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Bacopa monniera protects rat heart against ischaemia–reperfusion injury: role of key apoptotic regulatory proteins and enzymes

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Abstract

Objectives Rat isolated hearts were perfused in a Langendorff model to study the cardioprotective effects of *Bacopa monniera*, a medicinal herb used in the Indian system of medicine, on cardiomyocyte apoptosis and antioxidant status following ischaemia–reperfusion (I-R) injury.

Methods Forty-eight rats were randomly divided into four groups (12 in each group): sham group (no ischaemia–reperfusion injury), *B. monniera* control group (orally fed *B. monniera* at a dose of 75 mg/kg, for three weeks); ischaemia–reperfusion control group-(subjected to ischaemia–reperfusion-induced myocardial injury) and *B. monniera*-treated group (same protocol as ischaemia–reperfusion control group except that rats also fed *B. monniera*).

Key findings Post-ischaemic reperfusion injury resulted in significant cardiac necrosis, apoptosis, depression of heart rate, decline in antioxidant status and elevation in lipid peroxidation. Oral administration of *B. monniera per se* for three weeks to healthy rats caused augmentation of myocardial antioxidants, superoxide dismutase, catalase and glutathione, along with induction of heat shock protein 72 (HSP72). Ischaemia–reperfusion-induced biochemical and histopathological perturbations were significantly prevented by *B. monniera* (75 mg/kg) pre-treatment. Interestingly, *B. monniera* also restored the antioxidant network of the myocardium and reduced myocardial apoptosis, caspase 3 and Bax protein expression.

Conclusions Histopathological studies and myocardial creatine phosphokinase content further confirmed the cardioprotective effects of *B. monniera* (75 mg/kg) in the experimental model of ischaemia–reperfusion injury. The study provides scientific basis for the putative therapeutic effect of *B. monniera* in ischaemic heart disease.

Keywords antioxidants; *Bacopa monniera*; medicinal herbs; myocardial infarction; oxidative stress

Introduction

Over the last decade, increasing evidence has suggested that apoptosis is an important mechanism involved in the development and progression of cardiovascular disease.[1] In the myocardium, apoptosis has been detected in a number of cardiac pathologies including ischaemia followed by reperfusion and myocardial infarction.[2] The progressive loss of cardiomyocytes by apoptosis in a heart that is already compromised leads to further deterioration of cardiac function.[3] Apoptosis is a genetically regulated process, hence a better understanding of the cellular mechanisms that control apoptosis could lead to defining novel and effective therapeutic strategies to limit the amount of tissue damage in patients with ischaemia–reperfusion.[4]

Various studies have shown that these programmed cell death pathways can be inhibited by antioxidants.[5] Several medicinal plants are known to possess antioxidant activity.[6,7] It would therefore be interesting to study whether they could attenuate apoptosis. Recently, there has been keen interest in medicinal plants for cardioprotection because of their numerous possible cardioprotective mechanisms. There has been an upsurge in the use of medicinal plants for the treatment of various diseases. A concept is now emerging of 'adaptogenic drugs' – drugs that increase non-specific resistance of the user to a variety

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of stresses.[8] One of the proposed mechanisms of action of such drugs is the enhancement of cellular antioxidant enzymes and nucleic acid biosynthesis.[9] Thus, a major opportunity exists in using our natural resources for identifying and selecting efficacious, inexpensive and safer approaches for cardioprotection against ischaemic and reperfused hearts. As few systematically designed studies are currently available, these medicinal plants need to be investigated scientifically.

In this investigation, modification of the condition of reperfusion has been achieved with the use of *B. monniera*, a medicinal herb, giving new insight into advanced therapeutic targets and strategies for the treatment of myocardial ischaemia–reperfusion injury. *B. monniera*, commonly known as 'brahmi', has gained worldwide recognition as a memory booster and is used for the treatment of epilepsy and bronchial asthma in the Indian system of medicine. The whole plant is used therapeutically and the active ingredients bacosides are mainly responsible for its antioxidant, immunomodulatory and adaptogenic properties.[10]

There is no data on the cardioprotective effects of *B. monniera* and its effect on apoptotic mechanisms in the heart until now. Therefore in this study the therapeutic potential of *B. monniera* in the Langendorff model of ischaemia–reperfusion injury was evaluated. In addition, to elucidate the potential clinical implications of such actions, the relationship of the detrimental effects of key oxidants and apoptotic signals with ischaemia–reperfusion injury was studied. Biochemical parameters evaluated included: lipid peroxidation product thiobarbituric acid reactive substances (TBARS); endogenous antioxidant glutathione (GSH); antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx); and myocardial enzyme creatine phosphokinase (CPK). To correlate the biochemical derangement and altered cardiac performance during ischaemia–reperfusion, changes in the haemodynamic variables heart rate (HR) and coronary perfusion pressure (CPP) was measured at pre-set time points during the study protocol. Myocardial apoptotic index (AI) was determined using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method. The expression of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax, heat shock protein (HSP) and enzyme: caspase 3 in cardiac myocytes was detected by immunohistochemistry.

Materials and Methods

Chemicals

All chemicals were of analytical grade, purchased from Sigma Chemical Co. (St Louis, USA). The ABC staining kit and primary (Bax mouse monoclonal IgG2b and Bcl-2 mouse monoclonal IgGI) and secondary antibodies (anti-mouse IgG) were procured from Santa Cruz Biotechnology (Santa Cruz, USA). HSP 72 mouse monoclonal IgG1 primary antibody was procured from Biogenex Life Sciences Private limited (Secunderabad, India). Caspase 3 mouse monoclonal IgG primary antibody was procured from Allied Scientific Products (Kolkata, India). The TUNEL assay kit was purchased from Roche Diagnostics (Indianapolis, USA).

B. monniera plant extracts analysed in this study were supplied by Dabur Research Foundation, India. The extractive values (1 g sample) in water was 64.15% w/w and in methanol was 53.94% w/w. The pH of 1% w/v aqueous solution was 8.23 and loss on drying value at 105°C by IR balance was 3.1% w/w. The total ash content was 14.06% w/w. Total saponin content (on dried basis) was 24.44% w/w. Identity and purity (95%) of the isolated compounds were confirmed by chromatographic (HPLC) data.

Experimental animals

Adult male Wistar rats, 10–12 weeks old, 150–200 g, were used in the study. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee and conformed to the Indian National Science Academy Guidelines for the Use and Care of Experimental Animals in Research. Rats were obtained from the Animal Facility of Mahatma Gandhi Mission Medical College, Navi Mumbai, India. Rats were housed in polyacrylic cages ($38 \times 23 \times 10$ cm) with not more than four rats per cage. They were housed in an air-conditioned room and were kept under natural light and dark cycles (approx. 14 h light/10 h dark) and maintained at 60 ± 5%humidity and an ambient temperature of 25 ± 2^{o}C. All experiments were performed between 0900 h and 1600 h. The rats were allowed free excess to standard diet (Ashirwad, Chandigarh) and tap water and allowed to acclimatise for one week before the experiments. Commercial pellet diet contained 24% protein, 5% fat, 4% fibre, 55% carbohydrates, 0.6% calcium, 0.3% phosphorous, 10% moisture and 9% ash w/w.

Analysis of *B. monniera* plant extract by HPLC method

B. monniera powdered plant material, 1–5 g, was weighed into a 100-ml round-bottom flask, about 30 ml of 70% (v/v) methanol was added and then refluxed on a water bath for 30 min. After filtration, the extraction was repeated twice (30 ml) with 70% (v/v) methanol. All the alcoholic fractions were combined and made up to 100 ml with 70% (v/v) methanol and filtered through a 0.45-mm membrane filter.

Five hundred milligrams of the sample was dissolved in a medium of sodium sulfate (0.05 M) buffer, pH 2.3, and acetonitrile (50 : 50, v/v), sonicated for 10 min, diluted to 100 ml with the same medium and filtered through a0.45-mm membrane filter.

Calibration standard solutions, containing 10 mg/ml of the standards, were bacopasaponin (E,F,C), bacoside A3 and bacopaside (I-V,N1-2, X), in a medium of sodium sulfate (0.05 M) buffer, pH 2.3, and acetonitrile (50 : 50, v/v) (Stock solution).

HPLC experiments were performed on a Shimadzu HPLC system equipped with Phenomenex Luna C18, 5 mm (4.6–250 mm) column, LC10AT VP pumps, SCL-10AVP system controller, SIL-10 AD VP auto injector, SPD-M10 AVP photodiode array detector and class VP software was used.

For analysis, the mobile phase was a mixture of 0.05 M sodium sulfate buffer, pH 2.3, and acetonitrile (68.5 : 31.5, v/v) at flow rate of 1 ml/min and the column temperature was

maintained at 30°C. The detection wavelength was set at 205 nm. The injection volume was 20 ml. The chromatography system was equilibrated by the mobile phase. The separation and quantification of the samples were conducted when the same retention time and peak areas of the repetitive injection of the standard solutions were obtained. The total run time was 75 min.

The specificity of the method was ascertained by analysing the standards and the samples. The peaks for compounds bacopasaponin (E,F,C), bacoside A3 and bacopaside in the samples were confirmed by comparing the retention times of the peak with those of standards. The linearity of the method was checked with standards with the calibration curves in the range 1.0–60.0 mg/20 ml, using six different concentrations in six replicate assays.

The stability of the sample and standards were checked and found to be stable for 48 h. The results revealed that the compounds bacoside A3, bacopaside II, bacopaside I, bacopaside X and bacopasaponin C were major components and the remainder were minor, albeit in quantifiable amounts in *B. monniera* plant materials, and total saponins were 24.44%.

Dose selection studies: isoproterenol model of myocardial necrosis

The isoproterenol (85 mg/kg) model of myocardial necrosis was used for the evaluation of therapeutic intervention with *B. monniera* on the extent of jeopardised myocardium and to select the optimum cardioprotective dose of the herbal extract for further studies in the Langendorff model of ischaemia–reperfusion injury.[11] According to the experimental protocol, normal saline/*B. monniera* (25, 75 and 150 mg/kg doses) was administered orally, using an intragastric tube, to the rats for three weeks. Infarct-like myocardial lesions were developed by isoproterenol injection (85 mg/kg, s.c.) for two consecutive days (on 20th and 21st day of the experiment). On the 22nd day, the rats in all the experimental groups were sacrificed. The hearts were rapidly removed and processed for biochemical estimations and histological evaluation.

Myocardial ischaemia–reperfusion model: Langendorff heart preparation

The rats in all experimental groups were orally fed normal saline/*B. monniera* (75 mg/kg) for 21 days. On the 22nd day, the rats were anaesthetised. The diaphragm was cut and thoracotomy was performed and the pericardium was opened to expose the heart. The heart was gently elevated by cradling it in the fingertips to avoid contusion injury. The aorta, vena cava and pulmonary arteries were incised. Immediately after excision, the heart was placed into a beaker containing cold Kreb's–Hensleit buffer. The heart was then set on the Langendorff apparatus (Inco, India) by cannulating the aorta attached to a reservoir containing oxygenated Kreb's–Hensleit solution. The solution was then delivered in a retrograde direction down the aorta at constant flow rate of 10 ml/min delivered by an infusion pump. After a 10-min stabilisation period, global myocardial ischaemia–reperfusion was induced by completely stopping perfusion for 20 min followed by 30 min reperfusion at 37°C. Haemodynamic parameters HR and CPP were recorded at preset time points of the study (before ligation, at 5, 10 and 20 min post ischaemia and at 10, 20 and 30 min post reperfusion). The CPP was measured with a pressure transducer connected to another side port of the aortic perfusion cannula. All these data were recorded on a polygraph system (Inco, India). At the end of the reperfusion period, hearts were snap-frozen in liquid nitrogen for biochemical studies or in formalin for histopathological/immunohistochemical studies. Unstable hearts showing a significant (>10%) drop in HR during the baseline measurement were excluded from the study.

Experimental groups and treatment protocol

For baseline evaluation, healthy experimental rats without any pathologic challenge to the heart were assigned to the following experimental groups:

Saline control group (Sham): rats were administered 0.9% normal saline per orally using a feeding cannula for 21 days and then sacrificed on the 22nd day. There were 12 rats in this group.

B. monniera control group (Bm control): this group comprised of 12 rats. Hydro-alcoholic extract of *B. monniera* was dissolved w/v in 0.9% normal saline administered orally to healthy experimental rats once daily for 21 days at a dose of 75 mg/kg (Bm-75).

Control IR: rats were administered normal saline for 21 days and on the 22nd day, sacrificed and the heart mounted on Langendorff apparatus. After 10 min of stabilisation, global occlusion was undertaken for 20 min followed by 30 min of reperfusion. There were 12 rats in this group.

B. monniera-treated groups (Bm-IR). *B. monniera* (75 mg/kg) was dissolved in 0.9% normal saline and administered for 21 days. On the 22nd day, rats were sacrificed and the heart mounted on a Langendorff apparatus. After 10 min of stabilisation, global occlusion was undertaken for 20 min followed by 30 min of reperfusion. There were 12 rats in this group.

Biochemical studies

A 10% homogenate of myocardial tissue was prepared in 50 mM phosphate buffer, pH 7.4, and a sample was used for the assay of TBARS according to the method described by Ohkawa *et al*.[12] The homogenate was centrifuged at 7000 rev/min for 15 minutes and the supernatant was used for the estimation of the GSH,[13] GSHPx,[14] SOD,[15] CAT[16] and protein.[17] CPK was estimated spectrophotometrically using a kit from Randox Laboratories (Kearneysville, USA).[18]

Cardiac parameters

The time-course of changes in HR and CPP were monitored and recorded at preset time points during the study protocol in different experimental groups.

Determination of myocardial apoptosis

A monoclonal mouse anti-mouse caspase 3, Bcl-2 and Bax proteins as the primary antibody was used for caspase 3,

Bcl-2/Bax immunohistochemical staining. The ImmunoCruz Staining Systems utilises a horseradish peroxidase (HRP)–streptavidin complex for staining of formalin-fixed paraffin-embedded myocardial sections. Indirect immunoperoxidase staining was performed as described by Misao et al.[19] Briefly, 4- to 6-micron thick tissue sections were cut using microtome and applied to slides. The sections were deparaffinised. To unmask antigens by heat treatment, the slides were placed in coplin jars containing 10 mM sodium citrate, pH 6.0, covered and heated at 95°C for 5 min. All subsequent steps were carried out at room temperature in a humidified chamber. To quench endogenous peroxidase activity, the specimens were incubated in 1–3 drops of peroxidase block (3% H_2O_2 prepared in methanol) for 10 min. The specimens were then incubated in 1–3 drops of serum block for 2 h to prevent non-specific binding to collagen and connective tissue. The blocking serum was drained out. Antibody against Bax/Bcl-2 was pre-diluted (1 : 100) in serum block (5% normal goat serum in phosphate-buffered saline (PBS)) and caspase 3(1 : 1000). The specimens were incubated with one of the primary antibodies: Bax/Bcl-2/caspase 3. Precaution was taken so that the diluted primary antibody added was of sufficient volume to cover the tissues. The specimens were incubated for 24 h. The specimens were incubated for 30 min in 1–3 drops of biotinylated secondary antibody (goat anti-mouse IgG) and the slides were rinsed in PBS. The specimens were thereafter incubated in 1–3 drops of HRP–streptavidin complex for 30 min. During the incubation step, the HRP substrate was prepared in the substrate mixing bottle (1.6 ml de-ionised water, 5 drops of substrate buffer and 1 drop of 50 × DAB (3,3′-diaminobenzidine) chromogen and 1 drop of 50 × peroxidase substrate). One to three drops of HRP substrate–chromogen reaction mixture was added to each slide for 10 min for colour product formation at the site of reaction. The sections were rinsed in de-ionised water and transferred to de-ionised water wash and viewed under a light microscope (Nikon, Tokyo, Japan). As described earlier, the slides were counter-stained in hematoxylin and mounted in the permanent mounting medium (DPX).

Myocardial apoptosis was quantitatively analysed by detection of DNA fragmentation using the TUNEL technique.[18] Briefly, the enzyme terminal deoxynucleotidyl transferase was used to incorporate residues of digoxigenin nucleotide into 3′ OH ends of DNA with the aid of terminal deoxynucleotidyl transferase to the ends of DNA fragments. The signal of TUNEL assay was used to identify apoptotic cells using secondary reaction with antibodies or other detection system.

Total cell counts and TUNEL-positive cells in the specimens were determined by means of a light microscope. The cells with clear nuclear labelling were defined as TUNEL-positive cells. In this procedure, nuclei undergoing apoptosis were stained brown. Only cells with clear striations were scored as cardiac myocytes. The number of TUNEL-positive myocytes was manually counted and expressed as a percentage of the total number of cells in five randomly selected fields of view (magnification × 200) (0.22 mm^2) for each rat. Six rats were studied per group.

Immunostaining for the localisation of heat shock protein

Immunohistochemistry using the rabbit anti-cleaved polyclonal heat shock protein IgG1 antibody was performed on de-paraffinised tissue sections using a routine avidin–biotin–immunoperoxidase technique. Before incubation with the primary rabbit polyclonal antibody (1 : 1000 dilution), tissue sections were subjected to heat-induced epitope retrieval by incubation in a 0.01 M EDTA solution, pH 8.0, for 10 min in a vegetable steamer, followed by 20 min cool-down and treatment with 3% hydrogen peroxide before antibody application. Bound antibodies were detected using HRP–streptavidin complex. The target protein (HSP) was visualised by incubation in peroxidase substrate complex and DAB as the chromogen. Counter-staining was performed with Meyer's hematoxylin.

Immunohistochemical controls

The immunohistochemical negative tissue control included eliminating the primary antiserum and replacing the species-specific with normal serum of the appropriate species (normal goat serum), which did not contain the relevant tissue marker. However, positive tissue controls were also run. These controls were processed identically to the specimen and contained the target protein. These procedure controls (both positive and negative controls) served to ascertain primarily whether the staining protocols were followed correctly, whether day-to-day or worker-to-worker variation has occurred, whether reagents continued to be in good working condition, and if there is any background due to non-specific staining. Positive control sections of breast carcinoma tissue were also run. All descriptions and the pictures given in the manuscript are based on specific staining as adjusted against these controls.

Histopathological studies

At the end of the experiment, myocardial tissue was immediately fixed in 10% buffered neutral formalin solution. The tissues were carefully embedded in molten paraffin with the help of metallic blocks, covered with flexible plastic moulds and kept under freezing plates to allow the paraffin to solidify. Cross-sections (5 μm thick) of the fixed myocardial tissues were cut. These sections were stained with hematoxylin and eosin (H&E) and visualised under a light microscope (Nikon, Tokyo, Japan) to study the light microscopic architecture of the myocardium. The degree of necrosis, oedema and inflammation was graded and scored as follows: −, absence of any inflammation, oedema and necrosis; +, focal areas of inflammation, oedema and necrosis; ++, patchy areas of inflammation, oedema and necrosis; +++, confluent areas of inflammation, oedema and necrosis; ++++, massive areas of inflammation, oedema and necrosis.

Statistical analysis

All numerical data in text, figures and tables are expressed as the mean ± SD. Statistical analysis was performed by one-way analysis of variance or repeated measures analysis of variance when data were compared at different time points within a study group and for time courses between study

Table 1 Biochemical parameters in the different groups of rats

	Sham ($n = 9$)	**Bm-75** ($n = 9$)	**Control IR** ($n = 9$)	**Bm-IR** ($n = 9$)
GSH (umol/g tissue)	4.03 ± 0.3	5.23 ± 0.4	2.60 ± 0.19**	2.92 ± 0.11
TBARS (nmol/g tissue)	27.73 ± 5.7	18.33 ± 1.3*	80.48 ± 3.4	38.33 ± 1.3$^{\#}$
GSHPx (IU/mg protein)	0.33 ± 0.1	0.41 ± 0.1	0.18 ± 0.05*	0.21 ± 0.1
SOD (IU/mg protein)	7.94 ± 2.9	28.04 ± 1.2**	3.50 ± 1.07*	28.04 ± 1.2$^{\#\#}$
CAT (IU/mg protein)	21.1 ± 3.1	54.8 ± 8.0*	14.76 ± 2.60*	54.8 ± 8.0$^{\#}$
CPK (IU/mg protein)	6.7 ± 1.4	6.8 ± 0.4	1.7 ± 0.8	4.7 ± 0.4$^{\#}$

Sham, saline control group (no ischaemia–reperfusion injury); Bm-75, hydro-alcoholic extract of *B. monniera* was dissolved w/v in 0.9% normal saline and administered orally to healthy experimental rats once daily for 21 days at a dose of 75 mg/kg; Control IR, rats were administered normal saline for 21 days and on the 22nd day, sacrificed and the heart mounted on Langendorff apparatus. After 10 min of stabilisation, global occlusion was undertaken for 20 min followed by 30 min of reperfusion; Bm-IR, *B. monniera* (75 mg/kg) was dissolved in 0.9% normal saline and administered for 21 days. On the 22nd day, rats were sacrificed and the heart mounted on a Langendorff apparatus. After 10 min of stabilisation, global occlusion was undertaken for 20 min followed by 30 min of reperfusion. GSH, glutathione; TBARS, thiobarbituric acid reactive substances; GSHPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; CPK, creatinine phosphokinase. One unit of catalase activity represents 1 μmol of H_2O_2 decomposed/min. One unit of SOD is defined as the amount of enzyme required to produce 50% inhibition of adrenaline. One unit of enzyme activity is defined as 1 nmol of NADPH utilised per min at 37°C. One unit of CPK is defined as the amount of enzyme that will transfer 1 μmol of phosphate from phosphocreatine to ADP per min at pH 7.4 at 30°C. Data are means ± SD, n = No. of rats included in the study group. *$P < 0.05$, **$P < 0.01$ vs sham; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs Control IR.

groups, followed by the Bonferroni post-hoc test. $P < 0.05$ was considered statistically significant.

Results

Pilot study

In our laboratory, *B. monniera* at 25, 75 and 150 mg/kg doses was screened in the isoproterenol model of myocardial ischaemia (data published). Of the doses of *B. monniera* studied, only the 75 mg/kg dose preserved GSH levels. Both the 25 and the 75 mg/kg doses of *B. monniera* significantly prevented lipid peroxidation and restored the activity of the marker enzyme CPK, demonstrating a biochemical basis for the protective action of *B. monniera* against isoproterenol-induced myocardial injury. In the *B. monniera* (75 mg/kg)-treated group there was only occasional loss of myofibre and oedema and inflammation were minimal compared with other groups. However, with the *B. monniera* 150 mg/kg dose the degree of oedema and necrosis was nearly comparable with that of isoproterenol control group, with similar morphological changes. Among the various doses evaluated in this study, *B. monniera* 75 mg/kg demonstrated the optimum cardioprotective activity and was selected for further study in the ischaemia–reperfusion model of myocardial injury.[20]

Effect of *B. monniera* on biochemical parameters

Oral administration of *B. monniera* (75 mg/kg) *per se* to healthy controls for 21 days significantly enhanced the myocardial activity of CAT and SOD ($P < 0.01$) and reduced basal lipid peroxidation as compared with the sham group (Table 1).

In the Control IR group, following ischaemia–reperfusion a significant depletion in the antioxidant enzyme activity of SOD, GSHPx and CAT, GSH, myocardial CPK activity and increase in TBARS level was observed in comparison with the Sham group (Table 1). A significant decrease in myocardial TBARS level ($P < 0.01$) in the Bm-IR (75 mg/kg) treatment group in comparison with that of the Control IR group was observed. *B. monniera* treatment markedly prevented leakage of myocardial CPK ($P < 0.05$) in reference to the Control IR group. In addition, a significant restoration in SOD and CAT activity ($P < 0.01$) was observed in this group. However, *B. monniera* treatment failed to significantly protect leakage of GSH and antioxidant enzyme GSHPx as compared with the Control IR group (Table 1).

Cardiac parameters following ischaemia–reperfusion-induced myocardial injury

Initial value of HR in the control IR group was 343 ± 22.6 beats/min. Following occlusion of the aorta, at 5 min a significant ($P < 0.05$) fall in the value of this variable was recorded as compared with sham. Subsequently, the HR remained depressed during ischaemic duration as compared with sham. On reperfusion, a steady decline in HR, which was statistically significant at 20 and 30 min, was observed as compared with baseline sham values of this variable (Figure 1).

In the *B. monniera* (75 mg/kg)-treated group the baseline value of HR was 339 ± 35.3 beats/min. *B. monniera* treatment failed to significantly restore HR during ischaemic duration, as compared with control IR values at similar time points. However, the value of HR was significantly restored in the *B. monniera*-treated group at 30 min of reperfusion as compared with Control IR group (Figure 1).

Twenty minutes of ischaemia resulted in a significant increase in CPP in the Control IR group as compared with the Sham group baseline values. There was a transient decrease in CPP after 10 min of reperfusion. However, CPP did not significantly differ from sham at 20 and 30 min post reperfusion.

The baseline values of CPP in the Bm-IR (75 mg/kg) group was 60 mmHg. *B. monniera* treatment failed to correct the elevated CPP significantly during the entire period of global ischaemia–reperfusion as compared with Control IR group.

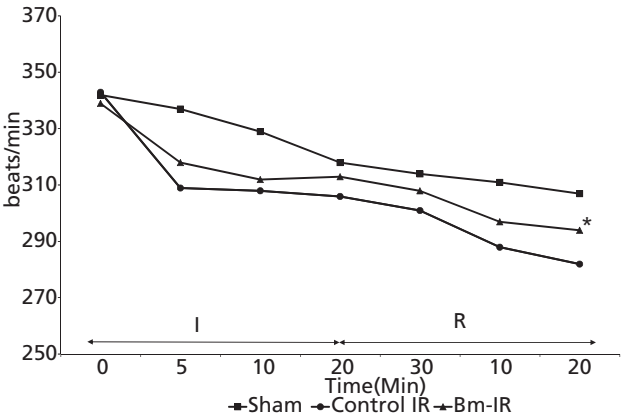

Figure 1 Time course of change in heart rate (HR) in the different experimental groups of rats. Sham, saline control group (no ischaemia–reperfusion injury); Control IR, rats were administered normal saline for 21 days and on the 22nd day, sacrificed and the heart mounted on Langendorff apparatus. After 10 min of stabilisation, global occlusion was undertaken for 20 min followed by 30 min of reperfusion; Bm-IR, *B. monniera* (75 mg/kg) was dissolved in 0.9% normal saline and administered for 21 days. On the 22nd day, rats were sacrificed and the heart mounted on a Langendorff apparatus. After 10 min of stabilisation, global occlusion was undertaken for 20 min followed by 30 min of reperfusion. Values are mean ± SD of 12 experiments. *$P < 0.05$ vs Control IR.

Myocardial apoptotic parameters following ischaemia–reperfusion-induced myocardial injury

Myocyte Bax protein expression

Slight brown Bax immunoreactivity (3.5 ± 0.4%) was observed in the myocytes of the Sham group. Ischaemia–reperfusion-induced myocardial injury significantly increased the expression of Bax protein ($P < 0.01$) compared with non-ischaemic tissue from 3.50 ± 0.40 to 9.80 ± 0.50% (Figure 2a). Bax expression was significantly attenuated to 4.04 ± 0.35% in the Bm-IR ($P < 0.05$) group as compared with Control IR (Figure 2b).

Myocyte Bcl-2 protein expression

Bcl-2 protein was clearly expressed in the Sham group as indicated by slight positive Bcl-2 immunoreactivity in the myocytes. The basal expression of Bcl-2 was found to be 1.86 ± 0.17%. Global ischaemia–reperfusion resulted in a reduction ($P < 0.05$) in Bcl-2 expression compared with non-ischaemic tissue (Figure 3a). Interestingly, treatment with *B. monniera* (75 mg/kg) was associated with increased (35.2 ± 2.8%) Bcl-2 expression ($P < 0.01$) as compared with Control IR group (Figure 3b).

Myocyte caspase 3 protein expression

Slight positive caspase 3 immunoreactivity (5.6 ± 0.17%) was observed in the myocytes of the Sham group. In the Control IR group significant immunostaining (63.2 ± 3.7%) of a subset of apoptotic cells within the germinal centre, as well as polymorphonuclear leucocytes within capillaries, was present (Figure 4a). However, caspase 3 expression was significantly attenuated (25.2 ± 2.2%) in the Bm-IR ($P < 0.05$) group as compared with Control IR (Figure 4b).

Table 2 Light microscopic changes in the myocardium observed in the different groups of rats

	Necrosis	Oedema	Inflammation
Sham	–	–	–
Control IR	++	+++	+
Bm-IR	+	+	+

Sham, saline control group (no ischaemia–reperfusion injury); Control IR, rats were administered normal saline for 21 days and on the 22nd day, sacrificed and the heart mounted on Langendorff apparatus. After 10 min of stabilisation, global occlusion was undertaken for 20 min followed by 30 min of reperfusion; Bm-IR, *B. monniera* (75 mg/kg) was dissolved in 0.9% normal saline and administered for 21 days. On the 22nd day, rats were sacrificed and the heart mounted on a Langendorff apparatus. After 10 min of stabilisation, global occlusion was undertaken for 20 min followed by 30 min of reperfusion. The relative cardiovascular effects are ranked as follows: –, No change; +, focal change; ++, patchy change; +++, confluent change; ++++, massive change.

TUNEL positivity

TUNEL positivity was expressed as percentage of total normal nuclei. Slight TUNEL positive staining was detected in the Sham group (0.2 ± 0.01%). However, the number of TUNEL-positive cells expressed as percentage of total normal nuclei was significantly increased subsequent to ischaemia–reperfusion-induced myocardial injury in the Control IR group (3.0 ± 0.2%, $P < 0.001$) compared with sham non-ischaemic myocardium as indicated by increased intensity of TUNEL staining (Figure 5a). The TUNEL positivity was significantly attenuated to 0.4 ± 0.03%, in the Bm-IR ($P < 0.05$) group as compared with Control IR (Figure 5b).

Heat shock protein expression following ischaemia–reperfusion-induced myocardial injury

HSP72 immunoreactivity in myocytes was granular and localised in the cytoplasm. HSP72 immunostaining in the myocardium was very faint in sham-operated controls (11.2 ± 4.2%). In the Control IR group post-ischaemic reperfusion led to increased intensity of immunostaining (71.2 ± 8.1%) in the ischaemic myocardium, and strong positive staining was seen in microvessels as well as myocytes (Figure 6a). In the *B. monniera*-treated groups, myocytes in the ischaemic and non-ischaemic areas were weakly positive for HSP72 (25.2 ± 3.1%) as compared with Control IR group (Figure 6b).

Histopathology following ischaemia–reperfusion-induced myocardial injury

Microscopic histology revealed that the non-infarcted myocardium in the Sham group was characterised by an organised pattern and showed normal architecture of the myocardium (Table 2). In contrast, on histological evaluation, rat hearts subjected to global ischaemia–reperfusion demonstrated marked oedema, confluent areas of myonecrosis, myofibre loss and mild inflammation as compared with those in the Sham group. In the *B. monniera* (75 mg/kg)-treated rats occasional focal myofibre loss, necrosis, oedema and inflammation was observed but to a lesser degree than in the Control IR group (Table 2).

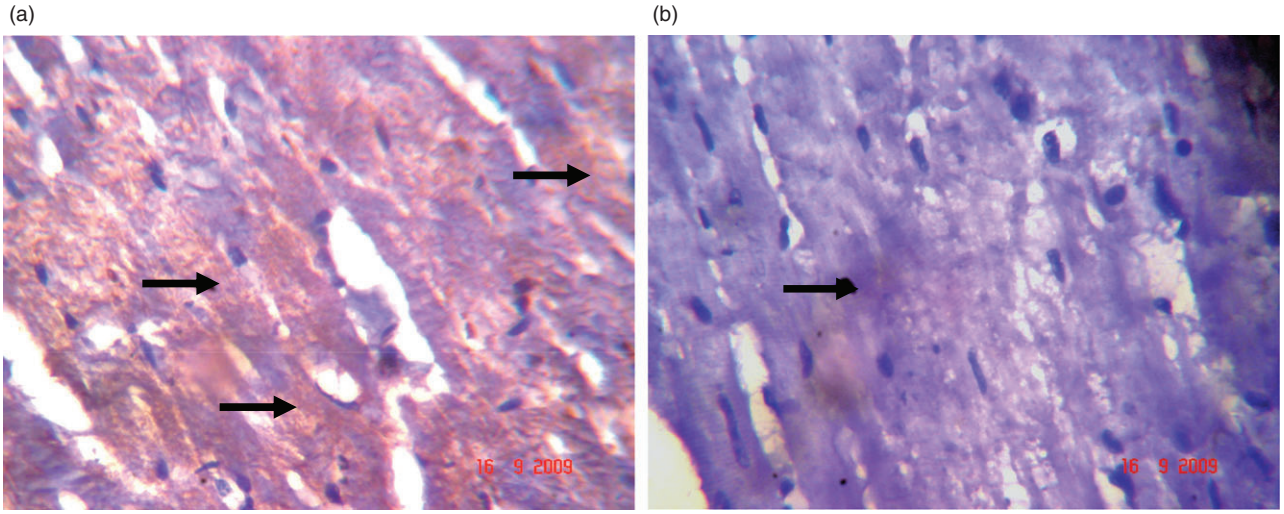

Figure 2 Immunohistochemical findings of Bax protein. (a) Ischaemia–reperfusion-induced myocardial injury significantly increased the expression of Bax protein compared with non-ischaemic tissue (b) *B. monniera* (75 mg/kg) treatment significantly attenuated the expression of Bax protein versus the Control IR group. The arrowhead indicates the localisation of Bax protein indicated by dark-brown positive immunoreactivity. Figures are representative of six separate experiments.

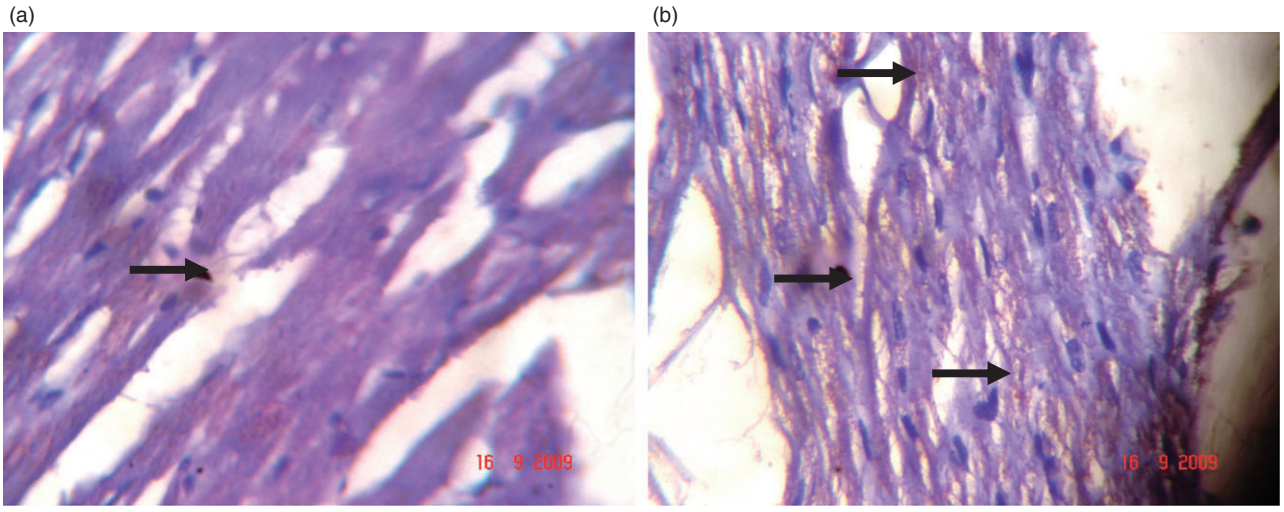

Figure 3 Immunohistochemical findings of Bcl-2 proteins. (a) Global ischaemia–reperfusion resulted in a slight reduction (non-significant) in Bcl-2 expression in the Control IR group compared with non-ischaemic tissue. (b) Upregulation in the expression of Bcl-2 as indicated by dark brown positive immunoreactivity is evident in the Bm treated group. The arrowhead indicates the localisation of Bcl-2 protein. Figures are representative of six separate experiments.

Discussion

Myocardial reperfusion may be viewed as a 'double-edged sword'; although it clearly exerts deleterious effects on the severely ischaemic cells, when reperfusion is carried out relatively early in the course of ischaemia its net effects are usually beneficial.[21,22] Thus, given the enormous potential clinical importance of early reperfusion in limiting infarct size, preserving antioxidant status, left ventricular function, and thus ensuing a significant decrease in patient morbidity and mortality, the development and identification of safe and effective interventions to reduce myocardial ischaemia and reperfusion-induced injury or optimise the benefit-to-risk ratio remains a fertile area for clinical and experimental investigation.

The present investigation was undertaken to determine the cardioprotective potential of *B. monniera*, a medicinal herb, and to elucidate the possible mechanisms of action on the basis of haemodynamic, biochemical and histopathological studies. In addition, the anti-apoptotic properties of the herbal extracts were studied using a combination of techniques of TUNEL positivity and immunohistochemical localisation of caspase 3 enzyme and Bax (an inducer of apoptosis) and Bcl-2 (an inhibitor of apoptosis) proteins.

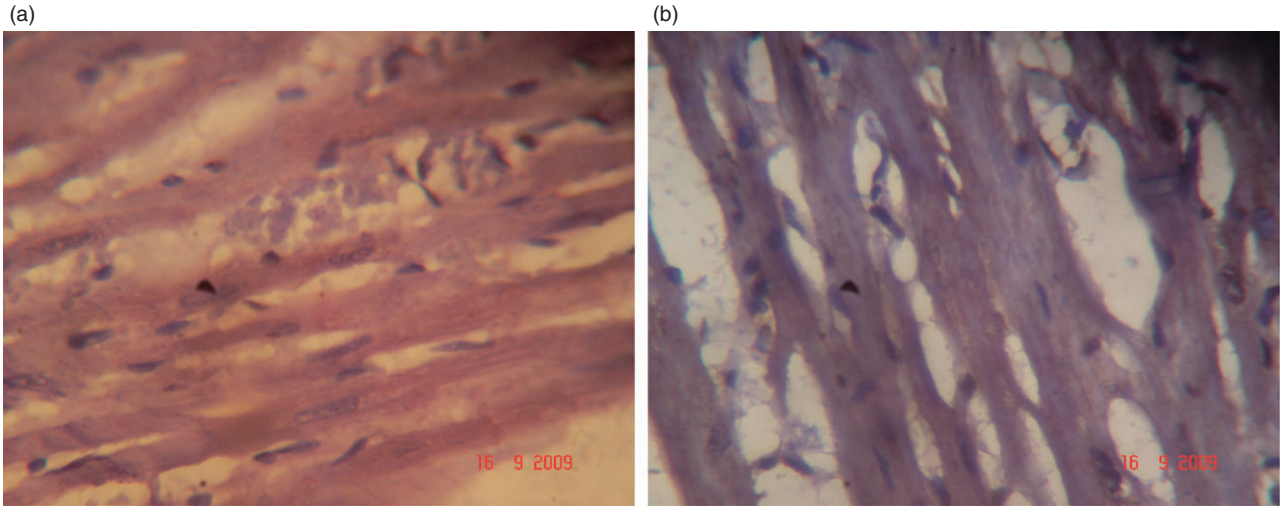

Figure 4 Immunohistochemical findings of caspase 3 proteins. (a) In the Control IR group significant immunostaining of subset of apoptotic cells within germinal centre, as well as polymorphonuclear leucocytes within capillary was present. (b) Caspase 3 expression was significantly attenuated in the Bm-IR group as compared with Control IR.

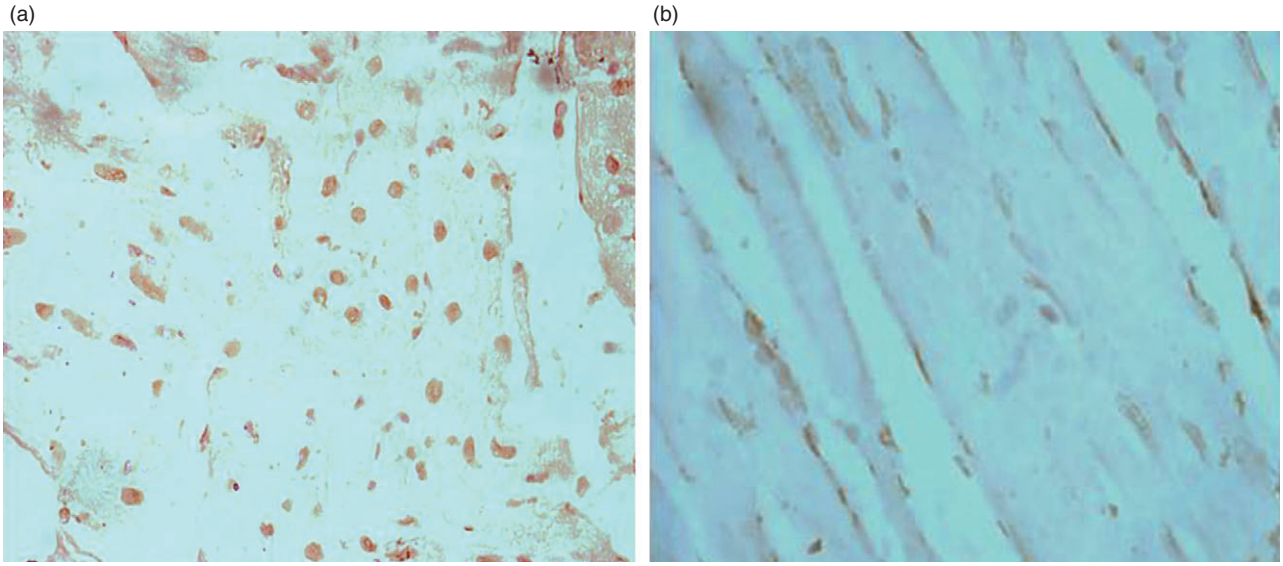

Figure 5 Representative photomicrographs of ventricular tissue stained for nick-end labelling (TUNEL) for DNA breaks. (a) TUNEL-positive cells were significantly increased subsequent to ischaemia–reperfusion-induced myocardial injury in the Control IR group. (b) Relative to the Control IR group the number of TUNEL-positive cells was significantly decreased by treatment with *B. monniera* (75 mg/kg). TUNEL-positive nuclei are indicated by arrowheads. Figures are representative of six separate experiments.

It is well known that one of the major causes of myocardial ischaemia–reperfusion injury, is an imbalance between oxidants and antioxidant defences.[23] Hence, it is possible to prevent or ameliorate disease progression by favoring the balance towards lower oxidative stress. Potential antioxidant therapy should, therefore, include exogenous supplementation of natural antioxidants that affect augmentation of endogenous antioxidants.[24]

In this study, oral feeding of rats with *B. monniera* for 21 days augmented basal endogenous antioxidants SOD and CAT and heat shock protein and reduced basal TBARS levels (i.e enhanced the antioxidant reserve), favorably modulating the antioxidant defence mechanisms of the myocardium in the healthy experimental animals. Any increase in SOD activity is beneficial in the event of increased free radical generation.[25] However, it has been reported that an augmented SOD activity, without a concomitant rise in the activity of CAT or GSHPx might be detrimental, since SOD activity generated hydrogen peroxide as a metabolite, which is more cytotoxic than oxygen radicals and must be scavenged by CAT or GSHPx. A simultaneous increase in CAT or GSHPx activity is essential for an overall beneficial effect of an increased SOD

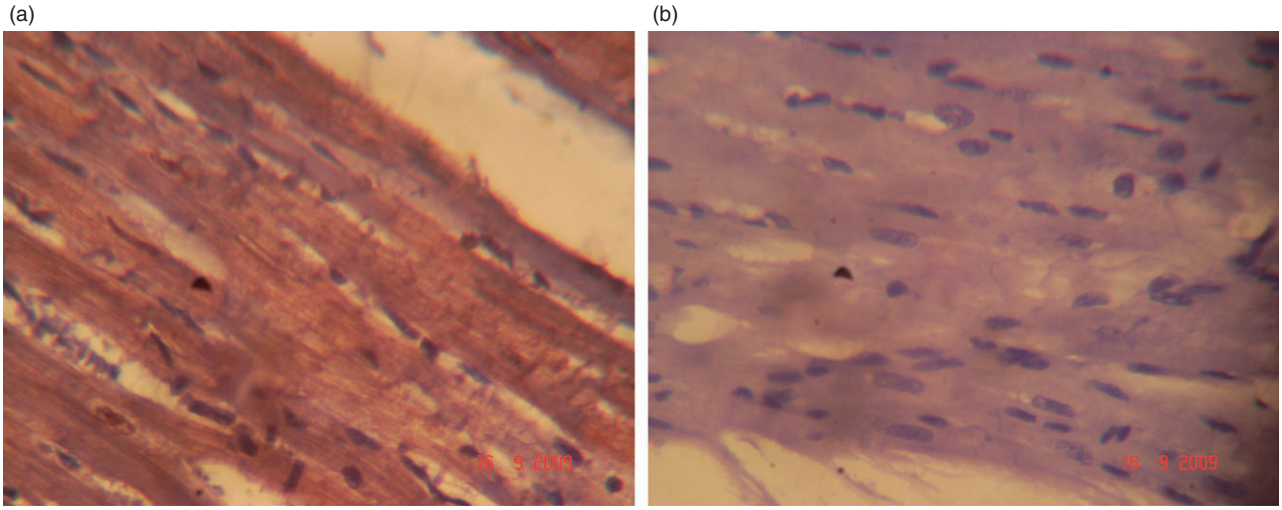

Figure 6 Immunohistochemical findings of caspase 3 proteins. (a) In the Control IR group post-ischaemia–reperfusion led to increased intensity of immunostaining in the ischaemic myocardium. (b) In the *B. monniera*-treated group, few of the myocytes in the ischaemic and non-ischaemic areas were weakly positive for HSP72 as compared with Control IR group.

activity.[24] Thus, the simultaneous increase in myocardial SOD, GSHPx and CAT activity observed in this study with *B. monniera* (75 mg/kg) underscores the distinct importance of enhanced beneficial effects of this herbal extract. However, a key question, which remains unanswered, is the mechanism by which *B. monniera* augments basal endogenous antioxidants. Subsequent to ischaemia–reperfusion-induced oxidative stress it was observed that *B. monniera* demonstrated a significant antioxidant property, which might contribute to its observed cardioprotective effects. The antioxidant effect of *B. monniera* has previously been reported in rat frontal cortex, striatum and hippocampus.[10]

In addition, in this study, *B. monniera* demonstrated significant anti-apoptotic potential as it upregulated the expression of the anti-apoptotic protein Bcl-2 and downregulated the expression of the pro-apoptotic protein Bax in association with a reduction in the expression of caspase 3 enzyme and percentage of TUNEL-positive cells in the ischaemic – reperfused myocardium. The exact mechanism by which *B. monniera* may reduce myocardial ischaemia–reperfusion-induced myocardial apoptosis is far from clear at present and may not be answered fully by this study. However, it can be speculated that it may attenuate apoptosis via a number of mechanisms: upregulation of Bcl-2 by *B. monniera* may result in the formation of heterodimers with Bax, resulting in no (or less) free Bax protein available for homodimerisation. If Bax homodimers predominate, cell death will occur, but when Bcl-2 and Bax heterodimererisation prevails cells can survive. Substantial evidence indicates that caspase 3 plays a critical regulatory role in the signal transduction pathway leading to apoptosis.[26–29] Caspase 3 is a central effector caspase in many cells and mediates the cleavage of itself, other downstream caspases and other caspase substrates.[30,31] *B. monniera* treatment significantly prevented the intense immunoreactivity observed in the Control IR group and this may contribute to its anti-apoptotic property. Moreover, free radicals have been demonstrated to directly activate calcium- and magnesium-dependent endonuclease (DNase I), thus resulting in DNA fragmentation and cell apoptosis.[32] *B. monniera* treatment, through its antioxidant mechanism, may prevent this DNAase activation and reduce myocardial apoptosis.

In this study, in the Control IR group strong expression of HSP72 appeared in microvascular endothelium after reperfusion for 30 min following a 20-min period of ischaemia. This may be associated with the damage to the microvascular endothelium that occurred in reperfusion.[33] In contrast to the ischaemic reperfused myocardium, the immunoreactivity in the *B. monniera*-treated group appeared much weaker and less frequent and was present mainly in the myocytes. These results indicate that *B. monniera* attenuates the HSP-induced ischaemia–reperfusion-mediated acute inflammation. This protective effect of *B. monniera* was supported by histopathologic examination in concert with preserved myocardial CPK content. The study also provides scientific rationale for the use of *B. monniera* in Ayurveda, the ancient Indian system of medicine.

Conclusions

Chronic oral pretreatment with *B. monniera* (75 mg/kg) exerted significant cardioprotective effects in the Langendorff experimental model of myocardial ischaemia–reperfusion injury in rats. The herbal extract of *B. monniera* was found to significantly ameliorate the ischaemia–reperfusion-induced cardiomyocyte apoptosis, compromised antioxidant status and histopathologic alterations as compared with the control ischaemia–reperfusion group. Cardioprotection afforded by *B. monniera* treatment may be attributed to its significant antioxidant and anti-apoptotic properties. This finding also suggests that *B. monniera* may attenuate HSP-induced ischaemia–reperfusion-mediated acute inflammation. Furthermore, *B. monniera* decreased the severity of pathological

changes and significantly preserved the myocardial CPK, confirming its myocardial salvaging effects.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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