Cardioprotective effect of root extract of *Picrorhiza kurroa* (Royle Ex Benth) against isoproterenol-induced cardiotoxicity in rats

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Normal rats pre-treated with *P. kurroa* (200 mg/kg) alone did not showed significant change, however, isoproterenol (ISP) administration resulted in hemodynamic and left ventricular dysfunction, oxidative stress, and lipid peroxidation. Such cardiac dysfunction was significantly prevented by *P. kurroa* root extract pre-treatment. Pre-treatment significantly attenuated the ISP-induced oxidative stress by restoring myocardial superoxide dismutase, catalase, and glutathione peroxidase enzymes except reduced glutathione content. *P. kurroa* pre-treatment markedly attenuated the ISP-induced rise in lipid peroxidation, thereby prevented leakage of myocyte creatine kinase-MB and lactate dehydrogenase enzymes. The results suggest that *P. kurroa* root extract possesses significant cardioprotective effect, which may be attributed to its antioxidant, anti-peroxidative, and myocardial preservative properties.

Keywords: Hemodynamics, Left ventricular dynamics, Lipid peroxidation, Myocardial injury, Oxidative stress

Picrorhiza kurroa Royle Ex Benth (Scophulariaceae) is popularly known as picrorhiza, kutki, and katuka. The root and rhizomes of *P. kurroa* are commonly used for medicinal purposes in the Indian system of traditional medicine (Ayurveda)[1] for its hepatoprotective[2,3], antidiabetic[4], and chemopreventive[5,6] activities. The major chemical constituents responsible for the biological effects include iridoid glycosides — picroside I-III[7]. The constituents are collectively known as kutkin and believed to offer multiple pharmacological effects. In traditional medicine, *P. kurroa* is acclaimed to be beneficial for cardiovascular diseases[7]. Oxidative stress, which is known to cause ischemic injury, has been demonstrated to exert deleterious effects on hemodynamic and left ventricular function of heart under ischemic conditions. Several medicinal plants possessing antioxidant property have been demonstrated to protect against heart diseases[8-10]. *P. kurroa* has also been believed to protect heart

owing to potent antioxidant activities[11]. However, its effect on hemodynamic, left ventricular function, myocyte injury markers, lipid peroxidation and histopathological, and ultrastructural alterations is unknown. This prompted us to further evaluate *P. kurroa* for its cardioprotective potential in the *in vivo* animal model of isoproterenol (ISP)-induced myocardial infarction (MI). In the present investigation, the effect of lyophilized hydroalcoholic extract of roots of *P. kurroa* was used.

Isoproterenol (ISP), a nonselective β-adrenergic agonist, produces "infarct-like" lesions in the heart similar to those found in MI and sudden death in man. Proposed underlying mechanism includes generation of highly cytotoxic free radicals through auto-oxidation of ISP. Such increased free radicals stimulate lipid peroxidation and cause irreversible damage to the myocardial membrane. In addition, ISP administration also reduces blood pressure, which triggers reflex tachycardia, thereby increasing myocardial oxygen demand. Moreover, positive inotropic and chronotropic actions of ISP further increase the myocardial oxygen demand, which leads to ischemic necrosis of myocardium in rats.

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To our knowledge, this is the first report that provides hemodynamic and structural mechanisms for the observed cardioprotective properties of *P. kurroa* extract in rat model of isoproterenol-induced MI.

Materials and Methods

Chemicals—All chemicals including (±)-isoproterenol hemisulfate procured from Sigma Chemicals Co. (St. Louis, MO, USA) were of analytical grade. Isoproterenol was dissolved in normal saline (0.9% NaCl) and used within 10 min of its preparation.

Preparation of plant extract—The roots of *P. kurroa* were collected from North-Western Himalayan region of India during May 2006 and authenticated by Dr. Santosh Kumari, Department of Plant Physiology, IARI, New Delhi on the basis of routine pharmacognostic studies, including organoleptic, macroscopic, and microscopic tests. A voucher specimen has been deposited in cardiovascular laboratory of AIIMS for future reference. The shadow dried roots of *P. kurroa* were grounded to fine powder (pass 80 mesh screen), subjected to Soxhlet extraction for at least 10-12 h by the methanol-water (50:50), and evaporated to dryness using rotary vacuum evaporator. The percentage extractive value in methanol was 60% (w/w). Extract contained total bitters and kutkin not less than (NLT) 8 and 7% (w/w) respectively.

Animals—Wistar male albino mature rats (150-200 g body weight; 10 to 12 weeks old) obtained from the Central Animal House Facility of AIIMS, New Delhi, were kept in polyacrylic cages (38 × 23 × 10 cm) with not more than four rats per cage and acclimatized to the laboratory conditions before randomization. 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. The animals were housed in an air-conditioned room and were kept in standard laboratory conditions under natural light and dark cycles (approximately 12:12 h L:D) maintained at 50±10% RH and an ambient temperature of 25±2 °C. They were allowed free access to pellet diet (Ashirwad Industries Ltd.; Chandigarh) and water *ad libitum.*

Experimental protocol—The rats (48) were randomly allocated to following 4 groups of 16 animals each: Gr. I (Control): physiologic saline orally administered once daily for 1 month; Gr. II (ISP control): in addition to saline, rats received ISP (85 mg/kg, sc) on days 29 and 30 at a 24 h interval;

Gr. III (*P. kurroa*-200 + ISP): *P. kurroa* (200 mg/kg) was orally administered once daily for 1 month and ISP (85 mg/kg, sc on days 29 and 30 at an interval of 24 h; and Gr. IV (*P. kurroa per se*): orally administered *P. kurroa* (200 mg/kg) once daily for 1 month.

The dose of *P. kurroa* (200 mg/kg) was selected on the basis of a pilot study in the ISP model of myocardial necrosis. Of the doses screened (100, 200, and 400 mg/kg), the 200 mg/kg dose exhibited significant ($P<0.05$) cardioprotective effects. The dose of ISP (85 mg/kg, sc) was selected according to the previous studies[12,13].

On day 31, 24 h after the second injection of ISP, the rats were anesthetized with pentobarbitone sodium (60 mg/kg, ip). Atropine (0.1 mg/kg) was administered along with anesthetic agent to reduce tracheobronchial secretions and maintain the heart rate (HR), during surgery. Body temperature was monitored and maintained at 37 °C during the surgical protocol by covering animal with warm cloth and putting under light. The neck was opened with a ventral midline incision to perform tracheostomy. The rats were 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. The left jugular vein was cannulated with a polyethylene tube for continuous infusion of 0.9% NaCl solution. The right carotid artery was cannulated and the cannula was filled with heparinized saline (50 units/mL heparin) and connected to the CARDIOSYS CO-101 (Experimentria, Budapest, Hungary) using a pressure transducer for the measurement of mean arterial pressure (MAP) and HR. A left thoractomy was performed at the fifth intercostal space, and the pericardium was incised to expose the heart. A wide bore (1.5 mm) sterile metal cannula was inserted into the cavity of the left ventricle from the posterior apical region of the heart and connected to a pressure transducer (model P231D, Gould Statham, Cleveland, OH, USA). Left ventricular end-diastolic pressure (LVEDP) was measured on a multichannel polygraph (model 7D, Grass, Quincy, MA). At the end of experiment, the rats were euthanized with an overdose of anesthesia (sodium pentobarbitone 100 mg/kg, iv). The heart were excised and washed with chilled phosphate buffer saline to process for biochemical, histopathological and ultrastructural studies. For biochemical analysis, the heart was further snap-frozen in liquid nitrogen until analysis.

In each group, eight rats were used for hemodynamic and biochemical studies, while the remaining eight rats were used for histopathological (4) and ultrastructural (4) studies.

Biochemical analysis—An aliquot of 0.5 mL of 10% homogenate (50 mM phosphate buffer, *p*H 7.4) was used for malonaldialdehyde (MDA)[14] and reduced glutathione (GSH)[15] estimation. The rest of the homogenate was centrifuged at 4,930 *g* for 15 min, and the supernatant was used for estimation of antioxidant enzymes: superoxide dismutase (SOD)[16], catalase (CAT)[17] and glutathione peroxidase (GPx)[18]. Myocyte injury markers i.e. lactate dehydrogenase (LDH)[19] and creatine kinase-MB isoenzyme (CK-MB isoenzyme)[20] were also estimated in the same supernatant. Protein content was estimated by using BSA as standard[21].

Histopathological studies—Excised hearts were immediately fixed in 10% buffered neutral formalin solution. The fixed tissues were embedded in paraffin blocks, and thin serial sections (5 μm thick) were cut. The sections were stained with hematoxylin and eosin and examined under the light microscope (Nikon, Tokyo, Japan). Representative area images were captured in an image analysis system. The Image Analyzer consisted of a BX-50 Research Microscope (Olympus, Tokyo), Coolsnap 10 bit Digital Camera (Media Cybernetics, Bethesda, MD), and Pentium 4 computer (Compaq, Delhi, India) with Image Plus Pro image analysis software (Media Cybernetics). To eliminate bias, the pathologist was blinded to the study groups and treatment regimen. The degree of myocardial necrosis mediated by ISP challenge was graded and scored as follows: Score (−): Absence of any inflammation, edema and necrosis, Score (+): Focal areas of inflammation, edema and necrosis, Score (++): Patchy areas of inflammation, edema and necrosis, Score (+++): Confluent areas of inflammation, edema and necrosis, Score (++++): Massive areas of inflammation, edema and necrosis.

Transmission electron microscopy—Small pieces of left ventricle (approximately 1 to 2 mm in thickness) were immediately fixed in ice-cold 3% glutaraldehyde. The tissue samples were washed in phosphate buffer (0.1 M, *p*H 7.4) and post-fixed for 2 h in 1% osmium tetroxide in the same buffer at 4 °C. The specimens were dehydrated with graded acetone and embedded in Araldite® CY 212 (TAAB, UK). Semithin as well as ultrathin sections (70-80 nm) were cut by ultramicrotome, stained with uranyl acetate and lead acetate and examined under a transmission electron

microscope (Morgagni 268D, Fei Co., Eindhoven, The Netherlands) operated at 80 kV.

Statistical analysis—The data were expressed as mean±SD and evaluated with SPSS (Chicago, IL) version 10 software using one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Range Test. Values of *P*<0.05 were considered statistically significant.

Results

The body weight of the rats in all groups was recorded weekly and no significant change was observed in the mean body weights of any of the groups studied. There was no significant mortality seen in the ISP control and drug-treated group as compared to the control. Overall, the mortality on account of anesthesia, experimentation and instrumentation was 6% (unpublished data).

Hemodynamic and left ventricular dynamic variables—During the experiment, hemodynamic (MAP and HR) and left ventricular dynamic functions [(+)LVdP/dt, (−)LVdP/dt, and LVEDP] were recorded for all groups (Table 1). Normal rats pretreated with *P. kurroa* (200 mg/kg) alone did not show significant changes however, ISP administration resulted in hemodynamic and left ventricular dysfunction as evidenced by significant fall in MAP (*P*<0.001), HR (*P*<0.001), (+)LVdP/dt (*P*<0.001) and (−)LVdP/dt (0.01), and rise in LVEDP (*P*<0.001). These changes were significantly (*P*<0.05) corrected except LVEDP on *P. kurroa* extract (200 mg/kg) treatment (Table 1).

Endogenous antioxidant milieu—Isoproterenol caused significant depletion in the level of myocardial endogenous antioxidants (SOD, CAT, and GPx enzymes and GSH content) as compared with the control group

Table 1—Effect of *P. kurroa* pretreatment on hemodynamic and ventricular dynamics

[Values are mean ± SD from eight experiments]

Hemodynamic parameters	Control	ISP control	*P. kurroa*-200+ISP
MAP (mmHg)	135±10	95±10[b]	111±13[c]
HR (beats/min)	385±25	311±30[b]	346±26[c]
(+)LVdP/dt (mmHg/s)	3269±110	2827±197[b]	3101±209[c]
(-)LVdP/dt (mmHg/s)	3137±101	2914±167[a]	3139±189[c]
LVEDP (mmHg)	3.10±0.10	5.19±0.16[b]	5.00±0.10

MAP=Mean arterial blood pressure; HR=Heart rate; (+)LVdP/dt=Rate of change in left ventricular peak positive pressure; (-)LVdP/dt=Rate of change in left ventricular peak negative pressure; LVEDP=Left ventricular end-diastolic pressure. *P* values: [a]<0.01 and [b]<0.001 versus control; [c]<0.05 versus ISP control.

(Table 2). Pretreatment with *P. kurroa* (200 mg/kg) significantly prevented the ISP-induced depletion of myocardial SOD (*P*<0.01), CAT (*P*<0.01), and GPx enzymes (*P*<0.05) except GSH content. However, only *P. kurroa* treatment in normal rats did not significantly alter the levels of endogenous antioxidants.

Lipid peroxidation and myocyte injury markers—A significant (*P*<0.001) rise in the content of MDA (a marker of tissue lipid peroxidation) with simultaneous depletion of CK-MB and LDH enzymes was observed in the heart tissues of ISP-treated rats. However, *P. kurroa* (200 mg/kg) pretreatment markedly (*P*<0.05) attenuated the ISP-induced rise in MDA and decline in CK-MB and LDH enzymes levels (Table 3). *P. kurroa per se* did not show any significant changes in lipid peroxidation and myocyte specific injury markers (data not shown).

Histoarchitectural perturbations—Histopathological examination (Table 4) of the myocardium of control group animals showed normal architecture with clear integrity of myocardium (Fig. 1A). Subcutaneous injection of ISP resulted in remarkable myonecrosis, edema and moderate infiltration of chronic inflammatory cells (Fig. 1B). However, chronic *P. kurroa* (200 mg/kg) treatment significantly preserved the myocardium as reflected by marked reduction in myonecrosis, edema and infiltration of inflammatory cells (Fig. 1C).

Ultrastructural perturbations—The ultrastructure of the myocardium from control group rats was normal in appearance showing sarcomere separated with rows of normal and abundant mitochondria (Fig. 2A). However, in ISP-challenged rat myocardium (Fig. 2B), there was significant myocardial damage marked by significant disruption of myofilaments, loss of cell membrane integrity, interstitial edema, appearance of intracytoplasmic vacuoles and smaller and irregular mitochondria. Nuclear chromatin was condensed and marginated. Perinuclear as well as sarcolemmal edema was also observed. Sections from the left ventricles of *P. kurroa* (200 mg/kg, Fig. 2C) treated hearts revealed that myofibrils and mitochondria were in general well preserved with only mild cytosolic and intermyofibrillar edema.

Discussion

Isoproterenol (ISP), a potent synthetic catecholamine with β-adrenergic activity, produces "infarct-like" lesions in the heart similar to those

Table 2—Effect of *P. kurroa* pre-treatment on myocardial antioxidant milieu

[Values are mean ± SD from eight experiments]

Antioxidants	Control	ISP control	*P. kurroa-*200+ISP
SOD (units/mg of protein)	7.94±2.90	3.40±1.40[b]	6.18±2.00[d]
CAT (units/mg of protein)	21.10±3.10	11.90±2.00[b]	16.01±3.01[d]
GSH (μmol/g of tissue)	1.86±0.69	1.22±0.50[a]	1.69±0.61
GPx (units/mg of protein)	0.59±0.12	0.17±0.09[a]	0.34±0.14[c]

SOD=Superoxide dismutase; CAT=Catalase; GSH=Reduced glutathione; GPx=Glutathione peroxidase.

P values: [a]<0.01 and [b]<0.001 versus control; [c]<0.05 and [d]<0.01 versus ISP control.

One unit of SOD inhibits the rate of auto-oxidation of adrenaline by 50% at *p*H 7 at 25 °C. One unit of CAT activity represents the amount of enzyme required to decompose 1 μmol of H_2O_2/min.

One unit of GPx activity is defined as the amount of enzyme required to utilize 1 nmol of NADPH/minute at 25 °C.

Table 3—Effect of *P. kurroa* pre-treatment on lipid peroxidation and myocyte injury markers

[Values are mean ± SD from eight experiments]

Biochemical parameters	Control	ISP control	*P. kurroa-*200+ISP
MDA (nmol/g of tissue)	66.50±13.90	99.90±9.50[b]	87.00±9.09[c]
CK-MB (IU/mg of protein)	162.40±27.30	59.4±21.30[b]	85.90±16[c]
LDH (IU/mg of protein)	210.30±36.90	113.7±16.90[b]	141.09±22.19[c]

MDA=Malonaldialdehyde; CK-MB=Creatine phosphokinase-MB; LDH=Lactate dehydrogenase.

P values: [b]<0.001 versus control; [c]<0.05 versus ISP control.

###<0.001 versus control; *<0.05 versus ISP control.

One unit of CK-MB isoenzyme is defined as the amount of enzyme that will transfer 1 μmol of phosphate from phosphocreatine to ADP per minute at *p*H 7.4 at 30 °C.

One unit of LDH is defined as the amount of enzyme required to reduce 1 μmol of pyruvate to D-lactate/minute at *p*H 7 at 25 °C.

Table 4—Light microscopic changes observed in the different experimental groups

Study groups	Necrosis	Edema	Inflammation
Control	–	–	–
ISP Control	++	+++	++
*P. kurroa-*200 + ISP	+	++	+

The relative pathological changes are ranked as follows

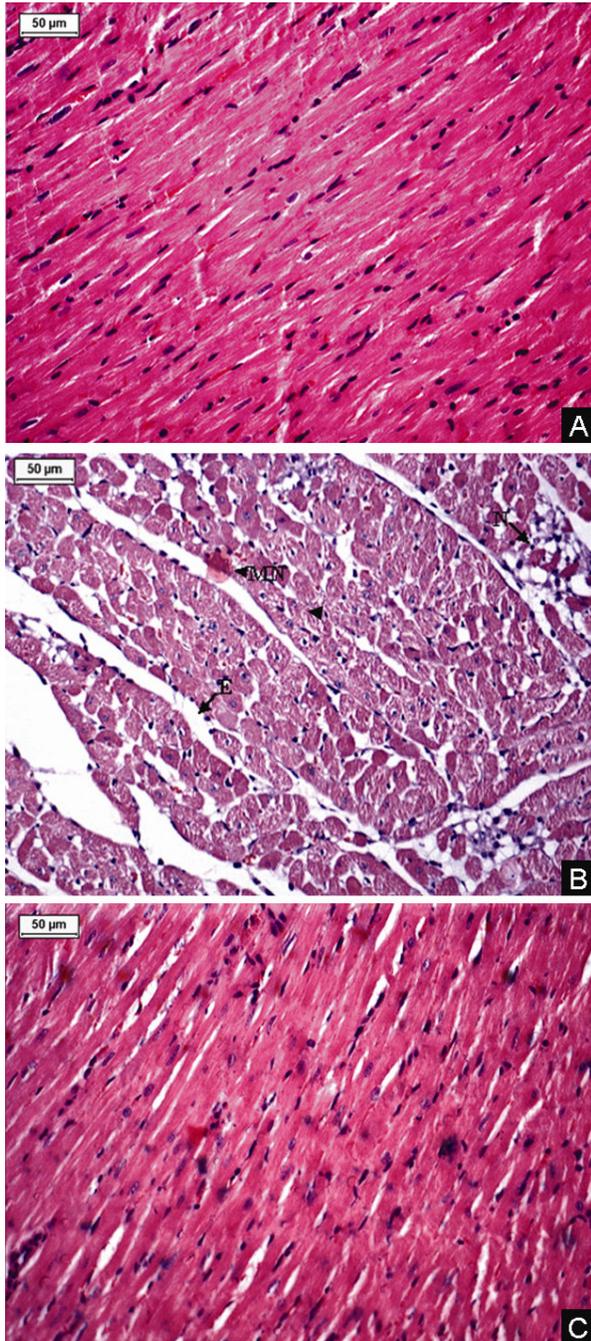– = No change; + = Focal change; ++ = Patchy change; +++ = Confluent change; and ++++ = Massive change

Fig. 1—(A): Representative light micrographs showing normal architecture of rat heart of the control group. Endocardium and pericardium are seen within normal limits with no infiltration of inflammatory cells. (B): Photomicrograph of rat heart administered ISP, showing focal loss of myofibers, myonecrosis (MN), infiltration of chronic inflammatory cells, and marked edema (E). In subendocardium vacuolar changes and prominent edema along with chronic inflammatory cells i.e. neutrophils (N) are clearly visible. (C): Photomicrograph (x100) of the heart of *P. Kurroa* pre-treated rat showed cardioprotection as evidenced by reduction in focal loss of myofibers edema and infiltration of inflammatory cells [A-C, x100].

found in MI and sudden death in man[22-24]. Proposed underlying mechanism includes generation of highly cytotoxic free radicals through auto-oxidation of ISP[23]. Oxygen derived free radicals are shown to be detrimental for carbohydrates, lipids and proteins thereby produce structural as well as functional damage of myocardium[25]. Such increased free radicals stimulate lipid peroxidation and cause irreversible damage to the myocardial membrane. Increased oxidative stress may also depress the sarcolemmal Ca^{2+} transport and result in the development of intracellular Ca^{2+} overload and ventricular dysfunction[26]. In addition, ISP administration also reduces blood pressure, which triggers reflex tachycardia, thereby increasing myocardial oxygen demand. Moreover, positive inotropic and chronotropic actions of ISP further increase the myocardial oxygen demand, which leads to ischemic necrosis of myocardium in rats. The present findings that ISP produces myocardial lesions and significantly alters biochemical and hemodynamic parameters concur with previous studies[27,28].

Myocardium contains an abundant amount of endogenous enzymatic (SOD, CAT and GPx) and non-enzymatic antioxidants (GSH) to protect cellular components from the deleterious effects of ROS and peroxides[9]. Depletion of these antioxidants along with a significant rise in myocardial MDA level in the present study indicates the occurrence of myocardial oxidative stress on following ISP administration. However, *P. kurroa* pretreatment showed significant antioxidant effect as illustrated by marked attenuation of ISP-induced depletion of antioxidant enzymes except glutathione. GSH acts as a substrate of GPx, therefore, decreased GSH levels even after *P. kurroa* pretreatment might be due to its enhanced utilization along with augmentation of GPx. Further, increased myocardial MDA levels also indicate excessive formation of free radicals and activation of the lipid peroxidation process. *P. kurroa* significantly ($P<0.05$) attenuated ISP-induced increase in myocardial MDA which could be by preventing formation of lipid peroxides.

In addition to antioxidants, myocardium contains CK-MB and LDH enzymes which are considered as gold standard diagnostic markers of myocyte injury. Structural and metabolic damage to myocardium leads to their release into the extracellular fluid and thereby depletion from myocardium[29]. The depletion
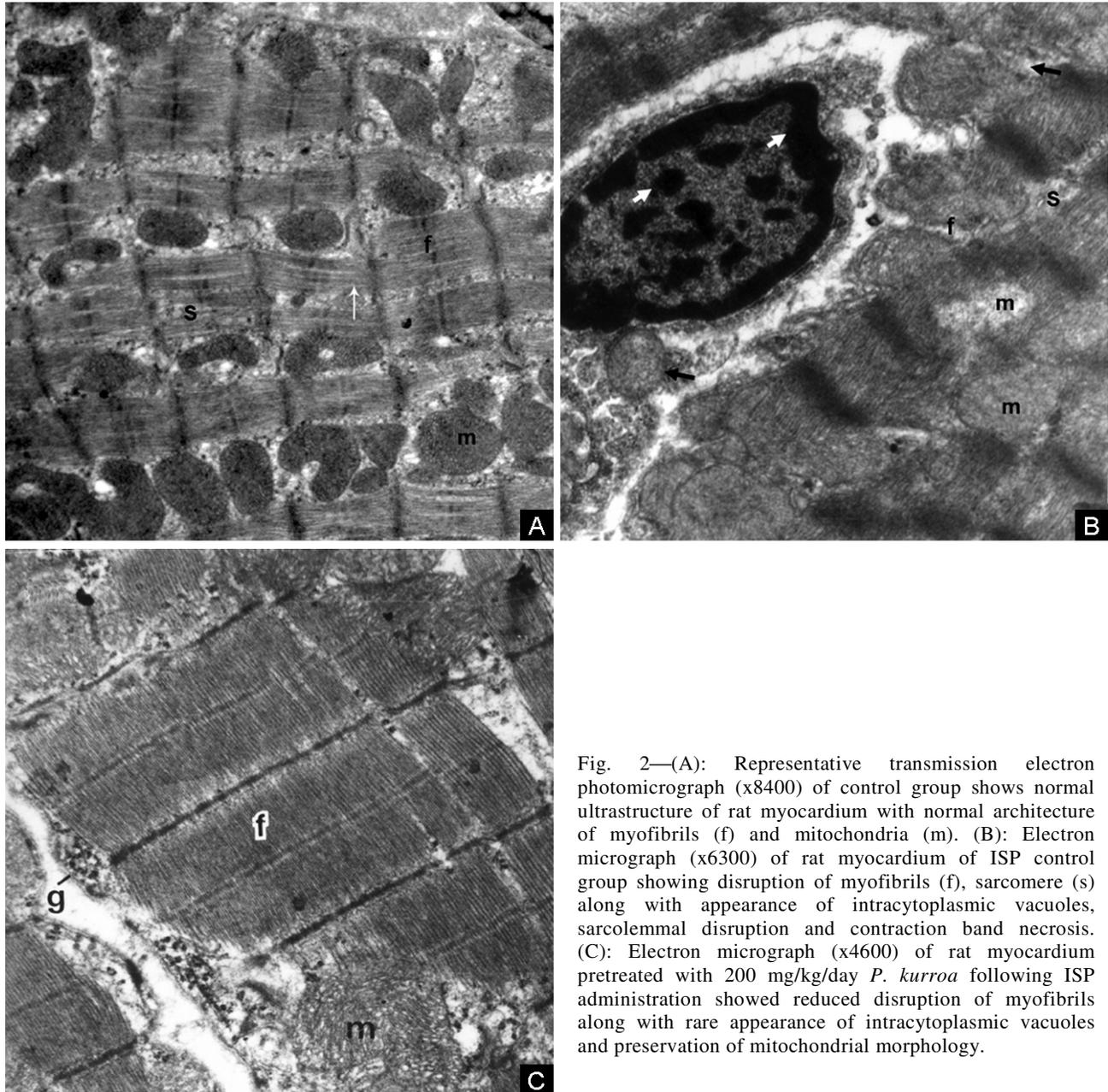
Fig. 2—(A): Representative transmission electron photomicrograph (x8400) of control group shows normal ultrastructure of rat myocardium with normal architecture of myofibrils (f) and mitochondria (m). (B): Electron micrograph (x6300) of rat myocardium of ISP control group showing disruption of myofibrils (f), sarcomere (s) along with appearance of intracytoplasmic vacuoles, sarcolemmal disruption and contraction band necrosis. (C): Electron micrograph (x4600) of rat myocardium pretreated with 200 mg/kg/day *P. kurroa* following ISP administration showed reduced disruption of myofibrils along with rare appearance of intracytoplasmic vacuoles and preservation of mitochondrial morphology.

of CK-MB as well as LDH levels in ISP control animals support the occurrence of ISP-induced myocardial injury and are in accordance with previous reports[30,31]. Subsequent to ISP challenge, myocardium was preserved by *P. kurroa* pretreatment as evidenced by marked restoration of myocardial CK-MB and LDH enzyme activities in drug treated rat hearts. Simultaneous attenuation of ISP-induced increase in lipid peroxidation and restoration of myocyte injury markers suggests that *P. kurroa* is able to maintain redox state of cell and the integrity and stability of cell membrane. It is further supported by significant

prevention of ISP-induced histopathological and ultrastructural perturbations.

Previous studies demonstrated that natural as well as synthetic antioxidants are able to attenuate myocardial oxidative stress and associated ventricular dysfunction[32-34]. These findings are in line with the present results where all ISP challenged rats showed myocardial dysfunction. It is illustrated by a marked fall in MAP, HR, (+) LVdP/dt, and (–) LVdP/dt and rise in LVEDP, which could be due to ISP-induced biochemical and structural perturbations. Oxidation products of catecholamines

also adversely affect contractile elements, and cardiac myocyte membrane that decline myocardial contractility[35]. Such a deteriorating contractile status of myocardium as evidenced by reduced (+)LVdP/dt values might be responsible for the significant decline in MAP. In ISP challenged rats, significant decline in (−)LVdP/dt indicates ISP-induced impairment in myocardial relaxation. Such an impaired myocardial relaxation could result in elevated filling pressure, low cardiac output, and elevation of LVEDP. Prereatment with *P. kurroa* prevented ISP-induced hemodynamic impairment and cardiac dysfunction.

Experimental and clinical studies have shown that herbal drugs and natural products with antioxidant and adaptogenic properties can be used prophylactically in various disorders[36-38]. They are gaining importance in therapeutic management of various disorders because of their effective and relatively safer nature[39]. In the present study, *P. kurroa* has shown strong antioxidant, antiperoxidative, and myocardial preservative effect against ISP-induced myocardial injury. Observed cardioprotective effect with *P. kurroa* treatment could be attributed to kutkin, iridoid glycosides (Picrosides I-III) believed to be responsible for its antioxidant, adaptogenic and anti-inflammatory activity. Kim *et al.*[40] colleagues demonstrated the cardioprotective activity of plantainoside D which was isolated from the leaves of *P. scrophulariiflora.* They demonstrated that plantaionoside D can inhibit adriamycin-induced apoptosis in H9C2 cardiac muscle cells via inhibition of ROS generation and NF-kappaB activation[40]. Picroliv, the active principle of *P. kurroa*, and its main components which are a mixture of the iridoid glycosides, picroside-I and kutkoside, were also studied *in vitro* as potential scavengers of oxygen free radicals[41]. They possess the properties of antioxidants which could be mediated through activity like that of SOD, metal ion chelators and xanthine oxidase inhibitors[41]. Picroliv has been studied for its hepatoprotective properties at a dose of 12 mg/kg/day, po for 7 days in rat model of hepatic ischemia reperfusion injury[42].

The present study demonstrates the cardioprotective effect of *P. kurroa* against ISP-induced myocardial injury and validates the traditional claim. However, further studies are warranted to support its clinical use in ischemic heart disease.

Conflict of interest

The authors report no conflict of interest.

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