Cardioprotection by *Inula racemosa* Hook in experimental model of myocardial ischemic reperfusion injury

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To evaluate the cardioprotective potential of *Inula racemosa* in myocardial ischemic-reperfusion injury, Wistar male albino rats were randomly divided into four groups. The group I and II animals were administered saline orally {(sham, ischemia-reperfusion (I-R) control group)} and animals of group III and group IV received *I. racemosa* extract (100 mg/kg) for 30 days. On the 30th day, animals of I-R control and *I. racemosa* treated groups were underwent 45 min of ligation of left anterior descending coronary artery and were thereafter re-perfused for 60 min. In the I-R control group, a significant decrease of mean arterial pressure (MAP), heart rate (HR), contractility, (+)LVdP/dt and relaxation, (-)LVdP/dt and an increase of left ventricular end diastolic pressure (LVEDP) were observed. Subsequent to haemodynamic impairment and left ventricular contractile dysfunction, a significant decline was observed in endogenous myocardial antioxidants; superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and reduced glutathione (GSH). Increased lipid peroxidation characterized by malonaldialdehyde (MDA) formation along with depletion of cardiomyocytes specific enzymes, creatine phosphokinase-MB (CK-MB) isoenzyme and lactate dehydrogenase (LDH) in I-R control group compared to sham group revealed I-R injury of heart. However, treatment with *I. racemosa* significantly restored the myocardial antioxidant status evidenced by increased SOD, CAT, GPx and GSH and prevented leakage of cardio-specific enzymes; CK-MB and LDH and favorably modulated the altered MAP, HR, (+)LVdP/dt, (-)LVdP/dt and LVEDP as compared to I-R control. Furthermore, I-R induced lipid peroxidation was significantly inhibited by *I. racemosa* treatment. These beneficial cardioprotective effects translated into significant improvement in cardiac function. In conclusion, our study has demonstrated that the cardioprotective effect of *I. racemosa* likely resulted to improved antioxidant status, haemodynamic and left ventricular contractile function subsequent to suppression of oxidative stress.

**Keywords**: Cardioprotection, *Inula racemosa*, Ischemia-reperfusion, Myocardial infarction

Myocardial ischemia (MI) due to sudden occlusion of a major coronary artery, leads to a complex series of cellular events that result in myocytes necrosis and functional impairment of heart1,2. Myocardial ischemia-reperfusion (M I-R) represents a clinically relevant problem associated with thrombolysis, angioplasty and coronary bypass surgery3. Myocardial damage induced by ischemia-reperfusion of heart has been proposed to be caused, at least in part, by generation of reactive oxygen species (ROS)2,3. Since experimental studies has implicated ROS in pathophysiology of ischemia and reperfusion, multitude of ROS generated during the oxidative stress associated with ischemia and reperfusion can damage major cellular component, including membrane lipids, protein, carbohydrate and DNA. Pathophysiological consequences of such uncontrolled injury are widespread tissue damage and associated contractile dysfunction, arrhythmias, depletion of endogenous antioxidant network, enhanced lipid peroxidation2,3. In addition, oxidative stress may also depress sarcolemmal Ca²⁺ transport and result in to the development of intracellular Ca²⁺ overload and heart dysfunction4. Antioxidants of natural or synthetic origin have been shown to significantly ameliorate myocardial injury and hence improve myocardial function5–8. In the current decade, much interest has been focused on the herbs which are traditionally acclaimed to possess therapeutic effect and as a source of novel therapeutic agents for long-term prevention of cardiovascular diseases9. Hence, a search of novel pharmacotherapeutic agents from medicinal plants for ischemic heart diseases is in progress. This is reflected by a large number of medicinal plants for which cardioprotective potential has been reported in a variety of animal models6–8.
Inula racemosa Hook, commonly known as Pushkarmoola, has therapeutic benefits in cardiorespiratory and cardiovascular diseases. In Ayurvedic, Chinese and Mediterranean traditional system of medicine, Inula species are used in anginal pain. The herbal formulation containing I. racemosa has shown protective effects in animal models of myocardial necrosis. Ability of I. racemosa, as β-blockers and antioxidant has generated interest to explore it as a cardioprotective agent. However, its cardioprotective potential is not known in in vivo animal model of myocardial ischemic reperfusion injury which closely resembles to human myocardial ischemic-reperfusion injury, a clinically relevant condition. Therefore, the present study was designed to evaluate the cardioprotective activity of I. racemosa using biochemical, haemodynamic and histopathological determinants in in vivo experimental ischemia and reperfusion model of myocardial infarction in rats.

Materials and Methods

Chemicals— All the chemicals used in the present study were of analytical grade and obtained from Sigma (St. Louis, MO, USA), Merck (Mumbai, India), Ranbaxy (New Delhi, India), Sisco (Mumbai, India) and Qualigenes (Mumbai, India).

Plant material, extraction and phytochemical analysis— Authentic dried roots of Inula racemosa Hook were obtained from Dabur Research Foundation, Ghaziabad, UP, India as a generous gift. The air dried roots (500 g) were chopped into small pieces and milled into fine powder mechanically. The cold extraction was done using methanol and water (50:50) at room temperature for 48 h following repeated cycling of the extract. The extract thus obtained was filtered and evaporated under reduced pressure to obtain a viscous mass. The extract was subjected to screening for determination of phytoconstituents. LD₅₀ of Inula racemosa extract in rats has been found to be 2100 ± 60 mg/kg body wt.

Experimental animals— A total of forty Wistar male albino mature rats, weighing 150 to 200 g, 10 to 12 weeks old were used in the study. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee and conforms to the Indian National Science Academy Guidelines for the Use and Care of Experimental Animals in research. Animals were obtained from the Central Animal House facility of All India Institute of Medical Sciences, New Delhi, India. The rats were housed in polyacrylic cages (38×23×10 cm) with not more than four animals per cage. They were kept in standard laboratory conditions under 14/10 h natural light and dark cycles, RH 55 ± 10%, and at 25° ± 2°C. All experiments were performed between 0900 and 1600 hr. The animals were allowed free access to standard commercial pellet diet (Ashirwad Industries Limited; Chandigarh) and tap water ad libitum. The commercial pellet diet contains protein (24%), fat (5%), fiber (4%), carbohydrate (55%), calcium (0.6%), phosphorous (0.3%), moisture (10%) and ash (9%).

Experimental protocol— The animals were allowed to acclimatize for one week before the experiments and randomized into three main experimental groups comprising six animals each. Haemodynamically unstable rats were excluded from the study. The animals of group I designated as sham control were administered normal saline (0.9%) once daily for 30 days and then sacrificed on day 30. Rats underwent the entire surgical procedure except the left anterior descending (LAD) coronary artery occlusion. The animals of group II were administered normal saline (0.9%) once daily for 30 days and in addition, underwent LAD coronary artery ligation for 45 min followed by 60 min of reperfusion. The animals of group III were administered hydroalcoholic extract of Inula racemosa (100 mg/day) dissolved in 0.9% of normal saline and administered orally once daily for 30 days. On day 30, rats underwent the entire surgical procedure except LAD coronary artery occlusion. The animals of group IV were administered hydroalcoholic extract of Inula racemosa (100 mg/day) dissolved in 0.9% of normal saline and administered orally once daily for 30 days. On day 30, animals of all groups underwent 45 min LAD coronary artery ligation and 60 min of reperfusion and haemodynamic parameters were recorded throughout ischemia and reperfusion period. After completion of reperfusion, the animals were sacrificed with an overdose of anesthetic agent; sodium pentobarbitone (100 mg/kg, iv). Heart was excised and processed for the biochemical estimations. The dose of Inula racemosa (100 mg/kg) used in present study was selected on the basis of a pilot study on isoproterenol model of myocardial necrosis. The doses screened were 50, 100 and 200 mg/kg/day and on the basis of biochemical, haemodynamic and histopathological study. Inula racemosa extract (100 mg/kg) exhibited maximum...
cardioprotective effects therefore this dose was selected for further evaluation in the coronary artery ligated ischemia and reperfusion model of myocardial infarction.

Induction of myocardial ischemia-reperfusion injury—All the animals were anesthetized intraperitoneally with pentobarbitone sodium (60 mg/kg) and supplemental dose of 4 mg/kg (iv) of pentobarbitone sodium was used as and when required. Atropine was administered subcutaneously once at a dose of 0.1 mg/kg before the start of surgical procedure to keep the heart rate elevated especially during the surgery protocol and to reduces broncho-tracheal secretions. The body temperature was monitored and maintained at 37°C throughout the experimental protocol. Neck was opened and tracheostomy was performed and the animals were ventilated with room air from a positive pressure ventilator (Inco, India) using compressed air at a rate of 90-strokes/min and a tidal volume of 10 ml/kg. The left jugular vein was cannulated with polyethylene tube for administration of supplemental anesthetic and saline (0.9%) infusion. The right carotid artery was cannulated for the measurement of haemodynamic variables; mean arterial blood pressure (MAP) and heart rate (HR). A left thoracotomy was performed at the fifth intercostal space and heart was exposed. A sterile metal cannula (1.5 mm bore) was introduced into the cavity of the left ventricle from the 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 development, (-)LVdP/dt, a marker of relaxation; and left ventricular end diastolic pressure (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). Anatomy of LAD coronary artery was examined and verified visually and then ligated 4-5 mm from its origin and ends of this ligature were passed through a polyethylene tube to form a snare. After stabilization for 10 min, the tracings were recorded on polygraph paper following baseline measurements at different standardized sensitivity and speed. The thoracic cavity was covered with saline-soaked gauze after the surgery to prevent the heart from drying. After completion of surgical procedure, heart was returned to its normal position in thorax. Electrocardiographic leads were attached to subcutaneous electrodes to monitor limb lead II and electrocardiogram (ECG) was recorded at various time intervals throughout the experimental duration on polygraph. The animals were then allowed to stabilize for 10 min before LAD ligation. Regional myocardial ischemia was induced by one stage occlusion of LAD by pressing polyethylene tube against the ventricular wall. This was designated time point 0. The animals then underwent 45 min of persistent ischemia, confirmed by the appearance of epicardial cyanosis and ST-segment elevation. Baseline haemodynamic parameters were measured before LAD occlusion and continued according to the experimental protocol throughout ischemia and reperfusion period. Thereafter, myocardium was reperfused by releasing snare gently for a period of 60 min. Successful reperfusion was confirmed by visualization of arterial blood flow through artery, disappearance of arterial cyanosis and rapid resolution of ST-segment changes. Brief episodes of ventricular arrhythmias frequently occurred within the initial 10 min of occlusion and first 5 min of reperfusion in few animals. At the end of the experiment, animals were sacrificed by an overdose of anesthesia and heart was excised for biochemical studies. Heart was washed with chilled phosphate buffer saline (pH 7.4) and rapidly snaps frozen in liquid nitrogen for biochemical analysis.

Preparation of tissue homogenate—Heart was brought back to room temperature weighed, minced and a 10% homogenate was prepared in 50 mM phosphate buffer (pH 7.4), and an aliquot of 0.5 ml was used for estimation of reduced glutathione (GSH)<sup>16</sup> and malonaldehyde (MDA)<sup>17</sup>. Rest of the homogenate was centrifuged at 7000 rpm for 15 min and the supernatant was used for estimation of protein<sup>18</sup> using BSA as a standard, myocytes injury marker enzymes, creatine phosphokinase-MB isoenzyme (CK-MB)<sup>19</sup>, lactate dehydrogenase (LDH)<sup>20</sup> and endogenous myocardial antioxidant enzymes, super oxide dismutase (SOD)<sup>21</sup>, catalase (CAT)<sup>22</sup> and glutathione peroxidase (GPx)<sup>23</sup>. One unit of SOD is defined as the amount of enzyme required to produce 50% inhibition of epinephrine auto-oxidation. One unit of CAT activity represents one μmol of H<sub>2</sub>O<sub>2</sub> decomposed per min at 25°C. One unit of GPx enzyme activity has been defined as 1 nmol of NADPH utilized per min at 37°C. SOD, CAT and GPx activities have been expressed as units/mg protein as compared to the standard. One unit of CK-MB isoenzyme has been defined as the
amount of enzyme that transfer 1 µmol of phosphate from phosphocreatine to ADP per min at pH 7.4, on 30°C. One unit of LDH is defined as the amount of enzyme required to reduce 1 µmol of pyruvate to D-lactate per min at pH 7, on 25°C.

Statistical analysis—Descriptive statistics such as mean and standard deviation were calculated for each variable in the experimental groups. The data were compared using One-way ANOVA with Bonferroni Multiple comparison test and differences were considered significant at $P < 0.05$.

Results

Phytoconstituents identified in the extract were alantolactone and isoalantolactone. The mortality rate in surgical protocol for recording haemodynamic parameter was 5% due to bleeding or in-coordination during anesthesia, surgery or cannulation. *Inula racemosa* administered per se did not show any significant change in haemodynamic and biochemical parameters as compared to sham group.

**Effect of *I. racemosa* on haemodynamic variables**—In the I-R control group, a continuous and significant fall in MAP was observed after coronary artery ligation and throughout the reperfusion period compared to sham group (Fig. 1a). However, *I. racemosa* significantly restored MAP throughout ischemia and reperfusion at same time points of the experimental duration. Similarly, in the control I-R group, HR rate was significantly depressed throughout ischemia. Following reperfusion, an improvement in HR was observed, but remained significantly depressed compared to sham group (Fig. 1b). However, treatment with *I. racemosa* significantly corrected HR in ischemia and reperfusion periods compared to I-R control.

**Effect of *I. racemosa* on ventricular function**—A significant fall in (+)LVdP/dt was recorded during ischemia and reperfusion (Fig. 1c). *I. racemosa* significantly improved (+)LVdP/dt as compared to control group during the reperfusion duration. Similar to contractility, a significant depression in (-)LVdP/dt was recorded in the control I-R group as compared to sham (Fig. 1d). The observed fall in diastolic function was significant compared to decline in the systolic function. *I. racemosa* treatment significantly restored (-)LVdP/dt as compared to control I-R group both during ischemia and reperfusion duration. In addition to contractile dysfunction, a significant elevation in LVEDP marked the onset of ischemia which remained elevated throughout the ischemia and reperfusion period in comparison to sham group (Fig. 1e). *I. racemosa* treatment significantly reduced LVEDP in consonance to other altered ventricular functions as compared to control I-R group.

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![Graphs showing effect of *I. racemosa* on haemodynamic and ventricular functions](image)

**Fig. 1**—Effect of *I. racemosa* (IR) on time course of change during ischemia and reperfusion injury period in (a) mean arterial pressure (MAP); (b) heart rate (HR); (c) contractility, (+)LVdP/dt); (d) relaxation, (-)LVdP/dt); and (e) left ventricular end diastolic pressure (LVEDP) [The values are expressed as mean ± SD of six readings. Significant at $^*P<0.05$ when compared to sham; and $^#P<0.05$ when compared to I-R control]
Effect of *I. racemosa* on antioxidant status—Coronary artery ligation-induced ischemia and further reperfusion caused a significant decrease in the activities of SOD, CAT and GPx (Table 1). Corresponding to a significant fall in activities of antioxidant enzymes, a significant rise in MDA level with a concomitant decrease of GSH content was observed in the control I-R group as compared to sham group (Table 2). Treatment with *I. racemosa* resulted in significant restoration of endogenous antioxidant enzymes, SOD, CAT, GPx and GSH level along with reduction of lipid peroxidation product, MDA in heart as compared to the control I-R group.

Effect of *I. racemosa* on myocytes injury marker enzyme—A significant depletion of CK-MB and LDH from heart in I-R control group was observed compared to sham group (Table 3). *I. racemosa* was found to prevent leakage of CK-MB and LDH from heart as compared to control I-R group.

Discussion
The present study showed that pretreatment with *I. racemosa* reduced ischemic-reperfusion injury in rat heart by improving haemodynamics (MAP and HR), left ventricular contractile function, (+)LVdP/dt, (-)LVdP/dt and LVEDP, endogenous antioxidant enzymes (SPD, CAT and GPx), antioxidant (GSH), cellular protein oxidation (increased activity of GPx), and decreasing lipid peroxidation (MDA) and also by inhibition of leakage of myocytes specific enzymes (CK-MB and LDH) and normalized myocardial histoarchitecture.

As described above, a burst of ROS occurs immediately after restitution of blood flow to previously ischemic myocardium. Free radicals are difficult to estimate directly because of its high reactivity and short half-life, therefore the level of product of lipid peroxidations MDA, was used to measure ROS generation in this study. ROS results in enhanced lipid peroxidation as indicated by an increase in MDA levels documented both in clinical and experimental studies in conditions of myocardial ischemia-reperfusion injury. In the present study, an elevated level of MDA in I-R control group indicated increased oxidative stress due to ischemia-reperfusion induced injury, as compared to sham group. On the other hand, *I. racemosa* treatment demonstrated decreased level of lipid peroxides and this could be due to reduced formation of lipid peroxides from fatty acids.

### Table 2—Effect of *I. racemosa* on lipid peroxidation (MDA) and reduced glutathione (GSH)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA (nmol/g tissue)</th>
<th>GSH (µmol/g tissue protein)</th>
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<tr>
<td>Group I (Saline; sham)</td>
<td>74.25 ± 2.51</td>
<td>10.48 ± 0.81</td>
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<tr>
<td>Group II (Saline; I-R control)</td>
<td>180.24 ± 1.28</td>
<td>18.62 ± 0.65</td>
</tr>
<tr>
<td>Group III (<em>I. racemosa</em> 100 mg/kg)</td>
<td>78.05 ± 2.42</td>
<td>21.32 ± 0.97</td>
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<tr>
<td>Group IV (<em>I. racemosa</em> 100 mg/kg + I-R)</td>
<td>91.61 ± 2.16</td>
<td>14.17 ± 1.02</td>
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</tbody>
</table>

Significant at *P*<0.05; when compared to sham; #*P*<0.05, when compared to I-R control

### Table 1—Effect of *I. racemosa* on antioxidant enzymes in heart

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SOD</th>
<th>CAT</th>
<th>GPx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Saline; sham)</td>
<td>8.70 ± 1.32</td>
<td>23.15 ± 2.46</td>
<td>1.23 ± 0.60</td>
</tr>
<tr>
<td>Group II (Saline; I-R control)</td>
<td>4.22 ± 0.88*</td>
<td>11.48 ± 1.76*</td>
<td>0.80 ± 0.23*</td>
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<tr>
<td>Group III (<em>I. racemosa</em> 100 mg/kg)</td>
<td>8.30 ± 1.12</td>
<td>24.05 ± 1.60</td>
<td>1.36 ± 0.40</td>
</tr>
<tr>
<td>Group IV (<em>I. racemosa</em> 100 mg/kg + I-R)</td>
<td>8.85 ± 1.50*</td>
<td>22.36 ± 1.78*</td>
<td>1.22 ± 0.66*</td>
</tr>
</tbody>
</table>

Significant at *P*<0.05, when compared to sham; #*P*<0.05, when compared to I-R control

Values are represented as units/mg protein
A significant decrease in myocardial endogenous antioxidants such as SOD, CAT and GSH, with a concomitant increase in the levels of MDA in the present study further confirmed the presence of oxidative stress in the I-R control group as compared to sham group. Due to disruption of endogenous antioxidant network, as observed in the study, the myocardium may be more susceptible to any ischemia-reperfusion injury. Thus, our study clearly demonstrated the antioxidant activity of *I. racemosa* as it significantly restored GSH levels; and SOD and CAT activity in the myocardium. The observed antioxidant activity of the extract in the present study is in agreement with previous reports show cardioprotection by restoring the level of endogenous antioxidants and energy substrates in isoproterenol-induced myocardial infarction in rats.

Besides antioxidant enzymes and physiological antioxidants, alterations in CK-MB isoenzyme have been considered as an important marker of myocardial injury. We observed a significant fall in myocardial CK-MB and LDH enzyme activity in the I-R control group as compared to sham group animals, consistent with the idea that CK-MB and LDH being the myocardial enzymes, leak out from the tissue to plasma on development of degenerative changes in myocardial cell membranes due to lipid peroxidation. The observation that *I. racemosa* treatment significantly restored CK-MB and LDH activity compared to I-R control demonstrated its myocardial salvaging effects.

In present study, a marked fall in MAP and HR was observed when ischemia-reperfusion injury was induced in rat heart by ligation of LAD coronary artery for 45 min and reperfused for 60 min. A significant increase in MAP at the onset of ischemia indicates the activation of sympathetic nervous system. This increase in MAP might be a compensatory mechanism of the myocardium to increase perfusion in order to meet the increased myocardial energy demand during the initial ischemic duration. However, with the progression of ischemia and throughout the reperfusion period, MAP decreased significantly indicating the deteriorated metabolic and functional state of the ischemic-reperfused myocardium. Heart rate was also depressed throughout the ischemia-reperfusion duration in the control I-R group as compared to sham, indicating an impairment of conduction (AV block) of the heart following I-R induced injury. Significant correction of MAP and HR which may increase blood flow through the subendocardial region of the ventricular muscle that bears the maximum brunt of ischemic insult may explain the possible mechanism of *I. racemosa* in cardioprotection from ischemic tissue injury. Though, the decrease in HR was less pronounced in the *I. racemosa* treated group as compared to I-R control. The decreased HR in present study was in line with previous study reporting that was reduced heart rate and cardiac output as well as prolonged mechanical systole resembles the properties of 

In addition, absence of positive chronotropic effect indicated by (+)LVdP/dt in the face of a reduced MAP suggests impairment of conduction (AV block) of heart following I-R injury. Normally, a fall in MAP due to I-R injury is expected to result in increase HR and myocardial contractility by activating the baroreceptor, which may subsequently result in reflex vasoconstriction thus, worsening the imbalance between myocardial oxygen demand and supply. However, none of these effects have been observed in the study due to ischemic injury to inotropic and chronotropic function of the heart. In normal physiology, the myocardium gets perfused during diastole phase of cardiac cycle through coronary arteries. These arteries are poor in collaterals therefore, under ischemic condition; the subendocardial region of heart is most vulnerable to ischemic necrosis because of disproportionate reduction in blood flow to subendocardial region, which is subjected to greatest extra-vascular compression during systole. Furthermore, increased LVEDP exerts an outward force on ventricular wall that reduces blood flow to the subendocardial region. By reducing LVEDP, a marker of preload and favourable alteration of inotropic {(+)}LVdP/dt, marker of myocardial contraction} and lusitropic {(-)LVdP/dt, marker of myocardial relaxation} functions of the left ventricle, *I. racemosa* might have improved perfusion to subendocardium thereby, reduced myocardial injury which may explain the cardioprotection.

Remarkable cardiodepressent ability of *I. racemosa* to augment contractile performance of myocardium injured by ischemia has been ascribed to its chemical constituents which resembles to β-adrenergic blockers, propranolol and antioxidant properties.
β-adrenergic receptor blocking activity bolsters cardiac performance and the antioxidant activity helps in the maintenance of cytosolic energy state, thereby providing energy to maintain cellular functions in the face of metabolic perturbations under the influence of oxidative stress. The present study supports the notion that *Inula racemosa* consists of herbal origin β-blockers which may provide a future drug lead for cardiovascular diseases.

The present study clearly emphasizes the cardioprotective effects of *Inula racemosa* and validates its traditional claims bracing the experimental and early clinical reports which have demonstrated its usefulness in myocardial infarction.

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**References**