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Protective effect of *Piper longum* L. on oxidative stress induced injury and cellular abnormality in adriamycin induced cardiotoxicity in rats

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Effect of methanolic extract of fruits of *P. longum* (PLM) on the biochemical changes, tissue peroxidative damage and abnormal antioxidant levels in adriamycin (ADR) induced cardiotoxicity in Wistar rats was investigated. PLM was administered to Wistar albino rats in two different doses, by gastric gavage (250mg/kg and 500mg/kg) for 21 days followed by ip ADR (15 mg/kg) on 21st day. ADR administration showed significant decrease in the activities of marker enzymes aspartate transaminase, alanine transaminase, lactate dehydrogenase and creatine kinase in heart with a concomitant increase in their activities in serum. A significant increase in lipid peroxide levels in heart of ADR treated rats was also observed. Pretreatment with PLM ameliorated the effect of ADR on lipid peroxide formation and restored activities of marker enzymes. Activities of myocardial antioxidant enzymes like catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase along with reduced glutathione were significantly lowered due to cardiotoxicity in rats administered with ADR. PLM pretreatment augmented these endogenous antioxidants. Histopathological studies of heart revealed degenerative changes and cellular infiltrations in rats administered with ADR and pretreatment with PLM reduced the intensity of such lesions. The results indicate that PLM administration offers significant protection against ADR induced oxidative stress and reduces the cardiotoxicity by virtue of its antioxidant activity.

Keywords: Adriamycin, Cardiotoxicity, Lipid peroxidation, Oxidative stress, *Piper longum*

Adriamycin (ADR), an antitumor antibiotic of the group anthracyclines, has been an effective agent against a variety of human cancers. However, the drug has acute and chronic serious side effects. Long term administration of adriamycin has been observed to cause a cumulative dose dependent cardiotoxicity. Several hypotheses have been proposed to account for the ADR cardiotoxicity, including free radical formation[1,2], impaired adrenergic regulation, the release of vasoactive amines, altered calcium handling, mitochondrial impairment, the suppression of muscle specific genes and ceramide generation[3-8]. The most widely accepted mechanism of ADR-induced cardiotoxicity is the formation of free radicals which is a rate limiting process in tissue peroxidative damage. In free radicals induced oxidative stress, the accumulation of lipid peroxides introduces hydrophilic moieties into membrane hydrophobic phase and thus alters membrane permeability and cell function[9]. This leads to loss of myocardial structural integrity and depressed cardiac function resulting in cardiotoxicity and congestive cardiac failure.

Protective effects of natural antioxidants against toxicity induced by chemotherapeutic agents, involving free radical generations have been reported[10]. Therapeutic strategies, designed to augment cellular indigenous defense systems have been identified as a promising approach to combat oxidative stress associated disease conditions [11,12].

Piper longum L. (Family: Piperaceae) commonly known as *Pippali* in Hindi or long pepper in English is widely used as a household remedy in treating respiratory disorders. Several biological activities of *P. longum* extract have been reported, including antiamoebic, antigiardial, immunostimulatory, antiulcer and anti-inflammatory properties[13-16]. The major chemical constituents of the plant are volatile oil, resin and alkaloids viz. piperine, piperlongumine, piperlonguminine etc. Methanolic extract of dried fruits of *P. longum* (PLM) was found to possess signi-

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ficant *in vitro* antioxidant potential and hepatoprotective activity[17]. Ayurvedic system of medicine recommends *P. longum* for treating cardiac disorders[18]. Hence, the present study has been undertaken to evaluate the cardioprotective potential of *P. longum* fruits against ADR induced cardiotoxicity.

Materials and Methods

Plant material and extraction—*Piper longum* fruits were procured locally, authenticated by Deptartment of Botany, Ruia College, Mumbai and extracted with methanol using Soxhlet apparatus. Methanolic extract was dried at 40°C using a vacuum evaporator and used for investigating cardioprotective activity.

Chemicals—Adriamycin (doxorubicin hydrochloride) injection was obtained from Biochem Pharmaceuticals, Mumbai, India. Thiobarbituric acid and glutathione (GSH) were purchased from Sisco Research Laboratory, Mumbai, India. The kits used for biochemical analysis were obtained from Merck India Limited, Mumbai. All other chemicals and solvents were of analytical grade and were procured from commercial sources locally.

Animals—Male albino rats of Wistar strain (150 ± 10g) used for the study were housed under standard hygienic conditions maintaining at 12:12 hr light and dark cycle and fed with standard pelleted diet and water *ad libitum*. The experiment was approved in accordance with the Institutional Animal Ethics Committee.

Experimental design—Rats were divided randomly into following 4 groups of 6 rats each.

Group I: Normal control rats which received only vehicle
Group II: Negative control receiving single ip injection of adriamycin (15mg/ kg)
Group III: PLM 250 mg/kg po for 21 days + ADR (day 21)
Group IV: PLM 500 mg/kg po for 21 days + ADR (day 21)

Animals were euthanized under light ether anesthesia 24 hr after the injection of ADR. Blood was collected and serum was separated by centrifugation. Heart was dissected out and immediately washed in ice cold physiological saline and homogenized in 100 m*M* Tris-HCl buffer (*p*H 7.4) to render a 10% homogenate. Aliquots of the tissue homogenate were suitably processed for biochemical assays, lipid peroxidation and antioxidant studies. A portion of heart tissue was preserved in 10% formalin (*p*H 7.2) and subjected to histopathological studies.

Biochemical analysis for assessment of oxidative stress—Diagnostic marker enzymes, such as alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK) which act as enzymatic indices of cellular damage were estimated in serum and heart homogenate using standard biochemical kits. Activities of ALT, AST and LDH were determined and expressed in terms of µ moles of pyruvate liberated/min/mg of protein at 37°C and CK was expressed as µ moles of phosphorus liberated/min/mg of protein at 37°C[19-21]. Lipid peroxidation in the heart tissue was determined by the method of Hogberg *et al*[22] and malondialdehyde (MDA) produced during peroxidation of lipids, served as an index of lipid peroxidation. Reduced glutathione (GSH) levels in heart were measured colorimetrically as protein-free sulfhydryl content using Ellman reagent[23]. The degree of inhibition of the autoxidation of pyrogallol at an alkaline *p*H by superoxide dismutase (SOD) was used as a measure of the total enzyme activity[24]. The activity of catalase (CAT) in myocardium was assayed by method of Sinha *et al*[25]. The activity of glutathione peroxidase (GPOX) in heart was assayed by monitoring the increase in the oxidized guaiacol at 470 nm for 1 min by the method of Chance and Maehly[26]. Glutathione reductase (GR) activity in heart was assayed by monitoring the oxidation of NADPH to $NADP^+$ at 340 nm by the method of Sheadle *et al*[27].

Statistical analysis—Values were expressed as mean ± S.D. The results were statistically evaluated using one-way ANOVA followed by Bonferroni comparison test using Graph Pad software with $P < 0.05$ considered significant.

Results

Effect of PLM on serum marker enzymes—Activities of ALT, AST, LDH and CK in serum of normal and experimental rats is presented in Table 1. Activities of these marker enzymes were increased in serum of ADR induced rats as compared to normal rats. Pretreatment with PLM at doses of 250 and 500 mg/kg for a period of 21 days significantly ($P < 0.05$) decreased the activities of these enzymes in

ADR treated rats as compared to rats treated only with ADR.

Effect of PLM on heart marker enzymes—Effect of PLM on heart marker enzymes of control and experimental rats is presented in Table 1. Activities of these marker enzymes were decreased in hearts of ADR induced rats compared to normal rats. Pretreatment with PLM at doses of 250 and 500 mg/kg for a period of 21 days significantly ($P < 0.05$) increased activities of these enzymes in ADR treated rats compared to rats treated only with ADR.

Effect of PLM on MDA and GSH content of heart—Activities of lipid peroxidation products (MDA) and reduced glutathione (GSH) content of heart in control and experimental animals are presented in Table 2. There was significant ($P < 0.05$) increase in myocardial MDA levels in the ADR treated group compared to control. A significant decrease ($P < 0.05$) in the levels of MDA was observed in PLM pretreated rats (Groups III and IV) compared to rats treated only with ADR. ADR treatment significantly decreased ($P < 0.05$) myocardial GSH compared to control. There was significant increase ($P < 0.05$) in GSH levels in the PLM pretreated groups after ADR administration compared to rats treated only with ADR.

Table 1—Effect of *P. longum* methanolic extract (PLM) pretreatment on adriamycin (ADR) induced changes in the activities of serum (S) and heart (H) alanine transaminase (ALT), aspartate transaminase (AST) lactate dehydrogenase (LDH) and creatine kinase (CK)
[Value are expressed as mean ± SD for 6 rats in each group]

Treatment group	ALT[a]	AST[a]	LDH[a]	CK[b]
Group I (Control)	(S) 70.25 ± 7.90 (H) 22.25 ± 0.88	(S) 12.26 ± 0.094 (H) 40.13 ± 4.51	(S) 140.25 ± 12.17 (H) 66.25 ± 4.12	(S) 120.25 ± 8.91 (H) 60.25 ± 4.55
Group II (Negative control)	(S) 136.98 ± 8.45[#] (H) 12.15 ± 0.98[#]	(S) 24.55 ± 0.242[#] (H) 17.25 ± 3.55[#]	(S) 225.12 ± 14.26[#] (H) 35.65 ± 3.25[#]	(S) 188.66 ± 9.18[#] (H) 31.22 ± 3.11[#]
Group III (PLM 250 mg/kg + ADR)	(S) 119.8 ± 6.68* (H) 14.55 ± 1.22*	(S) 18.05 ± 0.127* (H) 26.24 ± 3.44*	(S) 192.4 ± 12.33* (H) 42.30 ± 2.78*	(S) 173.5 ± 8.66* (H) 40.21 ± 3.24*
Group IV (PLM 500 mg/kg + ADR)	(S) 105.6 ± 7.5* (H) 15.84 ± 1.77*	(S) 16.93 ± 0.185* (H) 29.10 ± 4.12*	(S) 183.45 ± 11.22* (H) 47.25 ± 3.22*	(S) 165.20 ± 7.71* (H) 43.12 ± 4.55*

Units: [a]μmoles of pyruvate liberated/min/L. [b]μmoles of phosphorus liberated/min/L.
Significantly different as compared to [#]vehicle control, significantly different as compared to *negative control, One Way ANOVA, $P < 0.05$ considered significant.

Table 2—Effect of *P. longum* methanolic extract (PLM) pretreatment on adriamycin (ADR) induced changes in the activities of heart antioxidant enzymes, malondialdehyde (MDA) and reduced glutathione (GSH)
[Value are expressed as mean ± SD for 6 rats in each group]

Treatment Group	SOD[a]	CAT[b]	GPOX[c]	GR[d]	MDA[e]	GSH[f]
Group I (Control)	8.44 ± 0.45	19.75 ± 2.33	16.25 ± 1.98	12.98 ± 1.04	255.03 ± 6.10	1115.5 ± 12.65
Group II (Negative control)	4.12 ± 0.77[#]	7.98 ± 2.06[#]	8.98 ± 2.05[#]	7.88 ± 0.99[#]	340.19 ± 18.85[#]	568.03 ± 10.5[#]
Group III (PLM 250 mg/kg + ADR)	5.30 ± 0.55*	12.85 ± 3.01*	10.8 ± 1.22[ns]	9.66 ± 0.86*	310.78 ± 13.25*	802.10±10.11*
Group IV (PLM 500 mg/kg + ADR)	5.89 ± 0.41*	14.99 ± 2.11*	13.21 ± 1.85*	10.14 ± 1.12*	298.44± 16.60*	845.77 ± 9.25*

Units: [a]units (mg protein) -1, [b]μmol H_2O_2 consumed min-1 (mg protein) -1, [c]μmol GSH consumed min-1 (mg protein) -1, [d]nmol NADH oxidized min-1 (mg protein) -1, [e]nmol/g wet tissue, [f]μg/g wet tissue
Significantly different as compared to [#]vehicle control, significantly different as compared to *negative control, [ns] non significant as compared to negative control. One Way ANOVA, P value < 0.05 considered significant

Effect of PLM on heart antioxidant enzymes—Effect of PLM administration on antioxidant enzymes in heart tissue of control and experimental animals are shown in Table 2. ADR treated rats showed a significant ($P < 0.05$) decrease in the activities of SOD, CAT, GPOX and GR compared to control rats. PLM pretreated rats (Groups III & IV) showed a significant ($P < 0.05$) increase in the activities of these myocardial enzymes compared to rats treated only with ADR.

Effect of PLM on histopathology of heart—Figure 1a shows the light micrograph of control group heart with normal architecture. The ADR induced rat heart show the separation of muscle fibers with inflammatory infiltration (Fig. 1b). No significant changes in the light micrograph were observed in the groups administered with PLM at the dose of 250 mg/kg and 500 mg/kg groups (Figs 1c and 1d).

Discussion

Adriamycin (ADR) is one of the most frequently used chemotherapeutic drugs in the treatment of cancer[28]. Despite the wide use of ADR in the treatment of cancer patients, the mechanism of action not well established. Several mechanisms may account for the effects of anthracycline, both in terms of anticancer action and of cardiac and other organ toxicity[29]. Most widely reported and accepted aspect

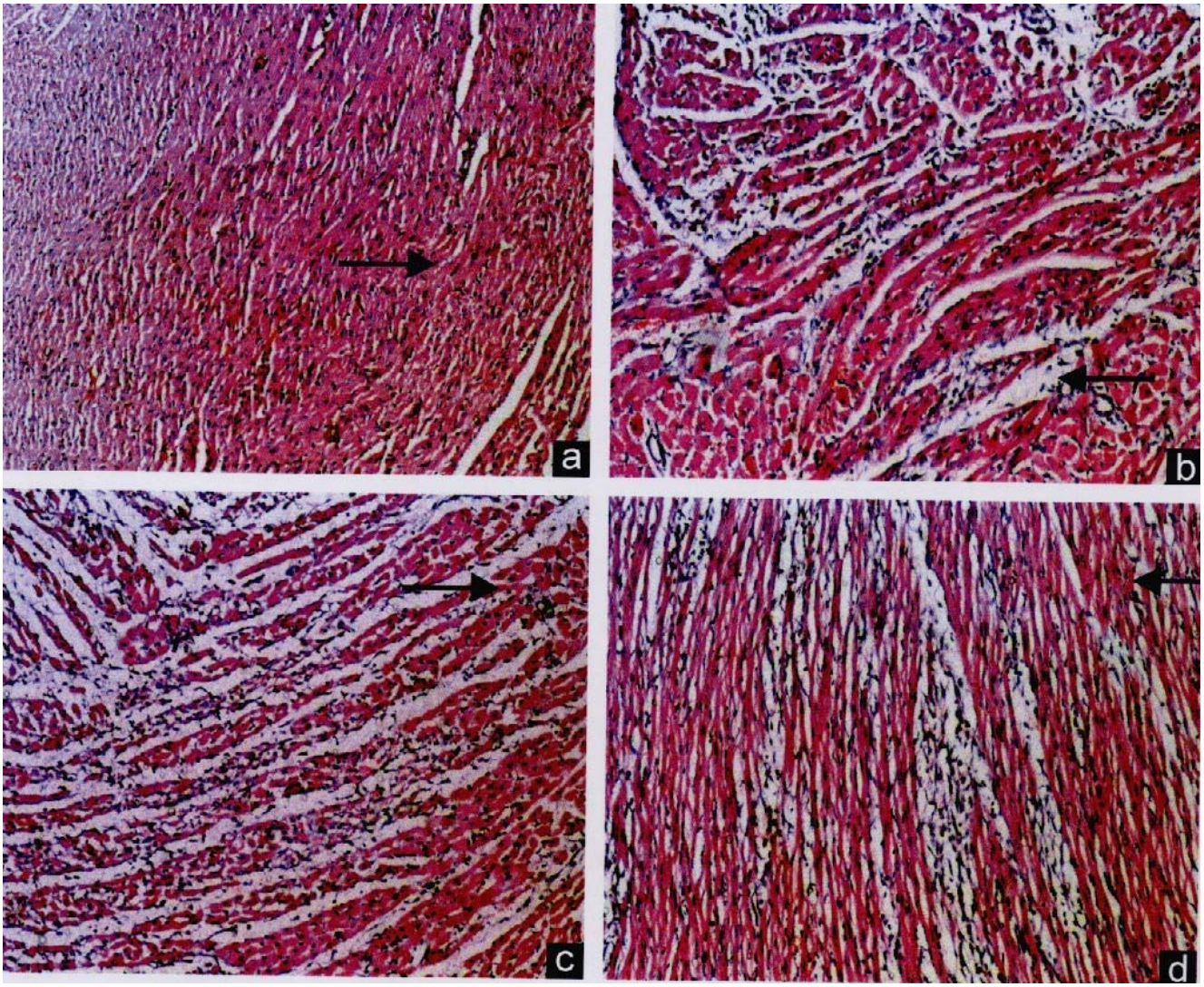

Fig. 1—(a) Light micrograph of control heart showing normal architecture. (b) ADR induced rat heart showing separation of muscle fibers with inflammatory infiltration. No significant change in the light micrograph was observed in the PLM 250 mg/kg (c) and PLM 500 mg/kg (d) groups × 200.

is the involvement of oxidative stress and the production of free radicals involved in ADR action, in relation to both anticancer effects and toxicity. Two different mechanisms of free radical formation by ADR have been described. The first implicates the formation of a semiquinone free radical by the action of several NADPH dependent reductases that produce one-electron reduction of the ADR to the corresponding ADR semiquinone. In the presence of oxygen, redox cycling of ADR-derived quinine-semiquinone yields superoxide radicals ($O_2^{\bullet -}$). In the second, ADR free radicals are produced by a non-enzymatic mechanism that involves reactions with iron. Iron-ADR complex can reduce oxygen to H_2O_2 and other active oxygen species[30,31].

Experimental studies in animals using ADR aid in the understanding of the drug's unfavorable cardiac toxicities, and several therapeutic strategies have been evaluated to counter the adverse effects which aim to limit free radical mediated cardiac injury. Myocardial marker enzymes viz. ALT, AST, LDH and CK serve as sensitive indices to assess the extent of cardiotoxicity due to ADR[32]. Results of the present study indicate that the high dose administration of ADR increased the serum enzyme content of these marker enzymes. This increase in enzymes in serum may be due to an increase in their release following ADR induced lipid peroxidation of cardiac membranes. It has been reported that as a result of lipid peroxidation, inflammatory cells accumulate in cardiac myocytes[33].

Increase in tissue enzyme activities, especially aminotransferases, has been reported in these experimental inflammatory conditions[34]. A significant increase in serum marker enzyme levels with their subsequent reduction in heart of the ADR treated rats as compared to the normal control rats was observed. Compared to rats treated with only ADR, rats pretreated with PLM restored these levels of enzymes indicating ameliorative effect of PLM on myocardium. The cardiotoxicity and oxidative damage induced by ADR administration are also manifested by a significant increase in the cardiac content of MDA along with a significant decease in levels of GSH. The association between elevated cardiac content of MDA and lowered cardiac content of GSH, found in this study, strongly proves the oxidative damage caused by ADR[35,36]. Pretreatment with PLM in ADR-induced rats restored the depleted GSH levels along with significant lowering of cardiac MDA content.

Pharmacological augmentation of endogenous myocardial antioxidants has been identified as a promising therapeutic approach in diseases associated with increased oxidative stress. CAT, which hydrolyses H_2O_2, has been found to be superior in improving cardiac function to SOD suggesting that H_2O_2 has a significant role in ADR-induced cardiotoxicity[37,38]. ADR directly stimulates the formation of superoxide anion radical, particularly in the heart mitochondria. In spite of the induction of defense enzyme, such as CAT, its abilities seem to be swamped by enhanced active oxygen radicals. In heart, GPOX is extremely important because of its ability to use and remove organic and inorganic peroxides. There was significant decrease in the myocardial endogenous antioxidants viz. SOD, CAT, GPOX and GR following ADR administration in rats owing to the compensatory mechanism of myocardium for removal of excess prooxidants.

In PLM pretreated rats, there was restoration of the levels of these antioxidant enzymes. This modulatory effect of PLM could be due to its ability to scavenge free radicals thus acting as an antioxidant by reducing prooxidant load and sparing antioxidant enzymes. Histopathological observations of the heart tissue of rats challenged with ADR showed confluent necrosis and separation of muscle fibers along with inflammatory infiltrations. This type of myocardial damage including myofibrillar degeneration, mitochondrial dilatation and cellular vacuolization is specific to all anthracycline antibiotics[39]. These legions were significantly reduced in groups pretreated with PLM. In conclusion, chronic oral administration of PLM 250 mg/kg and PLM 500 mg/kg prevented ADR induced alterations in marker enzyme activity, endogenous antioxidant levels and cellular damage. Thus methanolic extract of *Piper longum* L. fruits offers significant protection against adriamycin induced myocardial oxidative stress induced injury in rats by virtue of its anti-oxidative potential.

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