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Protective effect of *Piper longum* fruits against experimental myocardial oxidative stress induced injury in rats

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Abstract

The present study investigated the protective effect of methanolic extract of fruits of *Piper longum* L. (PLM) on isoproterenol (ISO) induced myocardial infarction in rats. PLM was administered to Wistar albino rats in two different doses, by gastric gavage for 28 days. Myocardial infarction was induced by subcutaneous administration of ISO for 2 days at the end of the dosing period. ISO administration resulted in significant decrease in the activities of marker enzymes aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and creatine kinase (CK) in heart with a concomitant increase in their activities in serum. A significant increase in lipid peroxide levels (TBARS) in hearts of ISO-induced rats was also observed. Activities of myocardial antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPOX) and glutathione reductase (GR) along with reduced glutathione (GSH) were significantly lowered owing to myocardial infarction in ISO treated rats. PLM pretreatment was found to ameliorate the effect of ISO on lipid peroxide formation and retained activities of marker enzymes. It also prevented ISO-induced decrease in antioxidant enzymes in the heart. The histopathological studies of heart revealed a protective role PLM in ISO treated rats. The results exhibited that the pretreatment with methanolic extract of *Piper longum* fruits may be useful in preventing the damage induced by ISO in rat heart.

Keywords: *Piper longum*, myocardial infarction, isoproterenol, lipid peroxides, antioxidant enzymes

1. Introduction

Cardiovascular disease remains the principal cause of death in both developed and developing countries, accounting for roughly 20% of all worldwide deaths per year [1]. Myocardial infarction, the most dreaded among ischemic heart diseases, is invariably followed by several biochemical alterations, such as lipid peroxidation, free radical damage, hyperglycemia, hyperlipidemia etc. leading to qualitative and quantitative alterations of myocardium [2]. The pathogenic mechanism of myocardial ischemic damage is still not completely understood, but the role of oxygen-derived free radicals in myocardial ischemia is

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established, although not completely characterized [3]. Oxidative stress incurred by free radicals is implicated in the etiopathogenesis of myocardial ischemia and has a major influence on the development of coronary artery diseases [4]. Isoproterenol, a synthetic catecholamine and β adrenergic agonist, which at higher doses has been reported to cause severe oxidative stress in the myocardium, resulting in infarct like necrosis and serves as a well-standardized model for studying certain physiological and pathological events during the course of acute myocardial infarction [5]. Several plant products are known to exhibit credible medicinal properties for the treatment of heart ailments and need to be explored to identify their potential application in prevention and therapy of human ailments. It might be possible to limit oxidative stress induced myocardial ischemic damage if medicinal plant extracts exhibiting antioxidant potential were administered. *Piper longum* L. (Family: Piperaceae) commonly known as Pippali or long pepper, is reported to be useful in the treatment of respiratory ailments, allergy, recurrent attacks of bronchial asthma etc. Several biological activities of *Piper longum* extract have been reported, including antiamoebic, antigiardial, immunostimulatory, antiulcer and anti-inflammatory properties [6-8]. The major chemical constituents of the plant are volatile oil, resin and alkaloids viz. piperine, piperlongumine, piperlonguminine etc. [9]. Methanolic extract of dried fruits of *Piper longum* (PLM) was found to possess significant *in vitro* antioxidant potential and hepatoprotective activity [10]. Ayurveda recommends *Piper longum* in the treatment of cardiac disorders. Hence the present study was undertaken to evaluate the cardioprotective potential of *P. longum* fruits against free radical induced oxidative stress in experimental animals.

2. Materials and Methods

2.1 Plant material and extraction

The authenticated fruits of *P. longum* were obtained from the local sources and extracted with methanol using soxhlet apparatus. The methanolic extract was dried at 40°C using a vacuum evaporator and then investigated for cardioprotective activity.

2.2 Chemicals

Isoproterenol hydrochloride was obtained from Sigma Chemical Co., USA. The biochemical kits used for biochemical analysis were obtained from Merck India Limited, Mumbai. All other chemicals were procured from standard local sources.

2.3 Animals

Male Wistar rats weighing between 150-180g were used for the study. Animals were housed under standard hygienic conditions, maintaining 12/12 h light and dark cycle and fed with standard pelleted diet and water *ad libitum*. All the experiments were performed in accordance with the Institutional Animal Ethics Committee (IAEC) constituted protocol as per directions of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), under the Ministry of Animal Welfare Division, Government of India, New Delhi.

2.4 Acute toxicity study

Oral acute toxicity study in Wistar rats were carried out for PLM in accordance with OECD guideline no.423 and extract was found to be safe at the dose 5000 mg/kg.

2.5 Treatment groups

The rats were divided randomly into four groups with six rats in each group.

Group I: Control rats received only water for injection.

Group II: ISO (85 mg/kg) was given by subcutaneous injection (*s.c.*) for 2 days at an interval of 24 hours (Day 28 & 29)

Group III: PLM 250 mg/kg *p.o.* for 28 days + ISO *s.c.* (Day 28 & 29)

Group IV: PLM 500 mg/kg *p.o.* for 28 days + ISO *s.c.* (Day 28 & 29)

2.6 Post treatment investigations

Twenty four hours after the last injection of ISO, the animals were euthanized under light ether anesthesia. Blood was collected and serum was separated by centrifugation. Hearts were dissected out and immediately washed in ice-cold saline and a homogenate was prepared in 0.1 M Tris HCl buffer (pH 7.4). The homogenate was centrifuged and the supernatant was used for biochemical estimations. A portion of heart tissue was preserved in 10% formalin (pH 7.2) and subjected to histopathological studies.

2.7 Biochemical Analysis

Marker enzymes, such as alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK) were estimated in serum and heart of experimental animals using standard biochemical kits. Myocardial thiobarbituric acid reactive substances (TBARS) were estimated as a marker of lipid peroxidation. Myocardial reduced glutathione (GSH) was estimated by the method of Ellman et al [11]. Superoxide dismutase (SOD) levels in heart were estimated by the method of Kakkar et al [12] and catalase (CAT) by the method of Chance & Maehly [13]. Other myocardial endogenous antioxidant enzymes viz. glutathione peroxidase (GPOX) and glutathione reductase (GR) were also estimated using standard assay procedures [14-15].

2.8 Statistical analysis

All values were expressed as mean ± S.D. The results were statistically evaluated using one-way analysis of variance (ANOVA) followed by Bonferroni comparison test using Graph Pad software with p value < 0.05 considered significant.

3. Results

3.1 Effect of PLM on serum marker enzymes

The effect of PLM on serum marker enzymes of control and experimental animals are shown in Table 1. ISO treated rats showed a significant (p<0.05) increase in serum marker enzymes AST, ALT, LDH and CK when compared with control. PLM pretreated rats showed significantly (p<0.05) decreased levels of serum marker enzymes activities as compared with Group II rats (Negative control).

3.2 Effect of PLM on heart marker enzymes

The effect of PLM on heart marker enzymes of control and experimental animals are shown in Table 2. ISO treated rats showed a significant (p<0.05) decrease in heart marker enzymes AST, ALT, LDH and CK when compared with control. PLM pretreated rats showed significantly (p<0.05) increased levels of heart marker enzymes activities as compared with Group II rats.

3.3 Effect of PLM on heart antioxidant enzymes

The effect of PLM on heart antioxidant enzymes of control and experimental animals are shown in Table 3. ISO treated rats showed a significant (p<0.05) decrease in the levels of SOD, CAT, GPOX and GR when compared with control rats. PLM pretreated rats showed significantly (p<0.05) increased activities of these myocardial enzymes as compared with ISO treated rats.

Table 1. Effect of PLM pretreatment on ISO-induced changes in the activities of serum AST, ALT, LDH and CK

Groups	Treatment	AST	ALT	LDH	CK
Vehicle Control	Water for injection	10.98 ± 0.072	46.463 ± 5.012	120.34 ± 11.57	245.6 ± 7.52
Negative Control	ISO 85 mg/kg	21.95 ± 0.187 #	88.076 ± 5.554 #	190.58 ± 12.35 #	464.81± 11.25 #
Test group 1	PLM 250 mg/kg + ISO 85 mg/kg	19.85 ± 0.154 ns	75.28 ± 5.39 **	168.9 ± 11.25 *	398.5 ± 13.65 ***
Test group 2	PLM 500 mg/kg + ISO 85 mg/kg	18.15 ± 0.165 **	70.25 ± 6.41 ***	161.25 ± 12.15 **	344.21 ± 11.22 ***

[The levels of LDH, ALT and AST are expressed as µmoles of pyruvate liberated/min/L. The levels of CK are expressed as ìmoles of phosphorus liberated/min/L.]
Values are expressed as Mean ± S.D. (n = 6) One Way ANOVA, with *P* value < 0.05 considered significant. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), # significantly different as compared to vehicle control, * significantly different as compared to negative control, ns non-significant as compared to negative control.

Table 2. Effect of PLM pretreatment on ISO-induced changes in the activities of heart AST, ALT, LDH and CK

Groups	Treatment	AST	ALT	LDH	CK
Vehicle Control	Water for injection	40.13 ± 2.90	133.90 ± 7.5	53.06 ± 1.464	48.15 ± 3.32
Negative Control	ISO 85 mg/kg	19.99 ± 2.42 #	75.90 ± 6.02 #	32.82 ± 1.203 #	28.67 ± 2.16 #
Test group 1	PLM 250 mg/kg + ISO 85 mg/kg	25.98 ± 3.22 *	99.20 ± 22.5 **	36.45 ± 1.95 **	35.51 ± 2.75 *
Test group 2	PLM 500 mg/kg + ISO 85 mg/kg	28.36 ± 2.98 ***	106.92 ± 12.82 ***	43.24 ± 1.35 ***	39.27 ± 3.44 ***

[The levels of LDH, ALT and AST are expressed as µmoles of pyruvate liberated/min/L. The levels of CK are expressed as ìmoles of phosphorus liberated/min/L.]
Values are expressed as Mean ± S.D. (n = 6) One Way ANOVA, with *P* value < 0.05 considered significant. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), # significantly different as compared to vehicle control, * significantly different as compared to negative control.

Table 3. Effect of PLM pretreatment on ISO-induced changes in the heart antioxidant enzymes

Groups	Treatment	SOD	CAT	GPOX	GR
Vehicle Control	Water for injection	5.62 ± 0.42	5.89 ± 0.33	13.18 ± 1.43	8.45 ± 0.88
Negative Control	ISO 85 mg/kg	3.21 ± 0.38 #	3.42 ± 0.27 #	6.52 ± 0.86 #	6.14 ± 0.59 #
Test group 1	PLM 250 mg/kg + ISO 85 mg/kg	4.12 ± 0.41 *	4.11 ± 0.17 **	9.25 ± 1.12**	7.24 ± 0.54 ns
Test group 2	PLM 500 mg/kg + ISO 85 mg/kg	4.83 ± 0.58 ***	4.65± 0.26***	11.2 ± 1.05 ***	7.75 ± 0.95 *

[Superoxide dismutase: units (mg protein) -1, Catalase: µmol H_2O_2 consumed min-1 (mg protein) -1, Glutathione peroxidase: µg of GSH consumed min-1 (mg protein) -1, Glutathione reductase: nmol NADH oxidized min-1 (mg protein) -1], Values are expressed as Mean ± S.D. (n = 6) One Way ANOVA, with P value < 0.05 considered significant. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), # significantly different as compared to vehicle control, * significantly different as compared to negative control, ns non-significant as compared to negative control.

Table 4. Effect of PLM pretreatment on ISO-induced changes in TBARS and GSH in heart.

Groups	Treatment	TBARS nmoles/mg protein	GSH nmoles/g protein
Vehicle Control	Water for injection	2.90 ± 0.107	5.22 ± 0.098
Negative Control	ISO 85 mg/kg	4.52 ± 0.176 #	3.75 ± 0.16 #
Test group 1	PLM 250 mg/kg + ISO 85 mg/kg	3.51± 0.186 ***	4.05 ± 0.08 ***
Test group 2	PLM 500 mg/kg + ISO 85 mg/kg	3.26 ± 0.127***	4.56 ± 0.15 ***

Values are expressed as Mean ± S.D. (n = 6) One Way ANOVA, with P value < 0.05 considered significant. (* P < 0.05, ** P < 0.01, *** P < 0.001), # significantly different as compared to vehicle control, * significantly different as compared to negative control.

Histopathology

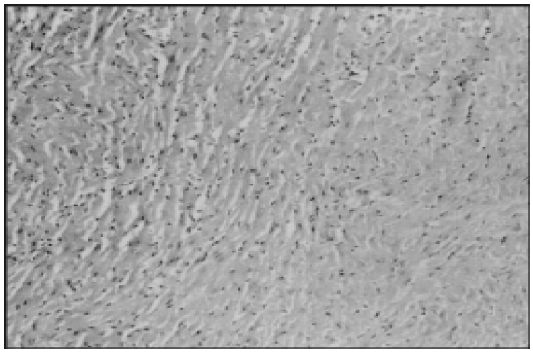

Figure 1

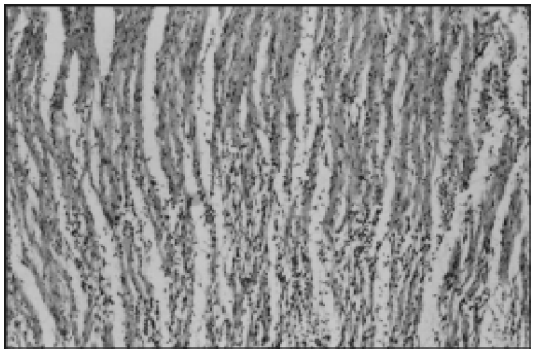

Figure 2

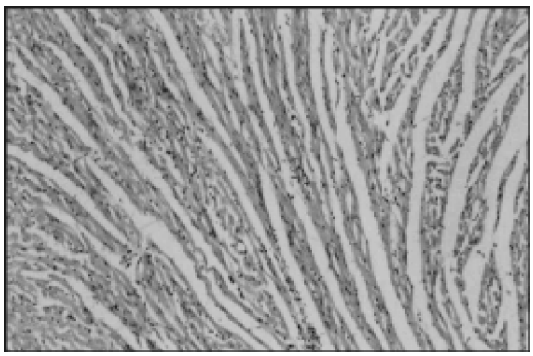

Figure 3

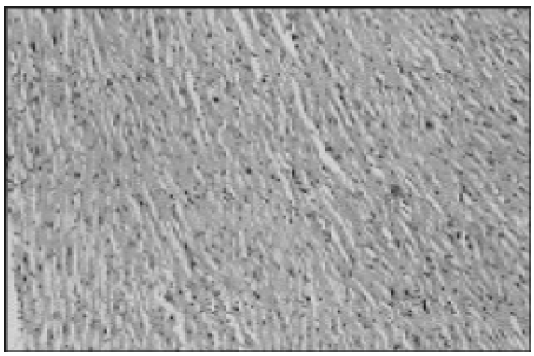

Figure 4

Photomicrographs of heart section from rat from normal group Figure 1, negative control group Figure 2, , PLM 250 mg/kg p.o. treatment group Figure 3 and PLM 500 mg/kg p.o. treatment group Figure 4.

3.4 Effect of PLM on TBARS and GSH content of heart

The activities of lipid peroxidation products (TBARS) and reduced glutathione (GSH) content of heart in control and experimental animals are shown in Table 4. The ISO treated rats exhibited decrease in myocardial GSH levels with consequent increase in TBARS levels, which were restored by pretreatment with PLM pretreated rats.

3.5 Effect on histopathology of heart

Figure 1 shows the light micrograph of control heart showing normal architecture. The ISO induced rat heart showed the separation of muscle fibers with inflammatory infiltration (Figure 2). No significant change in the light micrograph was observed in the PLM 250 mg kg and PLM 500 mg/kg groups (Figure 3, 4).

4. Discussion

It has been proposed that ISO-induced metabolic and morphologic aberrations in the heart tissue of the experimental animals have been reported to be similar to those observed in human myocardial infarction [16]. Oxidative metabolism of catecholamines produces quinones, which react with oxygen to generate superoxide anions and hydrogen peroxides. ISO thus, causes myocardial ischemia due to excessive production of oxygen derived free radicals resulting from this oxidative metabolism of catecholamines [17]. The free radicals generated by ISO administration initiate lipid peroxidation of membrane bound poly-unsaturated fatty acids, leading to an impairment of structural and functional integrity of myocardial membrane. The serum enzymes viz AST, ALT, LDH and CK serve as sensitive indices to assess the severity of myocardial infarction [18-19]. Due to impaired cellular integrity of myocardium, these marker enzymes get leaked in the blood stream. Hence, in ISO treated rats, the increased activities of serum ALT, AST, LDH and CK accompanied by their concomitant reduction in heart homogenate confirm the onset of myocardial necrosis [20]. In the present study, near normal activity of the diagnostic marker enzymes in the serum and heart tissue of rats treated with PLM and challenged with ISO is an indicative of the fact that PLM has significant cardioprotective effect. Lipid peroxidation plays an important role in the myocardial cell damage and the accumulated lipid peroxides reflect the various stages of diseases and its complications [21-22].

Increased levels of lipid peroxidation products injure blood vessels, causing increased adherence and aggregation of platelets to the injured sites [23]. ISO treated rats showed significantly elevated levels of lipid peroxides (TBARS) in heart, which were restored by PLM pretreatment thus, protecting heart from lipid peroxidative damage. The decrease in the activities of myocardial antioxidant enzymes following the oxidative stress is in close relationship with the induction of lipid peroxidation [24]. Myocardial GSH has a direct antioxidant function by reacting with free radicals, followed by formation of oxidized GSH and other disulfides [25]. It forms an important substrate for other antioxidants viz. GPOX, GR etc, which is involved in the free radical scavenging action [26-27].

Pretreatment with PLM restored the depleted GSH levels along with levels of GPOX & GR in heart thereby offering protection through antioxidant mechanism. In the present study, ISO-induced rats exhibited decrease activities of SOD and CAT in the heart. SOD plays an important role in protecting the cells from

oxidative damage by converting superoxide radicals into hydrogen peroxide, which is further metabolized by CAT to molecular oxygen and water. The decrease in the activities of these enzymes might be due to myocardial cell damage. Pretreatment with PLM significantly increased the activities of SOD and CAT in the hearts of ISO-induced rats, which demonstrated an important role of PLM in regulating anti-oxidative capacity.

Histopathological observations of the heart tissue of rats challenged with ISO showed confluent necrosis, separation of muscle fibers and inflammatory infiltrations. PLM pretreatment protected these morphological changes in a dose dependent manner, thus supporting the cardioprotective activity of PLM.

5. Conclusion

In conclusion, oral administration of PLM 250 mg/kg and PLM 500mg/kg prevented isoproterenol-induced alterations in marker enzyme activity, endogenous antioxidant levels and cellular damage. This overall cardioprotective effect was probably due to the effect of PLM as an antioxidant which acts by counteracting free radicals or by its ability to maintain the normal status of the activities of antioxidant enzymes. Combined effect of active principles present in the methanolic extract of *P. longum* might offer the protection against cardiac damage rendered by isoproterenol in rats. Thus, methanolic extract of fruits of *P. longum* exhibits significant cardioprotective potential against myocardial oxidative stress induced injury in rats.

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