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Cardioprotective effect of *Elepahnopus scaber* Linn. on the Isolated rat heart in Ischemia-Reperfusion induced myocardial damage

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ABSTRACT

The current study was carried out to determine the protective role of hydro-alcoholic extract of *Elephantopus scaber* Linn. (HAEES) on ischemia-reperfusion induced myocardial injury in rats. HAEES was screened for *in-vitro* antioxidant activity using standard methods, to investigate the free radicals scavenging activity which are produced by ischemia-reperfusion induced oxidative stress. Ischemia-reperfusion induced injury in isolated rat hearts by global no-flow ischemia (15 min) can be assessed by measuring the changes in Hemodynamic parameters of heart, level of cardiac marker enzymes in heart perfusate and HTH, antioxidant system and histological changes when compared to post-ischemic condition of ischemia-reperfusion control group. HAEES have shown good antioxidant activity in DPPH, nitric oxide, hydroxyl and superoxide radical methods. Pretreatment with HAEES and ischemia-reperfusion induced injury have shown good cardioprotective effect in terms of significant recovery in DT, changes in biochemical parameters in perfusate and HTH, antioxidants level and histological changes in isolated rat hearts.
Keywords: Antioxidant; Cardioprotective; *Elephantopus scaber* Linn.; Ischemic-reperfusion.

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INTRODUCTION

Myocardial infarction (MI) and Ischemia-reperfusion injury (IRI) are clinically relevant to situations such as coronary angioplasty, thrombolytic therapy, coronary revascularization and heart transplantation [1]. IRI is a pathological condition that results from an acute increase in oxidative stress during reperfusion after ischemia that triggers a cascade of pathophysiological events [2]. Several endogenous mediators are released during ischemic reperfusion that could potentially modulate intracellular signaling by acting on surface membrane receptors or on intracellular components of signaling pathways. Ischemia followed by reoxygenation is associated with oxygen free radical generation, which is one of the major factors in the induction of arrhythmias and myocardial damage in the ischemia and reperfusion syndrome. The cause of reperfusion-induced injury is apparently multifactorial. Several experimental studies have indicated that a burst of oxygen free radical occurs immediately after reperfusion of ischemic myocardium [3]. A major goal in the management of MI is to reduce post MI complications and mortality by reversing myocardial ischemia and limiting the infarct size.

The natural antioxidant defense system in our body was constructed to act against the free radicals generated in the cell. During ischemic condition the activity of antioxidant system will suppressed due to lack of Adenosine tri phosphate (ATP). Reperfusion after ischemia leads to tremendous increase in reactive oxygen species, which causes the cellular and tissue damage [4].

Many plants having the antioxidant activity have been reported to show the cardioprotective activity against IRI. In Indian traditional system, medicinal plants are used to treat human diseases including cardiovascular diseases. *Elephantopus scaber* Linn. is a plant found in semi evergreen forests of Western Ghats**,** Karnataka, Andhra Pradesh and Tamil Nadu. Leaves are used in applications for eczema, antitumor activity and ulcers. Roots are specially having cardioprotective activity. This plant is also used in insomnia, diabetes and urethral discharges, rheumatism and for filariasis. Molephantin and molephantinin possess cytotoxic and antitumor properties [5].

The present study has been designed to evaluate the cardioprotective activity of hydro-alcoholic extract *of Elephantopus scaber* Linn. in ischemia-reperfusion induced myocardial damage in rats and attempts to understand the molecular mechanism of its therapeutic effect with reference to biochemical markers and antioxidant enzymes, lipid peroxidation and Histopathological examination.

MATERIALS AND METHODS

Animals

Wistar albino rats of either sex weighing 180-200 g were used for the study. They were housed in clean polypropylene cages under standard conditions of temperature (23±2ºC), humidity (50±5%) and 12 h light-dark cycles and fed with standard diet (Amruth feeds and foods, Bangalore, Karnataka, India) and water *ad libitum*. All animals were handled with human care. All the studies conducted were approved by the Institutional

Animal Ethical Committee, Gautham College of Pharmacy, Uday vidya Kendra, Bangalore, India and according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (491/01/c/CPCSEA), Government of India.

Chemicals

All the chemicals used were of analytical grade and purchased from standard companies. Biochemical kits like CK-MB, LDH, AST and ALT were procured from Span diagnostics Ltd., Gujarat, India.

Plant material and extraction

The *Elephantopus scaber* Linn. plants were collected from Mollem National Park**,** Kulem, Goa, and the plant was identified and authenticated by Dr. M. D. Rajanna, Professor of Botany, Curator In-charge, University of Agricultural Sciences, GKVK, Bangalore, and voucher specimen of the plant was submitted to the college herbarium.

The roots were isolated from plant, and were washed under running tap water. The roots were shade dried and powdered; the coarse powder was subjected to successive extraction with petroleum ether for defatting for 6-8 h and dried. Then subjected to alcohol (70%) extraction in Soxhlet's extractor for 18 h and the extract thus obtained is evaporated to obtained the solid or semisolid mass and stored in air tight container [6]. The extract was dissolved in distilled water by using Tween 80 as suspending agent for Pharmacological studies.

Iv-vitro **antioxidant activity**

Determination of free radical scavenging activity by DPPH assay

The free radical scavenging activity of *Elephantopus scaber* Linn. was measured by DPPH the slightly modified spectrophotometric method. A solution of DPPH in methanol (6×10^{-5} M) was prepared freshly. A 3 mL aliquot of this solution was mixed with l mL of the HAEES samples 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 [7]. Ascorbic acid was used as the reference material. The antioxidant activity of the extract was expressed as IC_{50}, which was defined as the concentration (in μg/mL) of extract that inhibits the formation of DPPH radicals by 50%.

Determination of superoxide anion radical scavenging activity

About 1 mL of nitroblue tetrazolium (NBT) solution (156μM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL NADH solution (468μM in 100 mM phosphate buffer, pH 7.4) and 0.1 mL of sample solution of HAEES and standard in water was mixed. The reaction was started by adding 100μL of Phenazine methosulphate (PMS) solution (60μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 minutes and the absorbance at 560 nm was measured against blank. Ascorbic acid was used as the reference material. Decreased absorbance of the reaction mixture indicated

increased superoxide anion scavenging activity. Percentage inhibition of superoxide generation was evaluated by comparing the absorbance values of the control and experimental tubes [8].

Hydroxyl Radical Scavenging Activity

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell et al. [9]. Stock solutions of EDTA (1mM), $FeCl_3$ (10 mM), ascorbic acid (1mM), H_2O_2 (10mM) and deoxyribose (10mM) were prepared in distilled deionized water. The assay was performed by adding 0.1 mL of EDTA 0.01 mL of $FeCl_3$, 0.1 mL of H_20_2, 0.36 mL of deoxyribose,1.0 mL of HAEES (1-10μg/mL), 0.33 mL of phosphate buffer (50 mM, pH 7.4) and 0.1mL of ascorbic acid in sequence. The mixture was then incubated at 37 °C for 1hr. About 1.0 mL portion of the incubated mixture was mixed with 1.0 mL of 10% TCA and 1.0 mL of 0.5% TBA to develop the pink chromogen, measured at 532 nm. Sodium metabisulphite used as the reference compound. The hydroxyl radical scavenging activity of the extract is reported as percentage inhibition of deoxyribose degradation.

Nitric Oxide Radical Scavenging Activity

Nitric oxide (NO) radical were generated from sodium nitroprusside solution at physiological pH. Sodium nitroprusside (1mL of 10mM) were mixed with 1mL of HAEES of different concentration (20-100 µg/mL) in phosphate buffer (pH 7.4). The mixture was incubated at 25°C for 150 min. To 1 mL of the incubated solution, 1mL of Griess's reagent (1% sulphanilamide, 2% ophosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) was added. Absorbance was read at 546 nm. Decreased absorbance of the reaction mixture indicates increased nitric oxide radical scavenging activity [10]. Ascorbic acid was used as the reference material. The percentage inhibition of absorbance was calculated.

Experimental design

Treatment protocol

The experimental animals were divided in to five groups of six rats each.

Group I Ischemia-Reperfusion (IR) control rats were treated with vehicle.
Group II Rats were pretreated with Vit-C 20 mg/kg, p.o.
Group III Rats were pretreated with 200 mg/kg, p.o of HAEES.
Group IV Rats were pretreated with 400 mg/kg, p.o of HAEES.
Group V Rats were pretreated with 600 mg/kg, p.o of HAEES.

Groups I was pretreated with the vehicle, Group II, III, IV and V pretreated with Vit-c 20 mg/kg, p.o, HAEES 200, 400 and 600 mg/kg, p.o respectively for 30 days. The heart was isolated from each animal 2 hrs after the last dose of the drug(s) under Ketamine (70 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia, and was mounted to the Langendroff apparatus. The heart was perfused with K-H solution gassed with carbogen (95% O_2 and 5%

CO_2) at 37 ^{0}c. The heart was allowed to stabilize, regular recording was taken for a perfusion period of 15 min and then the heart was subjected to global no-flow ischemia by blocking the flow of K-H solution followed by 15 min of reperfusion [11]. Measurement of contractile force was done using force displacement transducer and recorded on a RMS Polyrite-2 (Chandigarh, India) by maintaining the resting tension 2 g [12]. Then measure the heart rate (HR) and developed tension (DT) during pre-ischemic and post-ischemic period and recovery percentage was calculated. LDH and CK-MB activity were measured in the perfusate during pre-ischemic and post-ischemic period. The heart was then homogenized to prepare heart tissue homogenate (HTH) using phosphate buffer (pH 7.0) and activity of LDH, CK-MB, Super oxide dismutase (SOD) and catalase was determined. Microscopic slides of myocardium were prepared by using hematoxylin and eosin (H & E) stain for histopathological studies after post ischemia.

Statistical analysis

Results are expressed as mean ± SEM. Statistical significance was assessed using One-way Analysis of variance (ANOVA) followed by Tukeys post-test.

RESULTS

In-vitro antioxidant activity

Different concentrations (50-250 µg/ml) of HAEES were tested for their antioxidant activity in various *in vitro* models. Free radicals were scavenged by the test compounds in a concentration dependent manner with the given range of concentrations in all the models. The half maximum inhibitory concentration (IC_{50}) in the DPPH, superoxide, hydroxyl and nitric oxide scavenging activities were 140.23, 203.41, 194.56 and 184.49µg/ml respectively (Table 1).

Effect on LDH and CK-MB

The activities of endogenous enzymes like CK-MB and LDH were evaluated in coronary effluent during pre-ischemic and post-ischemic period. In control group there was a elevated amount of endogenous enzymes leakage will occurs because of cell membrane damage. In pre-ischemic condition there was a significant elevation in these enzymes in group pretreated with HAEES-200 and depletion of these enzyme activities were observed in groups like Vit-C, HAEES-400 and HAEES-600 when compared to control group. In post-ischemic condition significant decrease in these enzyme activities were observed in HAEES pretreated groups when compared to control group (Table 2).

The endogenous enzyme activities like CK-MB, LDH, AST and ALT were evaluated in the HTH. These enzymes activities were decreased in the HAEES-200 group when compared to control group and elevation of these enzymes level were observed in the pretreated groups like Vit-C, HAEES-400 and HAEES-600 when compared to control group (Table 3).

Effect on SOD and Catalase activity

Tissue antioxidants like SOD and catalase levels were depleted in HAEES-200 group when compared to control. There was an increase in these antioxidants levels were observed in Vit-C, HAEES-400 and HAEES-600 groups when compared to control group (Table 4).

Heart rate and Developed tension

There was a short recovery of heart rate and developed tension in post-ischemic condition was observed in HAEES-200 group when compared to pre ischemic condition. In Vit-C, HAEES-400 and HAEES-600 groups significant recovery of heart rate and developed tension was observed in post-ischemic condition when compared to pre-ischemic condition (Table 5).

Histopathological changes

Histopathological examination of myocardial tissue obtained from Control rats showed severe myocytes damage, diffuse inflammatory process and huge vascular spaces. Animals pretreated with HAEES 200 mg/kg, p.o showed minute improvement in the myocardial integrity, but it shows some areas of necrosis and inflammatory cells process. The groups pretreated with Vit-C and HAEES (400 and 600 mg/kg, p.o) show reduction of myocardial spaces, necrosis and inflammatory process when compared to the control group (Figure 1).

Table 1: Free radical scavengibg activity of HAEES compared to standard drug.

Test	Sample	% Inhibition for concentrations (mean±SD)					IC_{50} µg/ml
		50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml	
DPPH	Ascorbic acid	28.85±0.026	42.63±0.012	64.41±0.012	74.86±0.006	91.85±0.006	127.48±0.65
	HAEES	22.88±0.015	34.36±0.02	55.78±0.011	62.37±0.026	73.12±0.01	140.23±0.53
Super Oxide	Ascorbic acid	16.12±0.02	28.25±0.026	45.55±0.013	72.25±0.02	93.43±0.023	162.99±0.86
	HAEES	13.45±0.02	19.98±0.01	33.56±0.011	47.68±0.01	78.73±0.01	203.41±0.67
Hydroxyl Radical	Sodium metabisulphite	11.52±0.01	27.67±0.012	39.16±0.01	58.82±0.032	77.65±0.02	177.85±1.23
	HAEES	4.21±0.032	21.42±0.02	30.61±0.006	51.62±0.02	65.62±0.032	194.56±1.54
Nitric Oxide	Ascorbic acid	19.25±0.02	38.68±0.01	46.63±0.011	70.39±0.01	82.28±0.02	165.84±0.36
	HAEES	13.12±0.015	28.32±0.01	37.56±0.02	66.72±0.01	71.28±0.003	184.49±0.45

Data represents the Mean ± SEM of three measurements.

Table 2: Effect of HAEES on CK-MB and LDH activities in rat heart perfusate.

Treatment	CK-MB(U/L)		LDH(U/L)	
	Pre-ischemia	Post-ischemia	Pre-ischemia	Post-Ischemia
Group-I (IR control)	41.12±1.625	68.38±1.74	222.3±2.07	501.6±4.202
Group-II (Vit-C 20 mg/kg, p.o)	27.76±0.68***	40.20±0.86***	159.0±2.76***	373.9±4.23***
Group-III (HAEES 200 mg/kg, p.o)	39.78±1.15	59.52±1.64**	202.6±5.14**	470.8±11.96*
Group-IV (HAEES 400 mg/kg, p.o)	33.53±1.01**	50.69±1.78***	185.3±2.8***	402.0±5.3***
Group-V (HAEES 600 mg/kg, p.o)	28.49±1.43***	40.71±1.52***	174.2±3.28***	386.8±6.0***

Each data represents the Mean± SEM (n=6). *p<0.05; **p<0.01; ***p<0.001 compared with IR control rats. One-way ANOVA followed by Tukeys post test.

Table 3: Effect on CK-MB, LDH, ALT and AST in heart tissue homogenate of isolated rat heart preparation.

Treatment	CK-MB (U/g wet tissue)	LDH (U/g wet tissue)	AST (U/g wet tissue)	ALT (U/g wet tissue)
Group-I (IR control)	49.43±2.639	558.0±5.249	22.54±0.84	40.22±0.73
Group-II (Vit-C 20 mg/kg, p.o)	90.19±3.387***	860.2±8.002***	16.15±1.15***	31.56±0.76***
Group-III (HAEES 200 mg/kg, p.o)	63.75±2.89*	585.6±7.18*	22.98±1.03	37.57±0.42
Group-IV (HAEES 400 mg/kg, p.o)	77.81±2.886***	697.4±5.38***	18.50±0.74**	35.16±0.36**
Group-V (HAEES 600 mg/kg, p.o)	90.19±3.387***	739±3.12***	17.39±0.45***	32.47±0.77***

Each data represents the Mean± SEM (n=6).*p<0.05; **p<0.01;***p<0.001 compared with IR control rats. One-way ANOVA followed by Tukeys post test.

Table 4: Effect of HAEES on SOD and Catalase and in heart tissue homogenate of isolated rat heart preparation.

Treatment	SOD	Catalase
Group-I (IR control)	1.605±0.042	2.673±0.06
Group-II (Vit-C 20 mg/kg, p.o)	4.36±0.17***	5.305±0.349***
Group-III (HAEES 200 mg/kg, p.o)	1.95±0.109	2.885±0.008
Group-IV (HAEES 400 mg/kg, p.o)	3.41±0.049***	3.63±0.085**
Group-V (HAEES 600 mg/kg, p.o.)	4.05±0.97***	5.138±0.034***

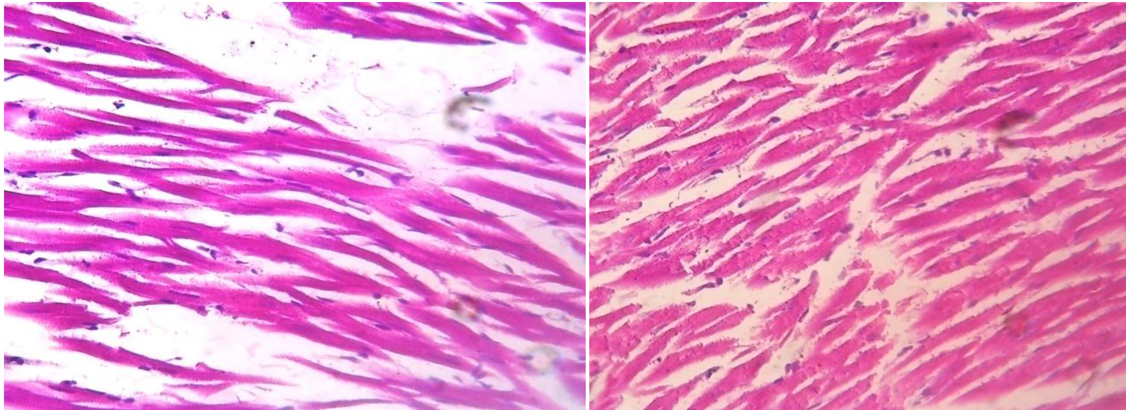
Each data represents the Mean± SEM (n=6). *p<0.05; **p<0.01; ***p<0.001 compared with IR control rats. One-way ANOVA followed by Tukeys post test.

Table 5: Effect of HAEES on Developed tension and Heart rate in Pre and Post Ischemic conditions in I/R induced myocardial damage.

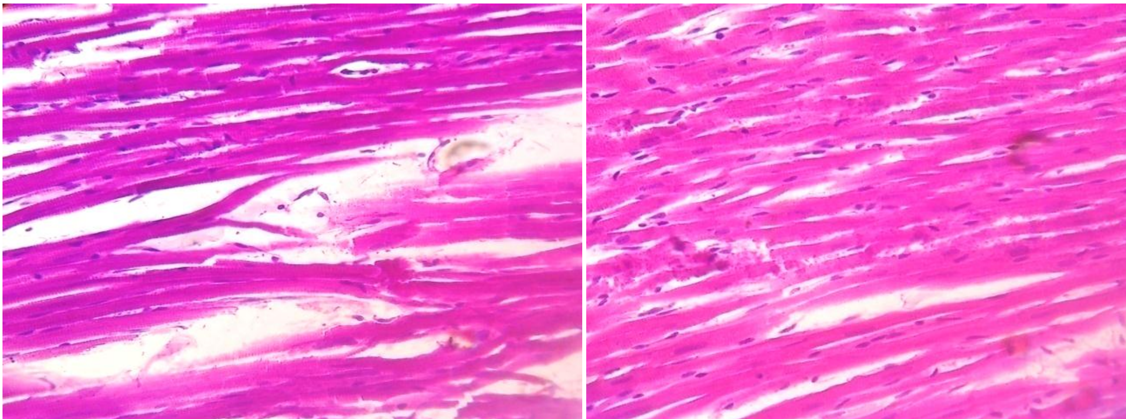
Pretreatment	Percentage recovery	
	Developed tension	Heart rate
Group-I (IR control)	66.89±3.39	61.97±3.99
Group-II (Vit-C 20 mg/kg, p.o.)	95.71±3.23***	93.20±1.37***
Group-III (HAEES 200 mg/kg, p.o.)	86.52±4.27**	63.95±4.07
Group-IV (HAEES 400 mg/kg, p.o.)	94.79±4.53***	83.64±2.75***
Group-V (HAEES 600 mg/kg, p.o.)	98.51±0.66***	89.8±1.64***

Each data represents the Mean± SEM (n=6).*p<0.05; **p<0.01;***p<0.001 compared with IR control rats. One-way ANOVA followed by Tukeys post test.

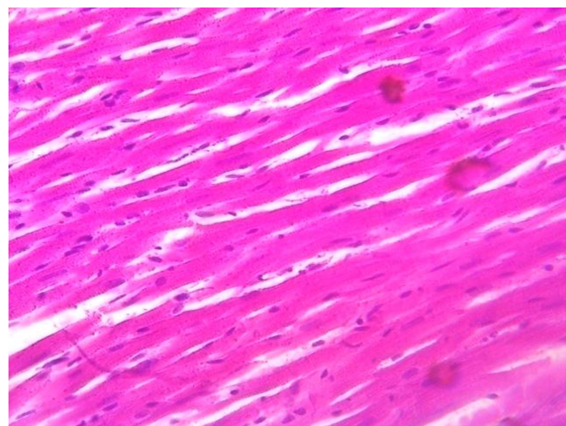
Figure 1: Histopathological study of heart in different group on Ischemic-reperfusion induced myocardial injury in isolated rat hearts.



Group-I: I/R control **Group-II: Vit-C 20 mg/kg, p.o.**



Group-III: HAEES 200 mg/kg, p.o. **Group-IV: HAEES 400 mg/kg, p.o.**



Group-V: HAEES 600 mg/kg, p.o.

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DISCUSSION

The DPPH method is independent of sample polarity for screening of many samples for radical scavenging activity, the radical scavenging activity increased markedly with increase in concentration. The IC_{50} value of extract for DPPH method was 140.23µg/ml. The superoxide, hydroxyl and nitric oxide radicals scavenging activities of the HAEES was increased as increase in the concentration, the IC_{50} values for superoxide, hydroxyl and nitric oxide radical scavenging activities are 203.41, 194.56 and 184.49 µg/ml respectively.

In this study, the Langendorff's heart perfusion model is used to simulate myocardial reperfusion injury during cardiac surgery. Ischemia induced by global no flow of perfusion leads to initiation of anaerobic respiration, which results in lactate accumulation. Further changes in biochemical alterations causes the increase in intracellular Na^+ and Ca^{2+} level that results in irreversible damage to myocardium [13,14].

Rapid reperfusion of the ischemic area is required to limit tissue damage and is necessary to reoxygenate and salvage remaining viable tissue. Conversely, reperfusion itself can cause additional damage; this phenomenon is termed lethal reperfusion-induced injury [15]. Such damage promotes further cell death, which contributes to an increase in irreversible tissue damage in the reperfused myocardium. Reperfusion of the ischemic myocardium causes an enhanced generation of reactive oxygenation species (ROS), which induces cellular damage. At the inner mitochondrial membrane a number of redox-reduction reactions occur along the electron transport chain to produce ATP [16].This form of respiration will result in the low level generation of ROS, such as superoxide (O_2-) and hydrogen peroxide (H_2O_2)[16]. Nevertheless, the cell is equipped with antioxidants, such as superoxide dismutase and catalase which can quickly convert the free radicals into H_2O [17]. However, during ischemia these antioxidants cease to function due to restrictions in the levels of ATP and the level of free radicals intensifies. Consequently, further changes occur to the mitochondria, such as additional swelling, membrane instability and release of Cytochrome *C* from the intramembrane space which contributes to mitochondrial membrane depolarisation. This makes the organelle unstable and initiates signalling for cell death [14]. The membrane damage further causes the leakage of intracellular contents and endogenous enzymes.

In the present study, increase in LDH and CK-MB activity in perfusate indicates more cardiac damage where, these are found to be elevated in control groups of hearts subjected to IR injury. The HAEES have shown significant reduction in LDH and CK-MB activity in perfusate compared to IR control, showing good cardioprotective effect. However, the resultant decrease in CK-MB, LDH, AST and ALT activities in heart homogenate indicates more cardiac damage, where maximum amount of enzymes are released into perfusate. In our study the groups pretreated with Vit-C and HAEES have shown more concentration of marker enzymes in heart tissue homogenate.

The recovery of cardiac contractile function correlates with the percent recovery in DT, heart rate, decrease in the release of cardiac enzymes LDH and CK-MB into the perfusate,

increase of LDH and CK-MB in heart homogenate, and prevention of tissue injury (infarction) which is consistent with amelioration of the myocardial injury [18].

Recent studies have reported that, the heart rate as a hemodynamic parameter in assessing cardiac injury. The heart rate is recorded before ischemia and after ischemia that is during reperfusion, at different time intervals. In the present study, IR control group shows prominent changes in HR, Vit-C and HAEES treated groups have not shown any drastic change in the heart rate during post-ischemic period, and are found to be showing cardioprotective effect.

It has been reported that, antioxidants showing good cardiac protection, may not show drastic change in post-ischemic heart rate (HR), whereas, in case of synthetic antioxidants, some may show a slight increase in post-ischemic HR compared to pre-ischemic HR, but they may not show drastic change in HR. Hence, the cardioprotective effect may not be assessed by recording change in post-ischemic HR, which lends support to our observation.

DT is the tension in grams developed during the contraction at the optimum resting tension of the heart and is a function of the work done by isolated heart. Increase in DT shows an improvement in cardiac contractions while a decrease in DT is the indication of injury to the cardiac musculature. DT is found to have been reduced in the IR control group during the reperfusion period following ischemia due to the changes occurring as a result of ischemia-reperfusion injury, while occurrence of ventricular arrhythmias are common. Lesser the recovery of DT, greater is the injury to the myocardium due to ischemia. However, in groups which are pretreated with Vt-C and HAEES shows significant recovery of DT and reduction in the incidence of ventricular arrhythmias showing protection from cardiac damage.

In addition, histopathological changes observed in IR induced injury were restored to the normal in HAEES pretreated groups. The changes like necrosis, separation of cardiac fibers and infiltration of inflammatory cells were not seen in these pretreated groups. Pretreatment with HAEES substantially decreased the interstitial cavity and kept the myocardial integrity, hence it is further confirmed the cardioprotective activity of *Elephantopus scaber* Linn.

CONCLUSION

The hydro alcoholic root extract of *Elepahntopus scaber* Linn. protects the myocardium against ischemia-reperfusion induced damage in rats. This effect might be due to augmentation of endogenous antioxidant enzyme synthesis.

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