoic acid, n- Hexadecanoic acid, 26 –Nor-5-cholestan- 3a-ol-25-one, Cholestan-3-ol, 2-Piperidinone, N-(4-bromo-n-butyl)-⁸. The rise in the activities of first line cellular defensive enzymes such as SOD and CAT in co treated ISO challenged group highlight the protection against the oxidative stress by increasing the removal of super oxide radicals. It is suggested that dose as low as 150 mg/kg of extract of *Microcosmus exasperatus* enhanced the antioxidant activity of SOD and contribute to the inhibition of free radical mediated cell injury.

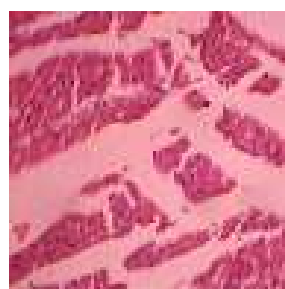
The status of GSH, a ubiquitous thiol – containing tripeptide, is a highly sensitive indicator of cell functionality and viability. It plays a central role in cell biology²⁹. A significant decrease in the level of GSH observed in the serum and heart tissue of ISO induced rats may be either due to decreased synthesis or increased degradation of glutathione. GPx activity was also reduced due to reduced availability of its substrate GSH. The major function of GPx is to reduce soluble hydrogen peroxide and alkyl peroxides which uses glutathione as its substrate³⁰. Therefore significant reduction causes accumulation of oxidised glutathione which in turn blocks protein synthesis by inactivating many enzymes containing the SH group. In pre treatment, GPx and GSH level was maintained to that of normal showing the prophylactic nature of extract in restoring the antioxidant properties. A gradual elevation was observed in the activities of GPx and GSH in co treated groups which highlight the antioxidant components present in the extract.

Histopathological studies showed intensive cardiomyocyte necrosis and increased edematous intramuscular space in ISO control. In *Microcosmus exasperatus* pre treated groups normal histoarchitecture of myocardium with mild edematous intramuscular space was evident. Group co treated with 50 mgkg⁻¹ bw, a moderate reduction in myonecrosis, oedema and infiltration of inflammatory cells was observed whereas in the groups which received 100, 150 mgkg⁻¹ bw the histomorphology of myocardium was normal with the absence of inflammatory cells (plate 2).

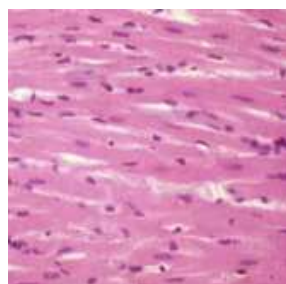
Plate II: Photomicrograph showing histoarchitecture of cardiac muscle



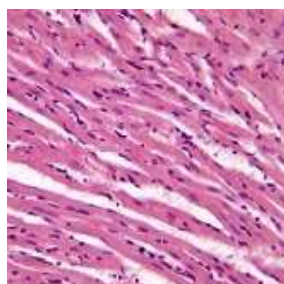
Group I – Normal control



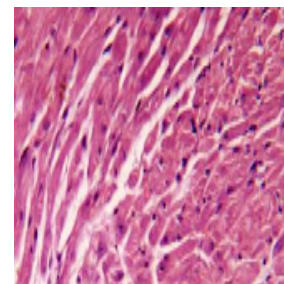
Group II – ISO control



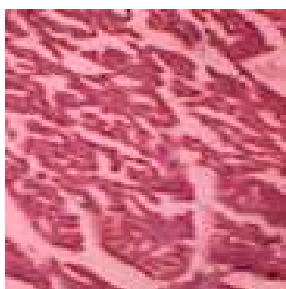
Group III- Extract of ME
(50 mg/kg bw)



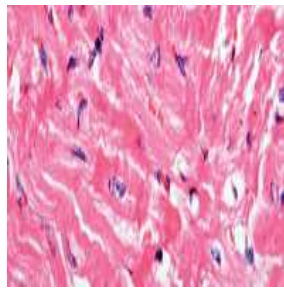
Group IV- Extract of ME
(100 mg/kg bw)



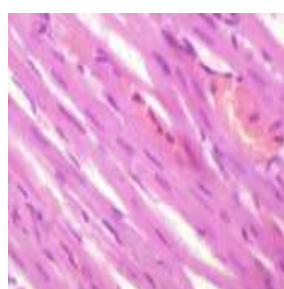
Group V- Extract of ME
(150 mg/kg bw)



Group VI- Extract of ME+ISO
(50 mg/kg bw)



Group VII -Extract of ME+ISO
(100 mg/kg bw)



Group VIII- Extract of ME +ISO
(150 mg/kg bw)

CONCLUSION

The present study projects the protective effect of *Microcosmus exasperatus* against isoproterenol induced myocardial infarction in rats as related to its effectiveness, inhibiting lipid peroxidation, preservation of antioxidant enzymes and scavenging of free radicals which is confirmed by histopathological examination. It is suggested that the antioxidant constituents in the extract might be responsible for its cardioprotective effect.

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