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Hepato-cardioprotective role of *Picrorhiza kurroa* against β-adrenergic agonists induced cytotoxicity in mice

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Abstract : The present study was undertaken to evaluate the cytoprotective potential of rhizomes of the Indian medicinal plant, *Picrorhiza kurroa* (kutki or katuka) against β-adrenergic agonists induced cytotoxicity in mice, its capacity to modulate oxidative stress and the levels of cytotoxic marker LDH during the process. Although it shows antioxidant, anti-inflammatory and immunomodulary activities, but is most valued for its hepato-cardioprotective effect. Heart and liver of Balb/c mice were processed for hepatotoxicity and cardiomyopathy by using different techniques such as fluorescent microscopy, transmission electron microscopy and electrophoresis. Release of intracellular marker enzyme LDH was also quantified biochemically and used as diagnostic cytotoxic marker enzyme. Our results clearly demonstrated that ethanol extract of *P. kurroa* with high antioxidant activity as established using different techniques, was effective in suppressing the deleterious effect of β-agonists. There was marked elevation in the activity of LDH and cell injury as evidenced from apoptotic and necrotic cells in treated groups. Oral administration of extract (50 mg and 150 mg/kg b.w.) resulted in significant reduction in level of enzyme and cell injury. Thus, it is evident that the rhizome of *P. kurroa* exerts therapeutic action on heart and liver by its antioxidant property thereby establishing its hepato-cardioprotective effect.

Key words: Cytotoxicity, β-agonists, TEM, LDH.

INTRODUCTION

β-Agonists are reported to induce pathophysiological changes, resulting in infarct like necrosis, comparable to that of human myocardial infarction[1,2]. Previously, the extracellular appearance of LDH has also been used to detect cell damage or cell death[3]. Due to its extraordinarily widespread distribution in the body, serum LDH is abnormal in a host of disorders[4]. It is released into peripheral blood after cell death, caused by ischemia, excess heat or cold, starvation, dehydration, injury, exposure to bacterial toxins, after ingestion of certain drugs and from chemical poisoning[3].

According to WHO, globally myocardial infarction is one of the leading causes of death both in men and women due to changing life style in developing countries, such as India and particularly in urban areas. Liver cancer is the fifth most common cancer in the world with a poor prognosis. 80% people of developing countries rely on traditional medicines; mostly plants derived drugs, for their primary health needs. Although modern drugs are effective in preventing these disorders, their use is often limited because of their side effects[5].

Picrorhiza kurroa is a well known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract. These days, research has been focused on its hepatoprotective, anticholestatic, antioxidant and immune modulating activity[6,7]. Present study will first assess the cytotoxic potential of β-agonist isoproterenol followed by evaluation of the cardioprotective and heptoprotective role of ethanol extract of rhizome of *Picrorhiza kurroa* on isoproterenol induced tissue damage in mice.

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MATERIALS AND METHODS

Animals

Adult male Balb/c Mice (20-25 g) procured from Central Research Institute (CRI), Kasauli (H.P.), were maintained in the animal house of the Department of Biosciences, Himachal Pradesh University, under suitable hygienic conditions. The mice were provided feed and water *ad libitum*. All procedures adopted in animal care and methodologies, had the approval from Institutional Animal Ethics Committee of the University (Registration No. IAEC/Bio/7/2004).

Experimental groups:

To study the effect of isoproterenol on normal healthy animals showing no sign of morbidity, mice were divided into two groups:

Group S: mice receiving saline

Group I: mice receiving a single oral dose of isoproterenol (100 mg/kg b.w.)

Effect of plant extract was also studied on normal mice and isoproterenol treated mice. For that, mice were administered with two doses of *Picrorhiza* rhizome extract for 30 days and divided in to four groups:

Group E_{50}: mice receiving 50 mg *Picrorhiza* rhizome extract

Group E_{150}: mice receiving 150 mg *Picrorhiza* rhizome extract

Group EI_{50}: mice receiving 50 mg *Picrorhiza* rhizome extract+100 mg isoproterenol

Group EI_{150}: mice receiving 150 mg *Picrorhiza* rhizome extract+100 mg isoproterenol

Lactate dehydrogenase assay

The hepatic tissues were obtained from each group of animals and homogenized separately in 0.2M Tris HCl buffer, (pH 7.3). Each homogenate was centrifuged at 4000 rpm. Serum samples were prepared by keeping the blood as such overnight at 8 C. Blood cells coagulated and serum was separated as a distinct layer. Protein concentration was determined by the method of Lowry *et al.*[8].

Total LDH activity of the liver and serum samples was determined by employing a standard LDH assay[9]. Reaction velocity was determined by a decrease in absorbance at 340 nm, following an oxidation of NADH. Change in absorbance was finally converted into units of LDH activity. Reaction mixture for LDH activity contained, 0.2 M Tris (pH 7.3), 6.6 mM NADH and 30 mM sodium pyruvate.

Native Polyacrylamide Gel Electrophoresis (PAGE)

The hepatic tissues were homogenised in 0.9% saline to get a 10% homogenate. Different homogenates were centrifuged at 4000 rpm for 30 minutes at 4°C. LDH isozymes were resolved on a 7.5% gel, employing native polyacrylamide gel electrophoresis (PAGE), according to the method of Cooper[10].

Tris HCl was used as buffer for separating (0.5 M; pH 8.8) as well as stacking (1.5 M; pH 6.8) gel. Tris glycine buffer (pH 8.3) was used as a running buffer. LDH isozymes were finally visualized by incubating the gels in a medium containing 100 mM Tris (pH 7.0), 0.4 mg/ml Nitro BT, 0.7 mg/ml NAD, 0.5 mg/ml phenozine methosulphate (PMS) and 100 mM sodium lactate (pH 7.0), for 20 minutes at 37°C. Different LDH isozymes appeared as dark (blue/violet) bands. Densitometry was also performed to evaluate the altered isozymic expression after drug administration.

Fluorescence microscopy

Tissue preparation

Mice were killed by cervical dislocation. Heart and liver tissue were excised and immediately immersed in cold buffered saline. Acridine orange and ethidium bromide staining was done in tissue sections of heart. Cell suspension was prepared for liver according to the method of Dawson[11], with few modifications. For making hepatic suspension liver slice was placed in a flask containing ice cold 0.05% (w/v) collagenase and 0.1% hyaluronidase in buffered saline. The flask was incubated at 37°C with intermittent shaking. After 70 minutes, the contents of the flask were poured on a wetted filter consisting of one layer of surgical gauze overlying two layers of stocking nylon. Filtration was usually facilitated by gently stirring the suspensions on the filter with a blunt glass rod. The filtered suspension was transferred to a plastic centrifuge tube and centrifuged at 100×g for 1 minute. The supernatant fluid was withdrawn by suction and cell pellet was gently resuspended in 10 ml of cold buffered saline and centrifuged again at 100×g for 1 minute. The washing procedure was repeated twice with buffered saline.

Fluorescent assay (Duke and Cohen)[12]

The purpose of this method was to simultaneously measure apoptotic cells, necrotic cells, and normal cells. Cell suspension and tissue sections were suspended in phosphate buffered saline (PBS) followed by 2 µl of acridine orange/ethidium bromide dye each and immediately examined under fluorescence microscope. A minimum of three sites of a slide were observed for following states: viable cells having normal nuclei yellow to orange chromatin with organized structure, necrotic cells having red chromatin with organized structure and apoptotic cells with fluorescent or highly condensed chromatin.

Electron microscopy

Transmission electron microscopy (TEM) was performed at All India Institute of Medical Sciences (AIIMS), Delhi. Mice liver tissue was carefully excised and fixed in fixative for 12 hr at 4°C. The fixed tissues were then washed four times in 0.2 M phosphate buffer and postfixed in 1% osmium tetraoxide for 2 hours. They were dehydrated in graded acetone steps and embedded in araldite CY212 and stained with uranyl acetate and lead citrate.

Extraction of plant materials

Rhizomes of *Picrorhiza kurroa* were obtained from Kullu district of Himachal Pradesh and identified in Himalayan Forest Research Institute (HFRI), Shimla, Himachal Pradesh. Plant extract was prepared according to the method of Ray *et al*.[13]. Fresh air dried rhizomes of *Picrorhiza kurroa* were powdered in a mechanical grinder. Crude powder was soaked in 95% ethanol for seven days with intermittent shaking to make a 25% solution. On 8th day, the whole material was filtered through muslin cloth. The filtrate collected and concentrated under reduced pressure. The residual solvent was removed under vacuum and a solid blackish brown mass was obtained.

During use, the dried material, after weighing was placed in a mortar and pestle, to which 2% w/v of gum acacia was added and macerated with double distilled water. It was transferred to a small tube and the volume was made up as required for the experiment.

Statistical analysis

Data was expressed as Mean±S.E.M. The statistical significance was determined by the application of Student's 't'-test and one way ANOVA to find the mean difference between the groups. Post hoc (Dunnett and Turkey), tests were also performed. The differences were assumed significant at $P<0.05$*.

RESULTS AND DISCUSSION

Selectively permeable lipid membranes are essential for maintaining the internal environment of the cell. Cell membranes disruption can lead to the release of cytoplasmic contents including various enzymes into extracellular space which serve as indicators suggestive of disturbances of the cellular integrity. Their presence in serum/plasma provides information about tissue or cellular damage that has occurred[14] Present study measured the levels of isoproterenol cytotoxicity in mice heart and liver after treatment with single high dose of 100 mg/kg b.w.

Measurement of lactate dehydrogenase (LDH) is important in diagnosis of myocardial infarction[15]. After the administration of isoproterenol, total ventricular LDH initially showed a spurt in its activity followed by a decline which was most prominent i.e. 43% at 48 hr of drug treatment (Fig. 1). While serum LDH was unaffected in the beginning, it displayed a burst in LDH activity later. This burst was most conspicuous i.e. 54% at 48 hr stage (Fig. 2). To have a clearer picture, electrophoresis was also performed, because according to earlier studies, the levels of serum LDH_1 and LDH_2 increase in acute heart muscle injury[16,17]. In the present study, ventricular LDH_2 demonstrated reduced expression of 12% at 48 hr stage along with a corresponding 72% enhancement in its serum levels (Fig. 3B & 3C).

Moreover isoproterenol administration resulted in an increase of 21% in the overall activity of total hepatic LDH after 4 hr (Fig.1). This enhancement might be correlated to the fact that the metabolism of the liver is dependent on glycolysis even in normal conditions. Because hepatocytes are under severe stress, the energy demands of the increased synthetic activities elicited by the primary injury may be met by enhanced glycolysis[18]. The increase in LDH activity might be favourable in this respect. Isoproterenol had resulted in significant decrease in the hepatic levels of lactate dehydrogenase in the later stages of the study (8, 20, 48 and 72 hr). A decline of as much as 31% was visible at 48 hr stage with subsequent increase in the activity in the serum compared to normal (Fig. 1). An increase of 54% in the serum levels of the enzyme may be attributed partly to its leakage from the liver as a result of

drug induced cytotoxicity and the amount of enzyme appeared in the serum was in proportion to the number of necrotic cells.

When liver samples were run on native PAGE, only one isozymic form of LDH i.e. LDH_5 was displayed (Fig. 3A). Prior Agostini *et al*.[19] have also reported mostly LDH_5 in whole liver cells. Aerobic isoforms like LDH_1 and LDH_2 were found to be completely absent in the liver thereby pointing it as an organ with anaerobic metabolism. Isoproterenol administration resulted in significant decline of 20% in the levels of hepatic LDH_5 after 48 hr of treatment (Fig. 3A), with subsequent rise of 37% in its serum levels at the same stage (Fig. 3C). As LDH_5 is found in abundance in the hepatic tissue and, serum LDH_5 is found to be high in acute liver injury[17], thereby proving that it has been released from this tissue after β-agonist induced tissue damage and necrosis suggesting role of isoproterenol in the process of apoptosis and necrosis, in mice hepatocytes.

From the apoptosis assay, it was deduced that heart and liver displayed excessive cellular injury after drug treatment. Even control mice tissues demonstrated cell injury/ death (Fig. 4A & 4C). Three types of cells were clearly recognized in liver, live, necrotic and apoptotic hepatocytes. Isoproterenol caused more instances of necrosis (53%) as compared to apoptosis (35%) after 48 hr of administration (Fig. 4D). Earlier, Honda *et al*[20] alleged that apoptotic cells undergo lysis after a period of time and called it secondary necrosis. The more incidences of necrosis compared to apoptosis in present study, suggested that some of the total necrosis measured after 48 hr of β-adrenergic agonist administration may in fact be secondary necrosis to the apoptosis measured at earlier time points.

From morphological viewpoint, the distinction between apoptosis and necrosis was also described by electron microscopy. Cardiomyocytes undergoing apoptosis were characterized by cell rounding, the formation of sharply-delineated, uniform fine granulated masses that became marginated against the nuclear membrane and condensation of cytoplasmic organelles (Fig. 5C). Necrosis typically involves foci containing many cells that swell (especially the mitochondria and endoplasmic reticulum) because they lose their ability to maintain their fluid and electrolyte balance[21]. In contrast the mitochondrial morphology is normal in apoptosis just the nuclear chromatin forms clumps at the nuclear periphery[22]. In our study majority of mitochondria exhibited an extensive edema in mice treated with isoproterenol leaving aside the fact that some of the mitochondria also showed normal structure even after treatment with the drugs. These results were corroborated by endoplasmic reticulum to some extent as it showed dilation in heart only (Fig. D).

Ultrastructural analysis of cardiac muscle revealed degeneration of the mitochondria and disappearance of cristae after β-agonist administration (Fig. 5E). This appearance was considered "atrophy of the striated muscle". The major mitochondrial pathology in present study was edema, and was the main ultrastructural sign of cellular injury[23]. There was extensive swelling of the organelle leading to structural damage to cristae. Some mitochondria had lost their outer membrane therefore presented a disrupted structure (Fig. 5E). Permeablization of the outer membrane of mitochondria is a common feature of both apoptosis and necrosis and this process of simultaneous induction of apoptotic and necrotic death, has also been termed as 'necrapoptosis'[24]. After permeabilzation of the outer mitochondrial membrane, the subsequent phenotype of cell death (i.e. apoptosis or necrotic) may be determined by the availability of ATP[25], as cells depleted of ATP undergo necrosis rather than apoptosis.

Disrupted mitochondrial structure may interfere with the cells ability to utilize oxygen to generate adequate amount of ATP[26]. This in turn may impair the ability of cell to utilize the nutrients to synthesize structural and functional proteins necessary for maintaining the cell. Depletion of ATP can shift the metabolism towards anaerobic glycolysis. In addition of being less efficient in terms of energy production, glycolysis is also accompanied by the accumulation of inorganic phosphate and lactic acid which lowers the pH inside the cell. This acidosis can interfere with enzyme functioning and can damage nuclear DNA, thus leading to cell death[27]. This has previously been studied in developing brain[26].

Hepatocytes undergoing apoptosis were confirmed by chromatin condensation, nuclear fragmentation, and extensive surface blebbing of the nuclear membrane and finally appearance apoptotic bodies (Fig. 7). No such cy-

tological observations were characterized in liver sections from control mice. After treating mice with β-adrenergic drugs, the hepatic tissue experienced significant cell damage because smooth endoplasmic reticulum of the liver cells, is the principal site of drug metabolism[28,29] and many enzymes which are involved in this process are bound to ER membrane.

Further we wanted to evaluate the healing potential of a medicinal plant *Picrorhiza kurroa* (Family: Scrophulariaceae) which is also known as kutki (vernacular name). There are evidences of this plant being used in the Ayurvedic system of medicine for the cure of liver disorders which indicated its hepatoprotective nature[13,30]. Lactate dehydrogenase was used as a marker of cellular injury to evaluate the cytoprotective potential of kutki in mice heart and liver, after administering single oral dose of isoproterenol hydrochloride (100 mg/kg b. w.) to mice for 48 hr. It was found that mice receiving no pretreatment of extract showed massive loss of marker enzyme lactate dehydrogenase (LDH) from the tissues and an enormous increase in the serum LDH after treatment with isoproterenol proving its cytotoxic nature. Pretreatment of mice with plant extract followed by isoproterenol administration demonstrated loss of tissue LDH to a lesser extent.

Protection from cytotoxicity was observed in liver and heart at both doses of plant extract (Fig. 6 & 7C). Out of the two doses of *Picrorhiza* plant extract i.e. 50 mg and 150 mg/kg b. w./day, later was found to more effective in fighting against isoproterenol induced cell damage. *Picrorhiza* reduced the hepatic damage or LDH release by 20-25% (Fig. 8A). These cytoprotective effects could be possibly via the stimulation of nucleic acid and protein synthesis. Heart also witnessed more than 50% less cell injury (Fig. 8B). Serum complimented the above findings by showing tiny increase in its LDH activity in both the groups (Fig. 8C).

The results of the present study indicated and confirmed the deleterious effects of acute doses of β-agonists, isoproterenol (100 mg/kg b. w.). As far as the effect of *Picrorhiza* is concerned, it showed great cytoprotective potential. Our data suggested its cardioprotective potential besides its hepatoprotective properties. *Picrorhiza* seems to be a promising tool to explore therapeutic alternative in cardiovascular disease and encouraging dietary intake of this plant could prove beneficial to heart in addition to liver.

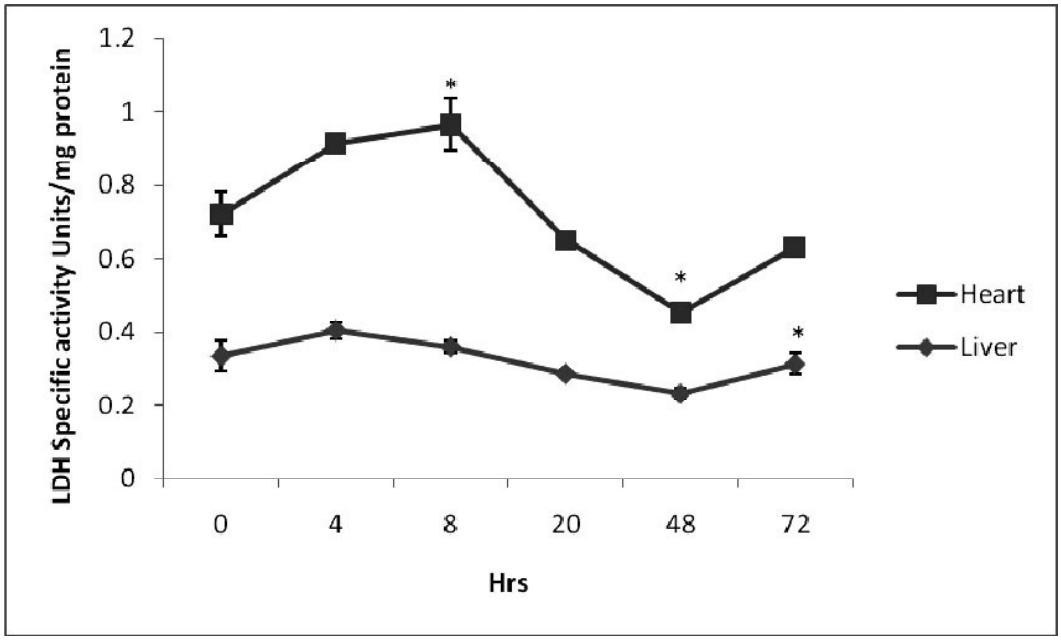


Figure 1

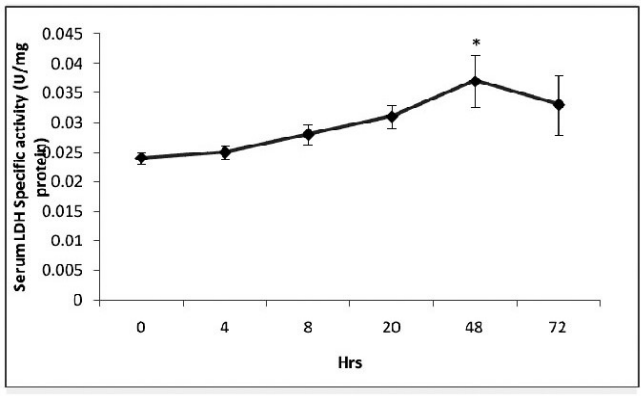


Figure 2

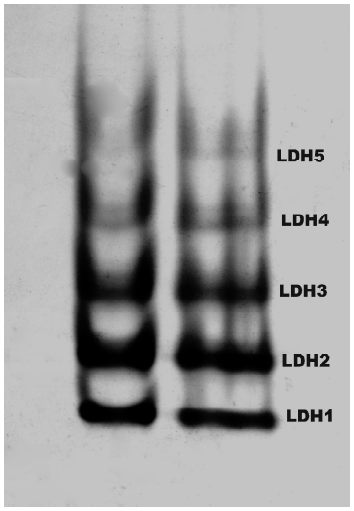


Figure 3B

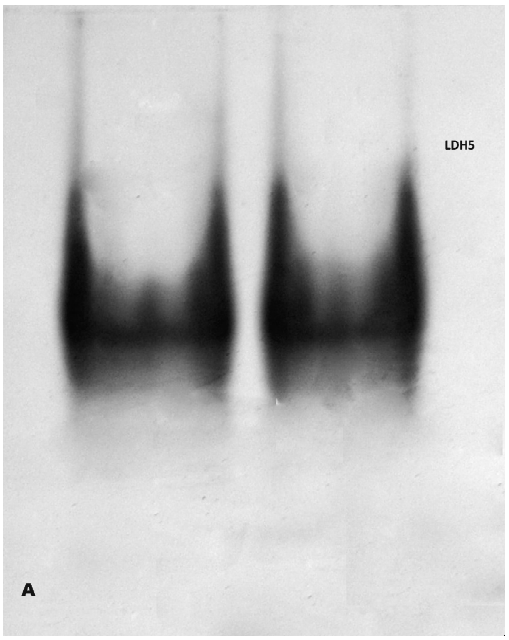


Figure 3A

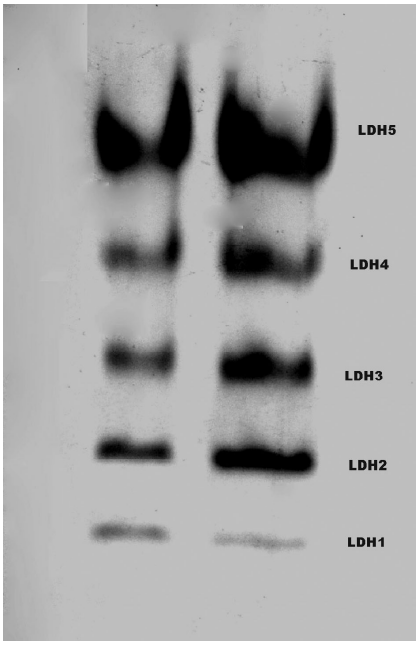


Figure 3C

LEGENDS

Fig. 1: Effect of acute dose of isoproterenol (100 mg/kg b. w.) on mice total heart and liver lactate dehydrogenase concentration (Units/mg protein). Values are Mean ± S.E.M. (n=6). $P < 0.05$*.

Fig. 2: Effect of acute dose of isoproterenol (100 mg/kg b. w.) on mice total serum lactate dehydrogenase concentration (Units/mg protein). Values are Mean ± S.E.M. (n=6). $P < 0.05$*.

Fig. 3: Native PAGE (7.5%), revealing distribution of lactic dehydrogenase (LDH) isozymes in control and isoproterenol (100 mg/kg b. w.) treated liver after 48 hr. Liver (A), Heart (B) and Serum (C).

Lane 1: Control

Lane 2: Isoproterenol treated

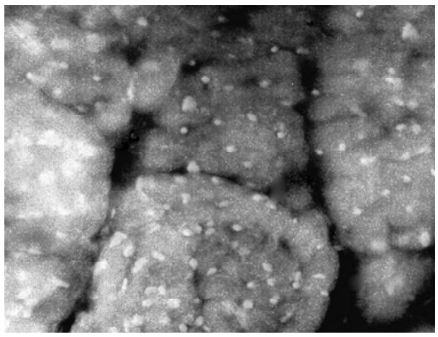


Figure 4A

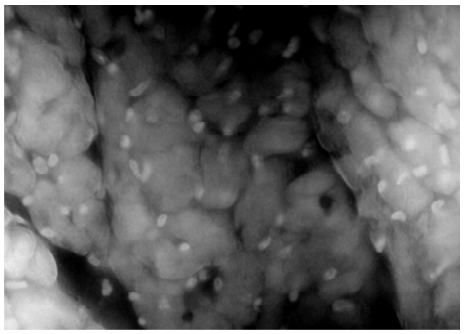


Figure 4B

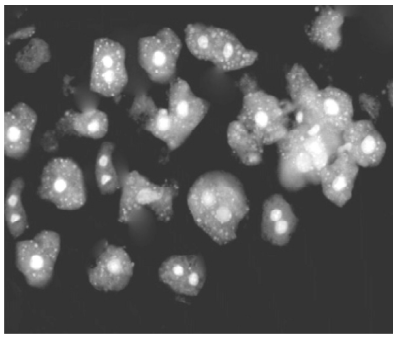


Figure 4C

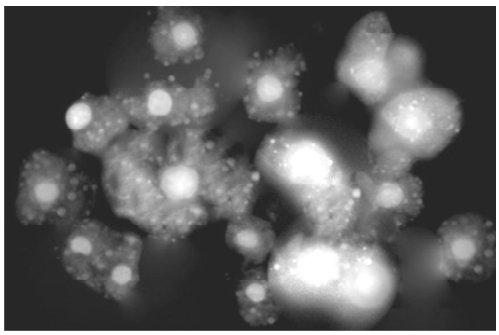


Figure 4 D

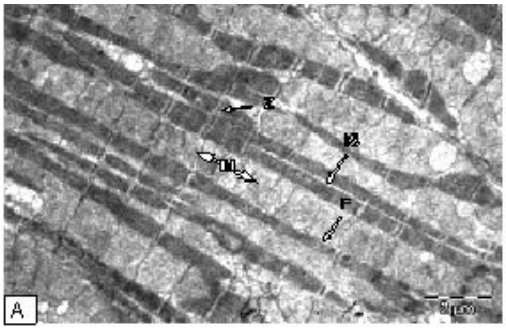


Figure 5A

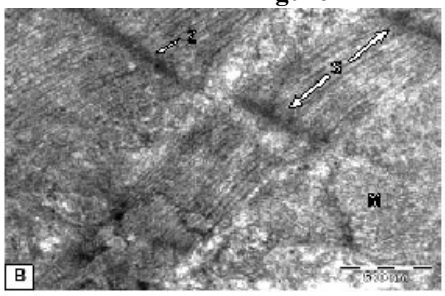


Figure 5 B

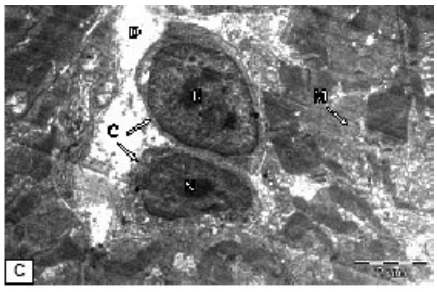


Figure 5 C

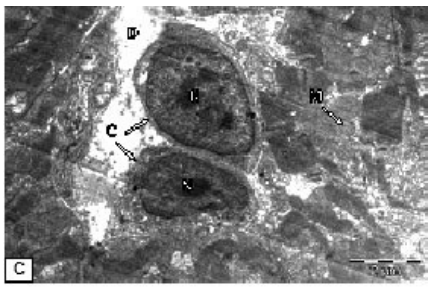


Figure 5 D

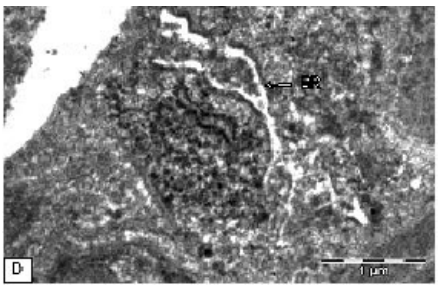


Figure 5 E

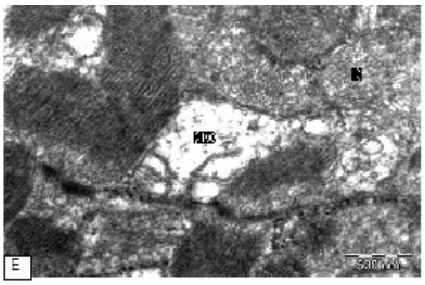


Figure 6A

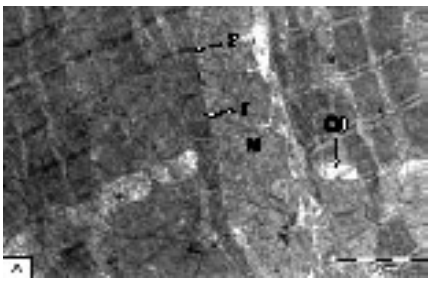


Figure 6B

Fig 4: Acridine orange/ethidium bromide stained mice control heart (A) and liver (C). Administration of â-agonist isoproterenol (100 mg/kg b. w.) for 48 hr resulting in widespread cell injury in mice heart (B) and liver (D). ×200 and ×400. **Fig. 5:** Transmission electron photomicrographs (TEM). **A:** Control mice heart (longitudinal sections) showing normal myofibrils (F) with prominent Z-bands (Z), intermyofibrillar space (IS) and mitochondria, **B:** Sarcomere (S) extending between two Z-lines (Z). **C:** Cardiomyocytes (C), demonstrating degeneration after isoproterenol administration. C (cardiomyocyte), N (nucleus), M (mitochondria) are clearly visualized.

D: Endoplasmic reticulum in cardiomyocyte, showing dilation after isoproterenol treatment. ER lost ribosomes which were present in the sarcoplasm freely. **E:** Mice heart demonstrating complete loss of mitochondrial cristae (MC) after drug treatment. M indicates mitochondria with intact structure.

Fig. 6: Tranmission electron photomicrograph of mice heart pretreated with plant extract 50 mg (A) and 150 mg (B), followed by administration of isoproterenol for 48 hr. Note the compact arrangement of the myofibrils (F) and mitochondria (M). Few mitochondria however are showing less matrix density and loss of cristae (CM). Z indicates Z-line and IS stands for interfibrillar space.

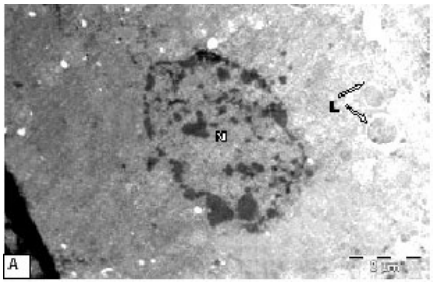
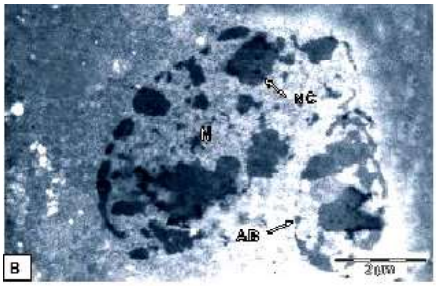


Figure 7 A



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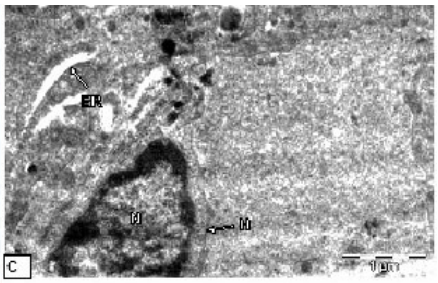

Figure 7 C

Fig. 7: A: Electronogram of control mice liver demonstrating N (nucleus) and lysosomes (L) in the hepatocyte cytoplasm. **B:** Hepatocyte nuclei (N) is demonstrating clumped chromatin (NC) along with formation of apoptotic bodies (AB) after 20 hr of isoproterenol administration.

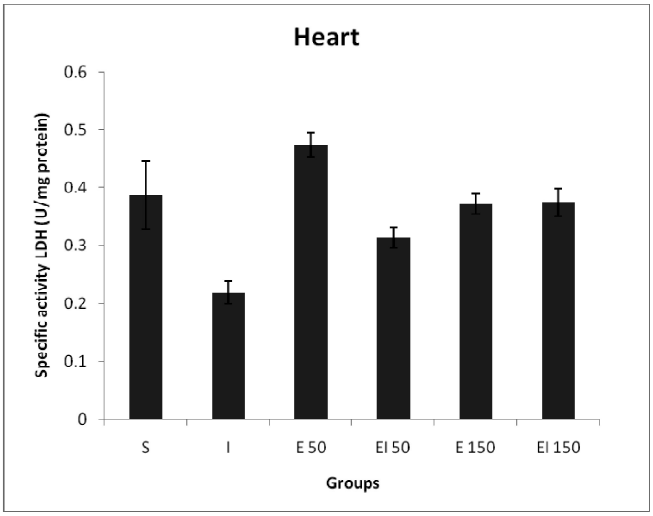

Figure 8 A

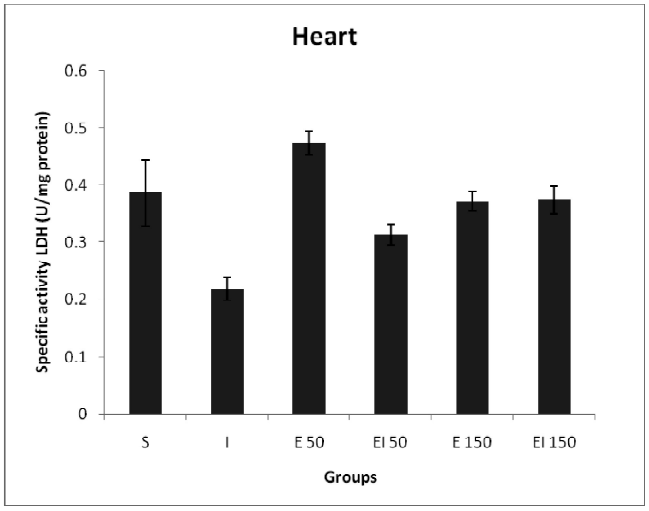

Figure 8B

Fig. 8: Activity of LDH (Units/mg protein) in liver (A), heart (B) and Serum (C) of mice. Group S (mice receiving saline only), group I (mice treated with isoproterenol alone for 48 hr), group E_{50} (mice treated with 50 mg extract for 30 days), E_{150} (mice treated with 150 mg extract for 30 days), group EI_{50} (mice pretreated with 50 mg extract for 30 days followed by isoproterenol 100 mg/kg b. w. for 48 hr), EI_{150} (mice pretreated with 150 mg extract for 30 days followed by isoproterenol 100 mg/kg b. w. for 48 hr). Value are expressed as mean±S.E.M. $p < 0.05$*.

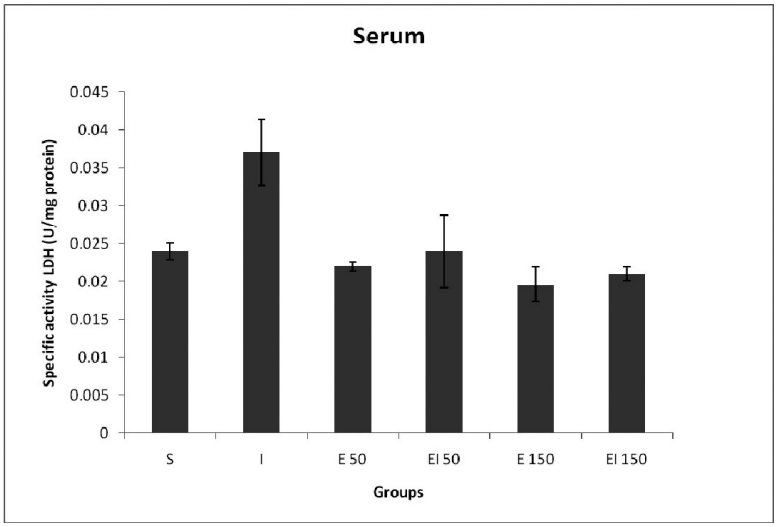

Figure 8 C

REFERENCES

1. **Nirmala C, Puvanakrishnan R.1994.** Isoproterenol-induced myocardial infarction in rats: functional and biochemical alterations. Med Sci Res; 22: 575-577.
2. **Koneri, R, Balaraman, R, Vinoth, K.M., Hariprasad. 2008.** Cardio protective effect of *Momordica cymbalaria* Fenzl against experimental myocardial injury induced by isoproterenol. The Internat J Pharmacol 2008; 5: 2.
3. Lott JA, Nemensanszky E. Lactate dehydrogenase. In: Lott JA, Wolf PL, eds. Clinical Enzymology, a case-oriented approach 1987; 213-244.
4. Henderson RF, Muggenburg BA. Bronchoalveolar lavage in animals. In: Baughman RB, eds. Bronchoalveolar Lavage. St Louis, Mosby Year Book 1992; 265-287.
5. Rajadurai M, Prince PSM. Comparative effects of *Aegle marmelos* extract and alpha-tocopherol on serum lipids, lipid peroxides and cardiac enzyme levels in rats with isoproterenol-induced myocardial infarction. Singapore Med J 2005; 46: 78-81.
6. Atal CK, Sharma ML, Kaul A, Khajuria A. Immunomodulating agents of plant origin. I: Preliminary screening. J Ethnopharmacol 1986; 18: 133-141.
7. Subedi BP. Plant profile: Kutki (*Picrorhiza scrophulariiflora*). Himalayan Bioresources 2000; 4.
8. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurements with the Folin-phenol reagent. J Biol Chem 1951; 193: 265-275.
9. Borgmann V, Moon T, Laidler K. Molecular kinetics of beef heart lactate dehydrogenase. Biochem 1974; 13: 51-52.
10. Cooper TG. Electrophoresis. In: The Tools of Biochemistry. A Willey-Interscience Publication, University of Pittsburgh, USA 1977; 228.
11. Dawson AG. Preparation and some properties of a suspension of fragmented tubules from rat kidney. Biochem J 1972; 130: 525-532.
12. Duke RC, Cohen JJ. Morphological and biochemical assay of apoptosis. In: Coligan JE, Krvisbeek AM, Margulies DH, Shevach EM, Strober W, eds. Current Protocol of Immunology. Wiley-Interscience, New York 1992; 31711-31716.
13. Ray A, Chaudhuri SR, Majumdar B, Bandyopadhyay SK. Antioxidant activity of ethanol extract of rhizome of *Picrorhiza kurroa* on indomethacin induced gastric ulcer during healing. Ind J Clin Biochem 2002; 17(2): 44-51.
14. Lott A, Nemensanszky E. Lactate dehydrogenase. In: Lott JA, Wolf PL, eds. Clinical Enzymology, a case-oriented approach. Field, Rich and Associates, New York 1987; 213-244.
15. Woolf N. Cell injury and its manifestations. In: Cell, tissue and disease: the basis of pathology. Elsevier Health Sciences 2000; 7-36.
16. Wroblewski F, Gregory KF. LDH isozymes and their distribution in normal tissue and plasma in disease states. Ann N Y Acad Sci 1961; 94: 869.
17. Lott JA, Stang JM. Serum enzymes and isoenzymes in the diagnosis and differential diagnosis of myocardial ischemia and necrosis. Clinical Chemistry 1980; 26: 1241-1250.
18. Tsunekawa N, Kitaura T. Effects of drinking administration of clenbuterol of Sol and EDL of mice. Japanese J Physiol 2000; 49: 149-156.
19. Agostini PS, Vergarn C, Villa L. Intracellular distribution of the different forms of lactic dehydrogenase. Nature 1966; 209: 1024-1025.

20. Honda O, Kuroda M, Joja I, Asaumi J, Takeda Y, Akaki S, Togami I, Kanazawa S, Kawasaki S, Hiraki Y. Assessment of secondary necrosis of Jurkat cells using a new microscopic system and double staining method with annexin V and propidium iodide. Internat J Oncology 2000; 16: 283-288.
21. Kroemer G, Golstein P. Cell death by necrosis: towards a molecular definition. Trends in Biochemical Sciences 2006; 32(1): 37-43.
22. Wyllie AH, Kerr JFR, Currie AR: Cell death: the significance of apoptosis. Int Rev Cytol 1980; 68: 251-305.
23. Kin HD, Rah BJ. Effects of diltiazem on isoproterenol or Ca-induced ventricular myocardial cell injuries in isolated perfused rabbit heart: an electron microscopic study. Anat Rec 1988; 222(3): 260-271.
24. Lemasters JJ, Qian T, Bradhan CA, Brenner DA, Cascio WE, Trost LC, et al. Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. J Bioenerg Biomem 1999; 31: 305-319.
25. Leist M, Jattela M. Four deaths and a funeral: from caspase to alternative mechanisms. Natural Reviews of Molecular and Cell Biology 2001; 2: 589-598.
26. Haddad GG, Yu PS. Postnatal hypoxia and the developing brain. In: Brain hypoxia and Ischemia (contemporary clinical neuroscience), Humana Press, New York 2008; 259.
27. Rubin R, Strayer DS. Cell injury. In Rubin's pathology: Clinicopathologic foundations of medicine. 5th edition. Lippincott Williams and Wilkins 2008; 1-35.
28. Hines RN, McCarver DG. The ontogeny of human drug metabolizing enzymes: Phase I oxidative enzymes. J Pharmacol Exp Ther 2002; 300: 355-360.
29. Kearns CF, Mckeever KH, Malinowski K, Struck MB and Abe T. Chronic administration of therapeutic levels of clenbuterol act as repartitioning agent. J Appl Physiol 2001; 91: 2064-2070.
30. Vaidya AB, Antarkar DS, Doshi JC, Bhatt AD, Ramesh VV, Vora PV, Perissond DD, Baxi AJ, Kale PM. *Picrorhiza kurroa* (Kutaki) Royle ex Benth as a hepatoprotective agent-experimental and clinical studies. J Postgraduate Med 1996; 42 (4): 105-108.

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