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RESEARCH ARTICLE

Cardioprotective Effect of *Banaba* on Myocardial Ischemia/Reperfusion Injury in Rats

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Abstract

The ethanol extract of *Banaba* (*Lagerstroemia speciosa* L.) leaves, was investigated for their effects on ischemia/reperfusion (I/R) injury induced by occlusion of the left descending coronary artery (LCA) for 1 h, followed by re-opening again for 1 h. *Banaba* ethanol extract (100 mg/kg) intraperitonially (i.p.) was administered 30 min after induction of ischemia. During I/R period, the hemodynamics and ECG parameters were measured. Oxidative stress markers like reduced glutathione (GSH), Superoxide dismutase (SOD), malondialdehyde (MDA), activities of myeloperoxidase (MPO), creatine kinase (CK) and myocardial infarct area were significantly ($P<0.05$) reduced in Wistar rats after *Banaba* treatment. The apoptotic activity and histological observations were influenced by *Banaba*. The cardioprotective effect of *Banaba* could be attributed to its ability to improve the antioxidant mechanism, anti-inflammatory and anti-apoptotic activity in ischemic animals.

Keywords: Antioxidant, Anti-inflammatory and Anti-apoptotic, *Lagerstroemia speciosa* L.

1. Introduction

In clinical interventions of the conditions such as myocardial infarction, percutaneous coronary interventions, thrombolytic therapy and coronary artery bypass grafting are linked with myocardial ischemia/reperfusion (I/R) injury[1]. Reperfusion of coronary blood flow is the primary requirement to salvage ischemic tissue. Brief periods of partial or severe ischemia can be tolerated by the myocardium without cardiomyocyte injury, but prompt arterial reperfusion results in myocardial ischemic injury which may be severe and irreversible. This injury due to reperfusion i.e. reperfusion injury could be intense if the period and the severity of ischemia increases[2]. The reperfusion injury results in myocardial damage due to the destabilization of the delicate endogenous antioxidant defense mechanism because there is excessive production of Reactive Oxygen Species (ROS), which leads to the generation of lipid peroxides by reacting with unsaturated free fatty acids. The lipid peroxides damages sarcolemma and alters membrane permeability resulting in elevated levels of malondialdehyde (MDA)[2,3]. The contractile dysfunction, the elevated activities of Creatine Kinase (CK) and myeloperoxidase are also the vital pointers of the myocardial damage in the event of reperfusion injury[3–7].

Banaba (*Lagerstroemia speciosa* L. of family Lythraceae), a traditional medicinal herb of Southeast Asia and widely used to treat diabetes mellitus and other aliments. The role of *Banaba* in obesity and diabetes has been extensively studied[8]. *Banaba* leaves contain various therapeutically important active constituents like corosolic acid[9] and lagerstromein which were characterized by using advance analytical techniques[8]. The cardioprotective potential of

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Banaba is not yet explored, and hence the current study was designed to investigate the implication of acute administration of *Banaba* on myocardial ischemia/reperfusion injury in rats.

2. Materials and Methods

2.1 Chemicals

Banaba leaves were collected from Botanical Society Garden, Pune, Maharashtra, India. Catalase (CAT), Superoxide dismutase (SOD), Epinephrine Hydrochloride, Malondialdehyde (MDA) and Triphenyl tetrazolium chloride (TTC) were procured from Sigma-Aldrich, USA. Thiobarbituric acid, 5,5'Dithiobis (-2 nitrobenzoic acid) (DTNB), Reduced Glutathione were purchased from Merck Ltd. India. Other chemicals were all of analytical grade.

2.2 Animals

The experimental procedures were performed using Wistar rats of either sex weighing 280-320g. The animals were purchased from Central Animal House facility of The Maharaja Sayajirao University, Baroda. The protocol of the animal study was approved by IAEC (The Institutional Animal Ethics Committee) (PH/IAEC/2k8/11). Animals were housed in 24±1 ºC, 12 h light and dark cycle. Animals were fed with standard pallet diet and water *ad libitum*.

2.3 Plant Material and Preparation of Extract

Leaves of *Banaba* were collected and shade-dried for preparation of the extract. The specimen was authenticated by BSI (Botanical Survey of India), Pune (Specimen number-BSI/WC/Tech/2007/811). One kg of raw material was macerated for 72h with 2.5 L ethanol (cold maceration). The extract was concentrated and dried under vacuum (10 % w/w).

2.4 Myocardial Ischemia/Reperfusion Injury Induction by Left Coronary Artery (LCA) Ligation

The myocardial ischemia/reperfusion injury was induced by left coronary artery ligation model[6]. Xylazine (7 mg/kg i.m.) and Ketamine (100 mg/kg, i.p.) were used to anaesthetize the rats. The rats were artificially ventilated (Ugo Basile Rodent Ventilator, Model 7025). The body temperature was maintained throughout the experiment at 37±1ºC. The thoracotomy in fourth intercostals area was performed. The oblong portion of chest wall and sternum was removed and the heart was exposed. The location was identified and a knot was placed around the LCA using silk suture (6.0). The knot was then pushed through a plastic cannula to allow occlusion (60 min) and reopening (60 min) of the vessel for the induction of ischemia and reperfusion respectively. The same procedure was followed in rats, except the tightening and the releasing of the cannula were considered as Sham operated animals. The carotid artery was cannulated using transducer (SL 13L, Biopac Systems) for measuring the hemodynamic parameters[10]. Parameters like systolic (SBP), diastolic (DBP) and mean arterial blood pressure (MAP) were observed. Biopac Student Lab. Pro. software (Version 3.6.7) was used to analyze the data.

2.5 Protocol

The rats were randomly segregated in to three groups (with 18 in each group). Sham-operated (SO) control group was treated with vehicle (2 ml/kg saline with 0.05 % polyethylene glycol i.p.), the ischemic groups were treated with vehicle and considered as I/R injury control, while the other group received *Banaba* (BNA 100 mg/kg, i.p.). The treatment was administered to the rats after 30 min of ischemia induction I/R animal groups. The chosen 100 mg/kg dose of *Banaba* extract was based on the results obtained from the isoproterenol induced myocardial infarction model of pilot study. The 100 mg/kg was found to be the most effective in restoring biochemical and histopathological markers of injury among the studied 50,100 and 200 mg/kg doses. *Banaba* leaves extract was suspended in 0.05% of Poly Ethylene Glycol (PEG). Every group was segregated in to three set of rats with six each. Infarct area was measured in the first set. The second set consisted of the measurement of the MPO activity and the oxidative stress parameters (SOD, GSH, CAT and LPO). The third set consisted of the measurement of DNA fragmentation analysis and histological observations.

2.6 Evaluation of Electrocardiographic and Haemodynamic Parameters

ECG pattern evaluation (SS2L, Biopac System) was used to measure the changes in ST elevation and ST segment prolongation. Data were measured before ischemia (baseline), after the ischemic period (end-ischemia), and on completion of the reperfusion period (end-reperfusion). To evaluate the effect on cardiac rhythm, we measured incidences of ventricular tachycardia (VT). During the I/R period, SBP, DBP, MAP, and maximal pressure change rate (±dp/dt) were recorded at intervals.

2.7 Evaluation of Myocardial Infarct Size and Area at Risk

The rats were sacrificed after 2 h of I/R injury and hearts were removed. In order to distinguish ischemic and non-ischemic areas of the heart, LCA was re-occluded and through aorta, 3ml of 2% Evans Blue was forced retrogradely. The heart was frozen and sectioned for 1 to 1.5 mm perpendicularly in the axis of apex-base for distinguishing the area at risk and the infracted area of myocardium. The slices were incubated for overnight period in TTC solution (0.5 mg/ml for 20 min at 37 °C) A computer-based Image J software 1.30 V (rsb.info.nih/ij.) was used to scan the area at risk and also the infracted area[6].

2.8 Determination of Myeloperoxidase Activity (MPO)

Ice cold 50 mM potassium phosphate buffer pH 6 containing 0.5% hexadecyl trimethyl ammonium bromide (HTAB, Sigma) mixed with heart tissue and homogenized. The frozen/ thawed (3 times) homogenate was centrifuged at 11000xg at 4°C for 20 min. The supernatant (34 µl), phosphate buffer (986 µl) containing 0.167mg/ml ortho-dianisidine dihydrochloride (Sigma) and 0.0005% hydrogen peroxide were mixed and absorbance was measured at 460 nm by spectrophotometer. Consumption of 1 nanomole of peroxide per minute at 22° C was measured as one unit of MPO activity. All results were expressed as milliunit/mg of protein[11].

2.9 Estimation of Oxidative Stress Biomarkers

At the end of experiment, heart was removed quickly. The heart was weighed and homogenized in cooled 10% w/v Tris buffer (10mM, pH -7.4). The homogenate was centrifuged at 10000×g for 20 min at 0°C. The clear supernatant were evaluated for alterations in biomarkers like SOD[12], Catalase[13], Reduced Glutathione[14] and Lipid peroxidation[15]. The proteins were estimated by method of Lowery et al[16]. Creatine kinase activity was estimated from the serum separated from the blood using Span Diagnostic kits.

2.10 DNA Fragmentation Analysis by Gel Electrophoresis

Sham, I/R control, and the animals treated with *Banaba* extract were investigated for the DNA fragmentation analysis. DNA was harvested from the heart isolated after 1 h of ischemia and 1h of reperfusion. 100 mmol/l of Tris-Hcl (pH 8.0), 200 mmol/l of NaCl, 5 mmol/l of EDTA and 0.2% of SDS with 5 mg/ml RNase A was used to digest heart samples followed by phenol-chloroform extraction. Each lane was loaded with 8 µg of DNA approximately and run at 70 V on a 0.8% agarose gel. It was stained with ethidium bromide (0.5 µg/ml) to separate the DNA[17]. A 1 Kb DNA (Fermentas, Germany) ladder was used as reference standard.

2.11 Histological Examination

Heart was removed, washed with saline, fixed in 10% formalin and embedded in paraffin. Sections of 5 µm thickness were cut and stained with hematoxyline (H) and eosin (E). Sections were observed for histological changes after induction of I/R injury under light microscope (Olympus BX40).

2.12 Statistical Analysis

The results are expressed in mean ± S.E.M. Statistical difference was obtained by ANOVA (One way Analysis of Variance) followed by the Tukey test. The occurrence of VT in various groups was analyzed by χ^2- test. $P<0.05$ was considered as the statistical significance.

3. Results

3.1 Effect on Hemodynamic Parameters

A significant decrease (P<0.05) in MAP, HR and ±dp/dt in ischemic control (I/R) groups were observed compared to the sham operated (SO) animals at the end of ischemia and reperfusion. Treatment with *Banaba* restored MAP but did not show any significant difference to SO group after I/R injury. The positive and the negative pressure difference (±d*p*/d*t)* values were significantly (*P*<0.05) increased as compared with I/R group. The fall in HR in the *Banaba* treated group was less pronounced as compared to I/R group but this treatment did not restore HR significantly (Table 1, 2).

3.2 Effect on Electrocardiographic Observations

After the completion of end-ischemic and end-diastolic interval, there was a significant prolongation and elevation of the ST segment in I/R group as compared to SO group. Marked change in ST segment propagation was significantly (*P*<0.05) reduced by *Banaba* treatment when compared to vehicle treated I/R animals (Figure 1,2).

3.3 Effect of Banaba on Arrhythmia

Administration of *Banaba* caused significant (*P*<0.05) reduction in incidences of occurrences of VT after I/R injury compared to the vehicle treated I/R animals (Table 3).

3.4 Effect on Myocardial Infarct Size after Banaba Treatment

I/R injury resulted in infarct formation (54.61 ± 3.59) in vehicle treated I/R group. This infarct area was significantly (*P*<0.001) reduced by the administration of *Banaba*. There was no significant alteration in risk area in all the groups after I/R injury (Figure 2).

Table 1. The changes in ± d*p*/d*t* of rats in different experimental groups

Group	+ d*p*/d*t* (mmHg/s)			- d*p*/d*t* (mmHg/s)		
	BL	EI	ER	Baseline	EI	ER
SO	2402 ± 215	2668 ± 108	2567 ± 180	2015 ± 328	2053 ± 326	1958 ± 127
I/R	2566 ± 166	1879 ± 148 *	1779 ± 102*	1811 ± 158	1146 ± 126*	1216 ± 93*
I/R+BNA	2629 ± 132	1951 ± 193	2190 ± 185	2002 ± 156	1655 ± 101	1791 ± 86

Values are expressed as mean ± S.E.M; n= 12. The values during the period of LCA occlusion was designated as BL; end of ischemia period was designated as EI; end of reperfusion period was designated as ER. Sham operated control - SO; vehicle treated I/R group – I/R; treatment with *Banaba* I/R group – I/R+BNA. * *P*<0.05 as compared to SO group.

Table 2. Changes in heart rate (HR) and mean arterial pressure (MAP) of rats in different experimental groups

Group	HR/min			MAP (mmHg)		
	BL	EI	ER	Baseline	EI	ER
SO	419 ± 19.52	396 ± 20.05	405 ± 15.09	107.13 ± 4.46	106.66 ± 3.98	108.5 ± 2.58
I/R	392 ± 18.21	325 ± 13.01*	308 ± 26.15*	107.52 ± 6.05	80.05 ± 3.56*	76.59 ± 5.98*
I/R+BNA	424 ± 22.21	339 ± 35.29*	317 ± 29.66*	100.01 ± 4.48	89.98 ± 8.47	91.11 ± 10.13

Values are expressed as mean ± S.E.M; n= 12. The values during the period of LCA occlusion was designated as BL; end of ischemia period was designated as EI; end of reperfusion period was designated as ER. Sham operated control - SO; vehicle treated I/R group – I/R; treatment with *Banaba* I/R group – I/R+BNA. * *P*<0.05 as compared to SO group

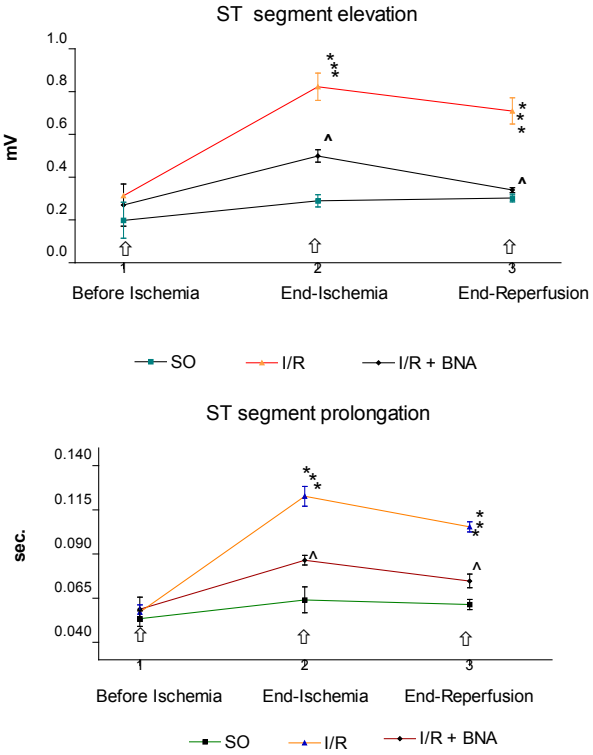


Figure 1. The change in the ST segment of experimental groups during ischemia and reperfusion period. 1**(a)** represents elevation in ST segment; 1**(b)** represents the prolongation in ST segment with respect to time. Values are expressed as mean ± S.E.M; n= 12. SO - Sham operated control; vehicle treated I/R group – I/R; treatment with *Banaba* I/R group – I/R+BNA. *** *P*<0.001 as compared to SO group. ^*P*<0.05 as compared to I/R group.

3.5 Effect on Inflammatory Reactivity

Neutrophil infiltration due to inflammatory reactivity was considerably elevated (*P*<0.01) after I/R injury, as compared to sham operated (SO) animals. Treatment with *Banaba* showed significant (P<0.05) decrease in MPO activity after induction of I/R injury (Table 4).

3.6 Creatine Kinase Activity

CK activity in I/R groups was significantly (P<0.001) increased after I/R injury as compared to SO group. The animals treated with *Banaba* showed significant (P<0.05) reduction in CK activity as compared to I/R group of animals (Table 4).

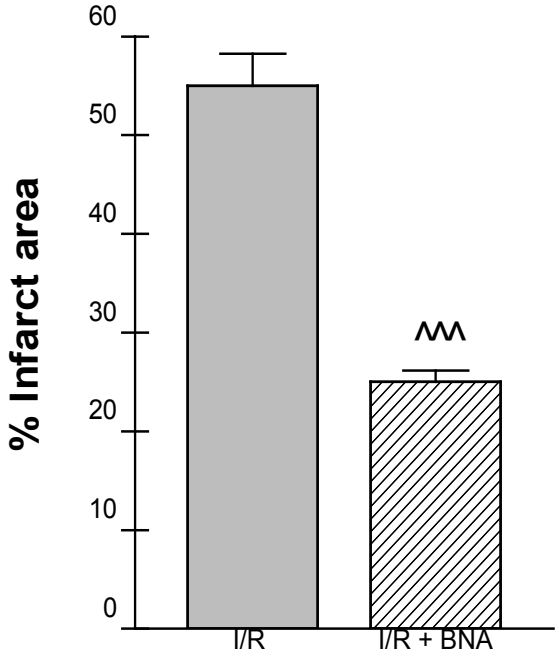


Figure 2. The percentage of infarct area with myocardial I/R injury with and without *Banaba* treatment. Vehicle treated I/R group – I/R; treatment with *Banaba* I/R group – I/R+BNA. ^^^*P*<0.001 as compared to I/R group.

Table 3. Ventricular tachycardia occurrence with myocardial I/R injury in experimental groups

Group	Occurrence of ventricular tachycardia	
	During ischemia period	During reperfusion period
I/R	11/12	10/12
I/R + BNA	4/12^	3/12^

Values are expressed in number of rats; n= 12. Vehicle treated I/R group – I/R; treatment with *Banaba* I/R group – I/R+BNA. ^*P*<0.05 as compared to I/R group.

3.7 Effect on Oxidative Stress

Treatment with *Banaba* has significantly (*P*< 0.05) elevated endogenous SOD activity and GSH pools as compare to I/R animals. LPO, measured by estimating formed MDA is significantly elevated in I/R group. *Banaba* treatment significantly (*P*< 0.01) reduced MDA levels in treated animals. There seemed to be no significant alteration of catalase activity in any of the study groups (Table 5).

3.8 Effect on Apoptotic Cell Death-Gel Electrophoresis

The apoptotic cell death induced by I/R injury was analyzed by observing the interneucleosomal DNA fragmentation. The sham control group did not show any prominent DNA fragmentation while the ischemic control group displayed a laddering pattern which is an indication of oligointernucleosomal DNA fragmentation. The *Banaba* treated group did not show any evidence of DNA laddering pattern (Figure 3). Vehicle treated I/R animals showed marked evidences of DNA damage, whereas treatment with *Banaba* substantially reduced the apoptotic cell death.

3.9 Histopathological Observations

Microscopic observation of the heart sections showed myofibrils with normal architecture in SO group whereas the I/R injury induced severe cellular edema, heavy neutrophil infiltration and cellular necrosis in vehicle treated animals. Treatment with *Banaba* considerably restored the normal cellular architecture and reduced the myocardial damage indicated by decreased edema and minimal neutrophil presence in the myofibrils (Figure 4).

4. Discussion

The results demonstrate that the treatment of *Banaba* (100 mg/kg) protects myocardial I/R injury in rats. Treatment with *Banaba* significantly decreased the inflammatory reactivity, enhanced scavenging activity of free radical, improvement in contractile function of the myocardium (increased ±d*p*/d*t*) and ECG stabilization (i.e., decreased ST elevation and VT incidences) after 1 h of ischemia followed by 1 h of reperfusion. *Banaba* treatment significantly improved the decreased maximal positive as well as the negative first derivative of pressure during ischemia and after the reperfusion of the myocardium in I/R animals. Elevation of ST segment is always a clear index of myocardial ischemia. The injury currents flowing between the region of ischemic myocardial to normal myocardium results in epicardial ST elevation in transmural ischemic condition[18,19]. Subendocardial ischemic injury at the onset of ischemia in all ischemic animals could be the reason behind induction of I/R and elevated ST segment. The ST segment disturbances in rats was considerably reduced by the *Banaba* treatment.

Oxidative stress induced injury due to the generation of free radical in reperfused myocardium can be accomplished by reestablishing the subtle equilibrium

Table 4. Changes in MPO (myeloperoxidase) and CK (creatine kinase) activity of rats in different experimental groups

Groups	MPO unit/mg of protein	CK (U/L)
SO	0.23 ± 0.91	1033.67 ± 36.23
I/R	0.82 ± 0.04**	4490.54 ± 120.99***
I/R + BNA	0.44 ± 0.02^	3507.83 ± 154.45^

Values are expressed as mean ± S.E.M; n= 6. SO - Sham operated control; vehicle treated I/R group – I/R; treatment with *Banaba* I/R group – I/R+BNA. ** $P<0.01$; *** $P<0.001$ as compared to SO group. ^$P<0.05$ as compared to I/R group.

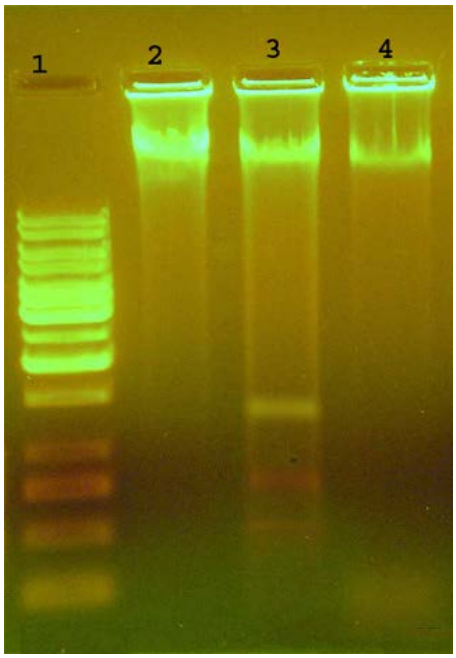
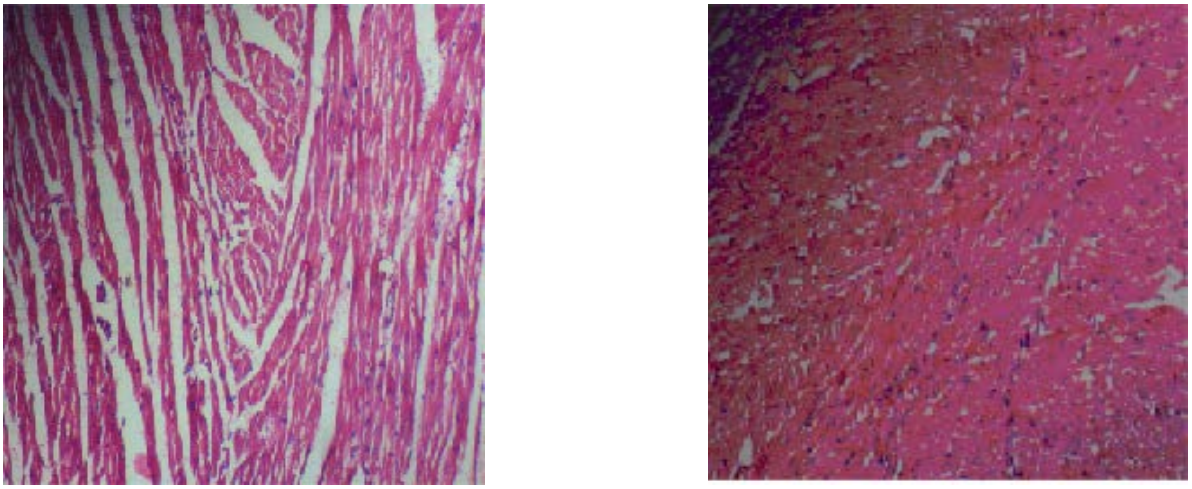


Figure 3. Showing electrophoresis of rat heart DNA after ischemia/reperfusion injury. (1) – 1 kb DNA standard; (2) - sham control group; (3) - ischemic control (I/R) group; 4 - *Banaba* treated (I/R + BNA) group.

Table 5. Changes in oxidative stress markers in different experimental groups

Groups	SOD IU/mg of protein	CAT H_2O_2 consu/min/ mg of protein	GSH µg /mg of protein	MDA nmol/mg of protein
SO	12.71 ± 0.89	442.35 ± 66.61	9.26 ± 0.84	1.11 ± 0.09
I/R	3.82 ± 0.62***	315.01 ± 43.23	3.89 ± 0.32**	3.14 ± 0.27**
I/R+ BNA	8.55 ± 0.83^	266.44 ± 41.83	8.13 ± 1.05^	1.51 ± 0.15^^

Values are expressed as mean ± S.E.M; n= 6. SO - Sham operated control; vehicle treated I/R group – I/R; treatment with *Banaba* I/R group – I/R+BNA. Superoxide dismutase – SOD; catalase – CAT; reduced glutathione – GSH; malondialdehyde - MDA. ** $P<0.01$; *** $P<0.001$ as compared to SO group. ^$P<0.05$; ^^ $P < 0.01$ as compared to I/R group.



(a) (b)

Figure 4. H&E stained photomicrographs (100 X). 4**(a)** - vehicle treated I/R rats; 4**(b)** - *Banaba* treated I/R.

of defensive antioxidant mechanism[20,21]. The decrease of endogenous antioxidant enzymes can be due to the combination of ROS generation and ischemia. The decrease in the level of endogenous antioxidant results in the dysfunction of cellular proteins of myocardium due to the peroxidation of lipids and the thiol group's oxidation[22]. There was a significant restoration of SOD and GSH levels in *Banaba* treated ischemic rats as compared to the vehicle treated I/R animals and moreover, there was a reduction in lipid peroxidation in treated animals. Free radical scavenging activity is directly related to the infarct area of the ischemic heart[23–25]. Inflammatory reactivity due various mediators induced by I/R, precipitates into myocardial functional suppression[26,27]. MPO is a biomarker index for neutrophil infiltration and inflammatory reactivity. MPO activity plays a pivotal role in myocardial reperfusion injury[5,6]. Elevated MPO activity in ischemic rats was significantly decreased by *Banaba* treatment. From the study, CK was a reliable marker of ischemic injury and also an early predictor of infarct formation. The increased level of CK in serum is due the leakage of enzyme into the blood[28]. CK is an important cellular enzyme mediating energy transduction in muscle cells. Infarction can be quantified by estimating CK release. The extent of myocardial damage depends on the proportionate increase of CK activity[28,29], which correlates with ECG and histological findings. *Banaba* treatment significantly reduced serum CK levels.

A potential indicator of apoptosis in the tissue, especially in myocardium, is interneucleosomal DNA fragmentation[30,31]. The varied neutrophil levels and oxidative stress influences apoptosis in the myocardial I/R injury[32–34]. Apoptosis is increased in reperfused myocardium due to increased accumulation of the neutrophil[35]. The agarose gel electrophoresis in the current study showed DNA fragments in reperfused rat myocardium as reported earlier by Fliss et al[36]. *Banaba* treatment in ischemic rats reduced the intensity of internucleosomal DNA fragmentation.

5. Conclusion

The cardioprotective effect of *Banaba* could be attributed to its ability to improve the antioxidant mechanism, reduction in the inflammatory reactivity and apoptotic activity in ischemic animals. One of the major phytoconstituent of *Banaba* is corosolic acid which is reported to exhibit potent antioxidant, anti-inflammatory and glucose reuptake activity[8,9,37]. Corosolic acid component of *Banaba* extract may influence the observed cardioprotective activity on myocardial I/R injury in rats. In view of efficacy and traditional acceptability of *Banaba*, large scale preclinical and clinical studies should be considered for establishing *Banaba* as a potent therapy for ischemic myocardial disorders.

6. Conflict of Interest

There is no conflict of interest among authors.

7. Acknowledgement

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