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Effect of *Inula racemosa* root extract on cardiac function and oxidative stress against isoproterenol-induced myocardial infarction

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The cardioprotective potential of *Inula racemosa* root hydroalcoholic extract against isoproterenol-induced myocardial infarction was investigated in rats. The rats treated with isoproterenol (85 mg/kg, s.c.) exhibited myocardial infarction, as evidenced by significant (P<0.05) decrease in mean arterial pressure, heart rate, contractility, relaxation along with increased left ventricular end diastolic pressure, as well as decreased endogenous myocardial enzymatic and non-enzymatic antioxidants. Isoproterenol also significantly (P<0.05) induced lipid peroxidation and increased leakage of myocyte injury marker enzymes. Pretreatment with *I. racemosa* extract (50, 100 or 200 mg/kg per day, p.o.) for 21 consecutive days, followed by isoproterenol injections on days 19th and 20th significantly (P<0.05) improved cardiac function by increasing the heart rate, mean arterial pressure, contractility and relaxation along with decreasing left ventricular end diastolic pressure. Pretreatment with *I. racemosa* also significantly (P<0.05) restored the reduced form of glutathione and endogenous antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase from the heart, which were depleted after isoproterenol administration. In addition to restoration of antioxidants, *I. racemosa* significantly (P<0.05) inhibited lipid peroxidation and prevented the leakage of myocytes specific marker enzymes creatine phosphokinase-MB and lactate dehydrogenase from the heart. Thus, it is concluded that *I. racemosa* protects heart from isoproterenol-induced myocardial injury by reducing oxidative stress and modulating hemodynamic and ventricular functions of the heart. Present study findings demonstrate the cardioprotective effect of *I. racemosa* and support the pharmacological relevance of its use and cardioprotection mechanism in ischemic heart disease as well as substantiate its traditional claim.

Keywords: Antioxidant, Cardiac function, Cardioprotective potential, *Inula racemosa*, Isoproterenol, Oxidative stress, Pushkarmool, Ventricular function.

Myocardial infarction (MI), the common presentation of ischemic heart disease is on rise worldwide in both men and women and is a leading cause of death and disability[1]. It is an acute condition of the necrosis in the myocardium which occurs as a result of imbalance between coronary blood supply and myocardial demand. Although, modern drugs are effective in treating ischemic- reperfusion condition, but there is still a need to develop newer therapeutic agents[2]. Growing evidences suggest that medicinal plants render resistance to heart from ischemic injury and protect from the deteriorated hemodynamic and ventricular function, a common accompaniment of ischemic myocardium[3-6]. The identification of such medicinal plants may be useful as a phytotherapeutic agent or adjuvant to prevent or limit myocardial ischemic injury and associated functional impairment. Therefore, it is desirable to explore such agents/therapies that prevent or attenuate ischemic injury in rat model of isoproterenol (ISO)-induced myocardial necrosis.

Isoproterenol, a synthetic catecholamine and a potent β-adrenergic agonist produces myocardial necrosis in rats comparable to those taking place in human AMI[7,8]. There are many mechanisms of ISO-induced myocardial infarction such as activation of β_1 and β_2-adrenoceptors, which leads to positive inotropic and chronotropic effects, and production of free radicals thru auto-oxidation of catecholamines[7-9]. Catecholamines play an important role in myocardial

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Abbreviations: AMI, acute myocardial infarction; CAT, catalase; CK-MB, creatine phosphokinase-MB; GPx, glutathione peroxidise; GSH, reduced glutathione; HR, heart rate; IR, *Inula racemosa*; ISO, isoproterenol; LDH, lactate dehydrogenase; LVEDP, left ventricular end diastolic pressure; (+)LVdP/dt, peak positive pressure development; (-)LVdP/dt, peak negative pressure decline; MAP, mean arterial pressure; MI, myocardial infarction; ROS, reactive oxygen species; SOD, superoxide dismutase; TLC, thin layer chromatography; TBARS, thiobarbituric acid reactive substances.

metabolism and function and their excess leads to cellular damage and cardiac dysfunction. A catecholamine (ISO-induced myocardial infarction) model of myocardial injury offers to explore into the possible mechanism of cardioprotection measuring hemodynamic and ventricular function and markers of oxidative stress, as well as myocyte injury marker enzymes[4].

In recent years, the interest in medicinal plants and phytochemicals has increased for their therapeutic properties in human diseases, including cardiovascular[10-12]. *Inula racemosa* Hook, commonly known as Pushkarmool (Hindi) is one of the reputed medicinal plant used in traditional system of medicine (Ayurveda) for its potential benefits in cardio-respiratory and cardiovascular diseases especially, angina pectoris[13-15]. It is a common ingredient of polyherbal formulations indicated for cardiovascular diseases[16-18]. Several experimental and clinical studies have demonstrated the potential of *I. racemosa* in cardiovascular diseases including hypertension, coronary heart disease, atherosclerosis, thrombosis and myocardial infarction[14-21]. Recently, muskone, a traditional medicinal preparation containing slender *Inula* root has been demonstrated to relieve ischemic pain and exhibit cardioprotective effect[22]. Sesquiterpenes (alantolactone, isoalantolactone and alloalantolactone) are the major constituents which accounts for bioactivity of this herb. It also contains several flavonol glycosides, terpenes, alantoides, germacranolides and eudesmenes etc[23]. The cardioprotective effects of *I. racemoa* have been attributed to its antioxidant activity and constituents resembling to beta-adrenergic blockers[24,25].

In view of β-blocking[24,25] and antioxidant[20] activities of *I. racemoa*, it is desirable to evaluate the pharmacological potential of *I. racemoa* in ISO-induced myocardial infarction and elucidate the mechanisms of cardioprotection. Thus, in the present study, we have evaluated the cardioprotective effects of *I. racemoa* pretreatment against ISO-induced myocardial infarction and elucidated the possible underlying mechanisms by recording hemodynamic parameters, mean arterial pressure (MAP), heart rate (HR) and ventricular function parameters, contractility; (+)LVdP/dt, relaxation; (-)LVdP/dt) and preload; left ventricular end diastolic pressure (LVEDP). To corroborate the functional changes with biochemical markers of oxidative stress, we also measured lipid peroxidation product, thiobarbituric acid reactive substances (TBARS), endogenous antioxidant reduced glutathione (GSH), antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and myocardial specific injury markers creatine phosphokinase-MB (CK-MB) and lactate dehydrogenase (LDH) in the heart.

Materials and Methods

Plant material and composition of extract

The roots of *Inula racemosa* were obtained as a generous gift from Dabur Research Foundation, Ghaziabad (U.P). Authenticated dried roots were chopped into small pieces and a fine powder was made by grinding the crushed material in the blender at Bioactive Natural Product Laboratory, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, New Delhi. Different extractive values of root powder was determined separately by maceration of drug for 48 h at room temperature with occasional shaking in like n-hexane (0.83% w/w), toluene (1.21% w/w), benzene (2.24% w/w), chloroform (4.87% w/w), ethyl acetate (8.40% w/w), methanol (10.97% w/w) and hydroalcoholic i.e. mixture of methanol and in the ratio of 70:30 (13.72% w/w).

The hydroalcoholic extract was selected for the study, as it contained maximum extractive value extracting maximum number of bioactive compounds. The extract was subjected to phytochemical screening for presence/absence of different secondary metabolites as per the standard protocol, which showed presence of steroids, flavonoids, phenolics, amino acids, and alkaloids. Thin layer chromatography (TLC) finger printing study of hydroalcoholic extract was also carried out in solvent system benzene: ethyl acetate (9:1). Five microlitre (10 mg/ml) of extract was applied on TLC silica gel $60F_{254}$ and developed in solvent system using twin trough glass chamber. It was developed in above-mentioned solvent system and sprayed with vanillin-sulphuric acid reagent. The developed and sprayed TLC plate was then heated at 105°C for 10 min. Chromatogram showed presence of nine violet coloured spots at different Rf values i.e. Rf 0.11, 0.23, 0.33, 0.38, 0.48, 0.52, 0.63, 0.72 and 0.95.

Chemicals

All chemicals of analytical grade were used for biochemical estimations. Isoproterenol hemisulphate

(Sigma Chemicals Co., St. Louis, MO, USA) was dissolved in normal saline (0.9% NaCl) and administered subcutaneously on two consecutive days, at the interval of 24 h for induction of myocardial infarction[4].

Experimental animals and experimental design

The study protocol was reviewed and approved by the Institutional Animal Ethics Committee of All India Institute of Medical Sciences, New Delhi and conducted in accordance with the Indian National Science Academy Guidelines for the Use and Care of Experimental Animals. Male Wistar albino rats (10-12 weeks old, weighing 150-200 g) were obtained from the Central Animal Facility of All India Institute of Medical Sciences, New Delhi, India. The animals were housed under standard laboratory conditions and fed commercial pellet diet and tap water *ad libitum*. They were randomly divided into five experimental groups, each containing 10 rats. After a week of acclimatization, rats were administered saline or *I. racemosa* extract using intragastric tube. Group I or vehicle control group were administered normal saline orally once daily for 21 days and on day 19 and 20, 0.5 ml of normal saline was injected subcutaneously at the interval of 24 h. Group II or ISO control group were administered normal saline orally once daily for 21 days and on days 19 and 20, ISO (85 mg/kg) was injected subcutaneously at an interval of 24 h. Groups III, IV and V were designated as *I. racemosa* pretreated group and orally received the 50, 100 or 200 mg/kg daily, respectively for 21 days. In addition, on days 19 and 20, rats of *I. racemosa*-treated group were administered subcutaneous injection of ISO (85 mg/kg) at an interval of 24 h.

Measurement of hemodynamic and left ventricular dynamics

Briefly, on day 21, rat was anesthetized with pentobarbitone sodium (60 mg/kg; i.p.) and atropine (0.1 mg/kg) was co-administered to reduce tracheobronchial secretions and maintain heart rate under surgical conditions. The body temperature was maintained with the help of a thermal lamp at dissection table during surgery. The neck was opened to perform tracheostomy and ventilated with room air from a positive pressure ventilator (Inco, Ambala, India) using compressed air at a rate of 90-strokes/min and a tidal volume of 10 ml/kg. Ventilator setting and oxygen were adjusted to maintain the arterial blood gas parameters within the physiological range. Left jugular vein was cannulated with polyethylene tube for continuous infusion of normal saline. Right carotid artery was cannulated with a heparinised saline filled polyethylene cannula and connected to CARDIOSYSCO-101 (Experimentria, Hungary) using a pressure transducer for measurement of mean arterial pressure (MAP) and heart rate (HR).

Left thoracotomy was performed at the fourth-fifth intercostal space on left side and after incising pericardium, the heart was exteriorized by gentle pressure on ribs. A sterile metal cannula (1.5 mm bore) was introduced into cavity of left ventricle from posterior apical region of heart for measuring left ventricular dynamics such as peak positive pressure development [(+)LVdP/dt, a marker of contractility], peak negative pressure decline [(-)LVdP/dt, a marker of relaxation] and LVEDP, a surrogate marker of preload. The cannula was connected to a pressure transducer (Gould Statham P23ID, USA) through a pressure-recording catheter on Polygraph (Grass 7D, USA). After stabilization of 10 min, tracings were recorded on polygraph paper, following baseline measurements at different sensitivity and speed. Thoracic cavity was covered with saline-soaked gauze after surgery to prevent heart from drying.

Evaluation of myocardial injury

After recording hemodynamic and ventricular function, rats were sacrificed with an overdose of anaesthesia using sodium pentobarbitone (100 mg/kg, i.v.). The heart was excised, rinsed with 0.9% chilled phosphate buffer saline (pH 7.4, 50 mM) and minced to prepare a 10% homogenate in phosphate buffer (pH 7.4, 50 mM). An aliquot of 0.5 ml heart homogenate was used for TBARS[26] and GSH[27] estimation. Rest of the homogenate was centrifuged at 4,930 g for 15 min and the supernatant obtained was used for estimation of antioxidant enzymes SOD[28], CAT[29], and GPx[30] and the myocyte injury markers CK-MB[31] and LDH[32]. Protein was estimated by the method of Lowry *et al*.[33].

Statistical analysis

Data were analyzed using one-way analysis of variance, followed by Bonferroni multiple range test. *P* value of less than 0.05 was considered significant.

Results

Effect on arterial pressure and heart rate

ISO produced a significant ($P<0.05$) fall in MAP and HR in comparison to vehicle-treated group

(Table 1). Pretreatment with *I. racemosa* (100 and 200 mg/kg) produced significant (P<0.05) restoration of MAP and HR in comparison to ISO control group. However, *I. racemosa* at 50 mg/kg failed to improve MAP and HR significantly (P<0.05) compared to vehicle-treated group.

Effect on left ventricular performance

A significant (P<0.05) increase in LVEDP was observed in ISO control group compared to vehicle-treated group (Fig. 1a). Pretreatment with *I. racemosa* (100 and 200 mg/kg) produced a significant (P<0.05) reduction of LVEDP, as compared to ISO control group. However, *I. racemosa* at 50 mg/kg did not significantly decreased LVEDP compared to vehicle-treated group.

In ISO control group, a significant (P<0.05) reduction in left ventricular peak positive and negative [(+)LVdP/dt and (-)LVdP/dt] pressure was observed compared to vehicle-treated group (Fig. 1b and 1c). Pretreatment with *I. racemosa* (100 and 200 mg/kg) significantly (P<0.05) prevented decline of (+)LVdP/dt, as compared to ISO control group. Pretreatment with I. *racemosa* at all doses (50, 100 and 200 mg/kg) significantly (P<0.05) prevented decline of (-)LVdP/dt in comparison with ISO control group.

Effect on myocytes injury marker enzymes and antioxidant enzymes in heart

Administration of ISO produced a significant (P<0.05) decrease in myocardial enzymes CK-MB and LDH by increasing the depletion of these enzymes from heart, as compared to vehicle-treated rats (Table 2). Pretreatment with *I. racemosa* at all doses significantly (P<0.05) prevented the depletion of myocardial enzymes, as evidenced by restoration of CK-MB and LDH in heart, following ISO administration as compared to ISO control group. However, *I. racemosa* at 50 mg/kg failed to significantly (P<0.05) prevent depletion of CK-MB isoenzyme.

Similar to myocyte injury marker enzymes, a significant (P<0.05) decrease in enzymatic antioxidants SOD, CAT, and GPx and non-enzymatic antioxidant GSH was observed in ISO control rats compared to vehicle-treated rats (Table 2). A significant (P<0.05) increase in endogenous antioxidants; SOD, CAT, GPx and GSH was observed in heart of rats pretreated with *I. racemosa* at all doses, except 50 mg/kg which failed to significantly (P<0.05) prevent depletion of SOD, CAT and GSH from heart, as compared to ISO control group.

Table 1—Effect of *Inula racemosa* (IR) on mean arterial pressure and heart rate

[Values are mean ± SD of 6 rats in each group]

Experimental groups	Mean arterial pressure (mm Hg)	Heart rate (beats/min)
Group I (Vehicle control; saline)	140 ± 17	369 ± 32
Group II (ISO control; saline + ISO)	104 ± 11*	220 ± 25*
Group III (IR 50 mg/kg + ISO)	111 ± 12	254 ± 26
Group IV (IR 100 mg/kg + ISO)	130 ± 14#	336 ± 28#
Group V (IR 200 mg/kg + ISO)	137 ± 10#	358 ± 36#

*P<0.05, when compared to vehicle control, #P<0.05, when compared to ISO control

Table 2—Effect of *Inula racemosa* (IR) on myocyte injury marker enzymes; CK-MB and LDH and on antioxidants in myocardium

[Values are mean ± SD of 6 rats in each group]

Experimental groups	CK-MB (IU/mg protein)	LDH (IU/mg protein)	SOD) (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)	GSH (µmol/g tissue)
Group I (Vehicle control; saline)	176.60 ± 22.45	230.50 ± 26.37	8.25 ± 2.12	22.73 ± 3.14	0.91 ± 0.32	2.93 ± 0.88
Group II (ISO control; saline + ISO)	71.58 ± 26.43*	97.56 ± 25.25*	3.76 ± 1.10*	12.80 ± 2.66*	0.31 ± 0.21*	1.10 ± 0.95*
Group III (IR 50 mg/kg + ISO)	80.44 ± 31.26	170.62 ± 28.45#	4.28 ± 1.83	13.59 ± 5.32	0.69 ± 0.38#	1.18 ± 0.68
Group IV (IR 100 mg/kg + ISO)	138.35 ± 26.34#	192.74 ± 17.32#	6.96 ± 2.05#	18.50 ± 4.72#	0.78 ± 0.20#	1.97 ± 0.75#
Group V (IR 200 mg/kg + ISO)	158.92 ± 41.18#	216.55 ± 13.50#	7.82 ± 2.12#	20.15 ± 4.58#	0.84 ± 0.26#	1.90 ± 0.69#

*P<0.05, when compared to vehicle control, #P<0.05, when compared to ISO control

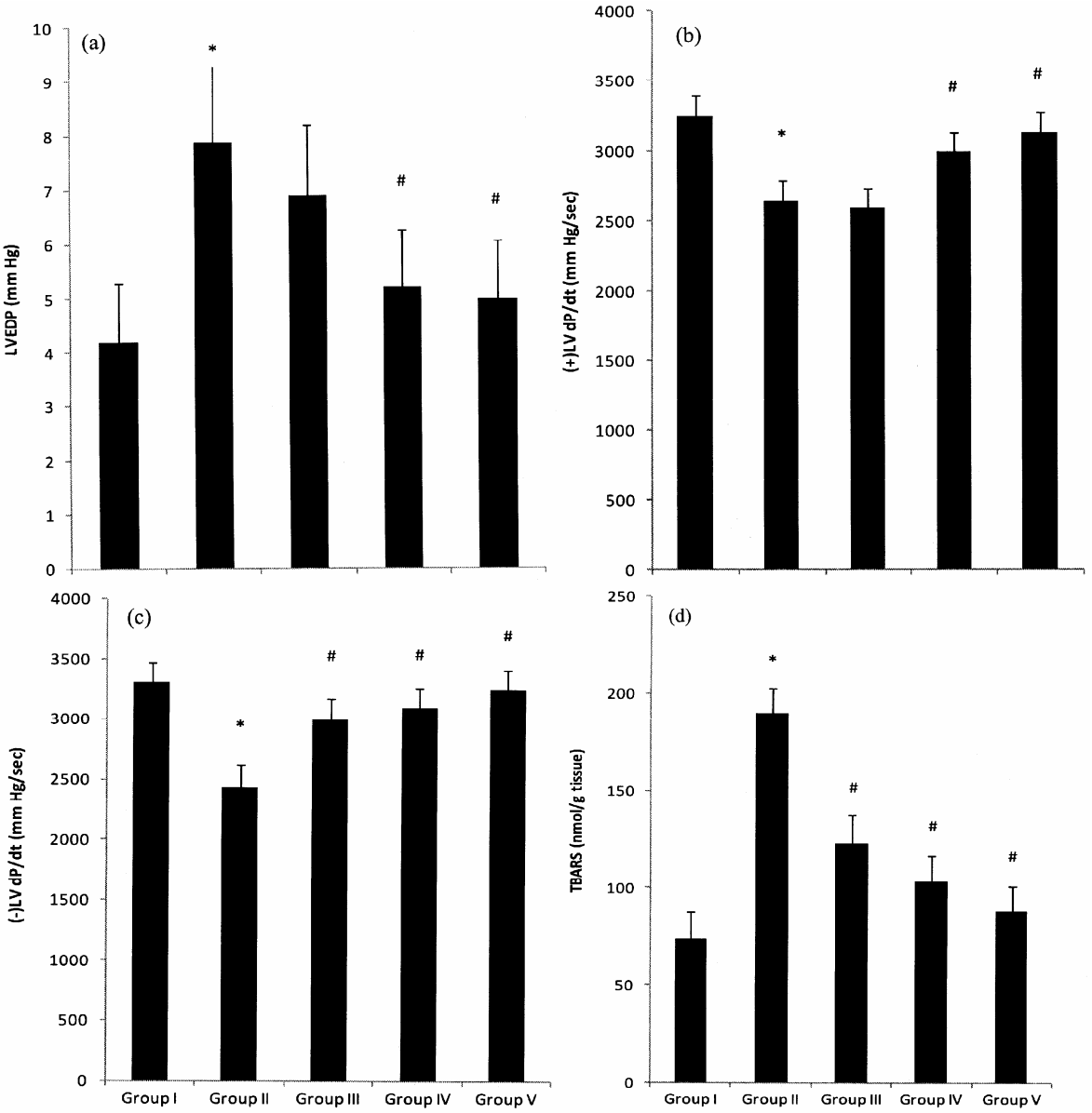

Fig. 1—Effect of *I. racemosa* (IR) on (a) left ventricular end diastolic pressure (LVEDP) (b) left ventricular peak positive pressure development {(+)LVdP/dt} (c) left ventricular peak positive pressure development {(-)LVdP/dt} and (d) lipid peroxidation product (TBARS) [The values are mean ± SD of 6 rats in each group. $^{*}P<0.05$, when compared to vehicle control, $^{\#}P<0.05$, when compared to ISO control]

Effect on lipid peroxidation in heart

ISO administration produced a significant ($P<0.05$) increase in lipid peroxidation product TBARS in heart compared to vehicle-treated (Fig. 1d). *I. racemosa* at all doses (50, 100 and 200 mg/kg) significantly ($P<0.05$) decreased TBARS, as compared to ISO control group.

Discussion

Present study demonstrates the cardioprotective effect of *I. racemosa* against ISO-induced myocardial infarction, as evidenced by attenuation of the hemodynamic impairment and contractile dysfunction along with improved antioxidant defence as well as inhibition of lipid peroxidation and reduced leakage of myocardial injury markers.

ISO has been reported to cause severe stress in the myocardium resulting in infarct like necrosis of the heart muscles[4,5]. Some of the mechanisms proposed to explain ISO-induced damage of cardiomyocytes include hypoxia due to myocardial hyperactivity and

coronary hypotension, Ca^{+2} overload, depletion of energy reserves and excessive production of free radicals, resulting from oxidative metabolism of catecholamines[7,8]. ISO-induced ischemic injury has been shown to produce contractile impairment and hemodynamic alteration consequent to the ISO-induced free radicals generated oxidative stress in myocardium[5,9]. Similar observations were also recorded in this study, when ISO (85 mg/kg) was administered subcutaneously for two days to rats. Following ISO administration, a significant decrease in MAP indicated an altered sympathetic reflex. This decrease in MAP might be a compensatory mechanism of the myocardium to increase perfusion in order to meet the increased myocardial energy demand. However, *I. racemosa* pre-treatment, following ISO administration was observed to increase the MAP which was translated into the recovery of cardiac function from ischemic conditions.

A significant decrease in heart rate, following ISO administration indicated the injured state of myocardium, in consonance to previous studies[4-6]. Pretreatment with *I. racemosa* significantly increased the heart rate, a determinant of myocardial oxygen consumption. In the face of restored MAP, an increase in heart rate indicated the conservation of cardiac efficiency and performance under ISO-induced myocardial injury. The favourable modulation of heart rate in present study supports previous studies[24,25], indicating the presence of chemical constituents similar to β-blockers. Restored mean arterial pressure in conjunction with heart rate might increased blood flow through the subendocardial region of the ventricular muscle, which bears the maximum brunt of ischemic insult, may be attributed to cardioprotective activity of *I. racemosa*.

Furthermore, *I. racemosa* pretreatment also improved left ventricular contractile function by restoring the inotropic [(+)LVdP/dt, a marker of myocardial contraction)] and lusitropic [(-)LVdP/dt, a marker of myocardial relaxation] states of the heart and attenuated ISO-induced increase in LVEDP, a marker of preload. Increased LVEDP exerts an outward force on ventricular wall that reduces blood flow to the subendocardial region, following ISO administration. Pretreatment with *I. racemosa* might have improved the perfusion to subendocardium and reduced the myocardial damage. Under ischemic conditions, the subendocardial region of heart is most vulnerable to ischemic necrosis because of disproportionate reduction in blood flow to the region, which is subjected to greatest extra-vascular compression during systole. Thus, in present study, improved hemodynamic and ventricular functions demonstrated the cardioprotective effect of *I. racemosa* against functional impairment in ischemic conditions of myocardium.

As described earlier, ISO-induced oxidative stress is characterized by decreased activity or depletion of antioxidant enzymes and increased lipid peroxidation, which leads cellular damage and leakage of myocyte enzymes[3-5]. In present study, increased myocardial TBARS content, following ISO administration indicated free radical-mediated membrane damage in agreement with previous studies[34]. A significant reduction in TBARS with *I. racemosa* pretreatment by reducing the formation of lipid peroxides from fatty acids is suggestive of its cardioprotective activity concurrent to previous studies[16,20]. Subsequent to ISO administration, a significant decrease in endogenous enzymatic antioxidants such as SOD, CAT, $GP_{X,}$ and non-enzymatic antioxidant GSH in heart indicated the occurrence of oxidative stress in accordance to previous studies[4-6]. However, pretreatment with *I. racemosa* significantly increased the activities of antioxidant enzymes in consonance to previous studies[14-20]. Furthermore, *I. racemosa* pretreatment also prevented depletion of GSH, an important soluble antioxidant, which acts both to replenish GPx and as a direct scavenger of ROS.

Besides endogenous physiological antioxidants, alterations in CK-MB isoenzyme and LDH have been considered as an important diagnostic marker of myocardial injury[35]. In present study, a significant fall in myocardial enzymes CK-MB isoenzyme and LDH indicated myocardial injury, following ISO administration. Decrease in CK-MB isoenzyme and LDH was consistent with the fact that CK-MB and LDH being the myocyte-specific enzymes leak out from the tissue to plasma, following myocardial damage concomitant to lipid peroxidation. The observation that *I. racemosa* pretreatment significantly prevented depletion of CK-MB and LDH enzymes demonstrated its myocardial salvaging effects. The recovery of compromised cellular status with *I. racemosa* was accompanied by decreased myocardial oxidative injury or improved redox state of the cell. In preliminary studies, *I. racemosa* has

been demonstrated to protect heart against ischemic injury ascribed to its antioxidant property and its chemical constituents resembling β-adrenergic blocker activity[15,20,25]. Present study findings also demonstrated the cardioprotective activity of *I. racemosa* against a β-adrenergic agonist, ISO by mitigating oxidative stress and β-adrenergic blocking property in concurrence with the previous reports[14-16,20]. The β-adrenergic blocking activity might have fostered cardiac performance and the antioxidant activity helped to maintain the redox state of cell to sustain cellular functions in oxidative conditions.

In conclusion, present study demonstrated that and *I. racemosa* prevented the depletion of myocardial antioxidants and restored cardiac function, following isoproterenol-induced myocardial infarction. Based on findings, it is suggested that *I. racemosa* may serve as an adjunctive therapy in patients of ischemic heart disease or as a preventive agent in delaying the progression of ischemic heart disease.

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