Eugenia jambolana Pretreatment Prevents Isoproterenol-Induced Myocardial Damage in Rats: Evidence from Biochemical, Molecular, and Histopathological Studies

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ABSTRACT Preventive effects of hydroalcoholic extract of fruit pulp of *Eugenia jambolana* (HEEJ) on isoproterenol (ISP)-induced myocardial damage in rats were evaluated. Rats were pre-treated with HEEJ (100, 200, and 400 mg/kg) daily for 30 days. ISP (85 mg/kg bw) was administered on the 28th and 29th days at an interval of 24 h. Ischemic control group exhibited significant increases in oxidative stress parameters, markers of inflammation, cardiac damage markers, and apoptotic markers. Oral pre-treatment with HEEJ (100, 200, and 400 mg/kg bw) provided cardioprotective activity by decreasing levels of malondialdehyde, cardiac markers (serum glutamate oxaloacetate transaminase, creatine kinase-myocardial band, cardiac troponin I), and markers of inflammation (interleukin-6, C-reactive protein, and tumor necrosis factor alpha); and increased levels of superoxide dismutase and reduced glutathione. HEEJ (400 mg/kg bw) was found to exert significantly greater effects in comparison to HEEJ (100 and 200 mg/kg bw). Apoptotic marker Bcl-2 was increased, while Bax was decreased in pre-treated rats, which was further confirmed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. The present study provides evidence that pre-treatment with HEEJ attenuates oxidative stress, apoptosis and improves cardiac architecture in ISP-induced rats and, hence, is cardioprotective.

KEY WORDS: • apoptosis • *Eugenia jambolana* • isoproterenol • myocardial infarction • oxidative stress

INTRODUCTION

ISCHEMIC HEART DISEASE (IHD) is the foremost cause of mortality globally. Prevention of myocardial infarction (MI) and decreases in mortality rates are of utmost importance and a major concern. The main culprit in MI is imbalance between coronary blood supply and myocardial demand, which leads to progression of MI. Isoproterenol (ISP)-induced cardiac necrosis results in increased oxygen consumption, insufficient oxygen utilization, increased calcium overload, alterations of membrane permeability, intracellular acidosis, and elevation in lipid peroxides.¹ The *in vivo* model of MI, which mimics human MI, has great importance.^{2–5} ISP-induced MI is a well-standardized model, because the pathophysiological changes after ISP administration are comparable to those taking place in hu-

man MI.⁶ There is an urgent need for drugs that can limit myocardial injury and protect the myocardium from toxic substances.

Natural resources, particularly medicinal plants, have been advocated for various disorders and been used since ancient times. *Eugenia jambolana*, commonly known as jamun, is an evergreen tree of the Myrtaceae family that has been traditionally used for the treatment of diabetes and cardiovascular ailments.⁷ Of the numerous herbal drugs in the Ayurvedic system of medicine of India, *E. jambolana* is being widely used to treat diabetes by traditional practitioners.⁸ *E. jambolana* is extensively used in various traditional systems of medicine such as in the Ayurveda, Unani, Siddha, and Homeopathy system of alternative and complementary medicine.⁹ *E. jambolana* fruit pulp is reported to contain 0.54% anthocyanins, 0.17% gallic acid/ellagic acid/ellagitannins, and 1.15% total polyphenolics.¹⁰ Vitamin E is a lipid soluble antioxidant that protects poly unsaturated fatty acids and cell and organelle membranes from oxidation of free radicals and reactive oxygen species (ROS).¹¹ Intake of vitamin E is associated with decreased incidence of

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IHD.¹² The protective effect of vitamin E treatment against myocardial ischemic injury in rats has recently been demonstrated.¹³

A wide range of validated pharmacological activities of different parts of *E. jambolana*, namely antibacterial,^{14,15} antifungal,¹⁴ free radical scavenging,^{16,17} anti-diabetic,¹⁸ anti-atherosclerotic,¹⁹ hypolipidemic,^{20,21} hypoglycemic,²² gastro-protective,^{18,23} hepatoprotective,²⁴ and anti-inflammatory,^{25,26} have been reported. However, no research work to date has been reported for screening of cardioprotective/anti-apoptotic activity of hydroalcoholic extract of fruit pulp of *E. jambolana* (HEEJ) in ISP-induced myocardial damage in rats. Based on the variable significant biological effects contributed by this plant, the present study was conducted to investigate the cardioprotective and anti-apoptotic effect of HEEJ on myocardial necrosis induced by ISP with reference to markers of inflammation, cardiac markers, apoptotic markers, and histo-architectural changes occurring during ischemic episode.

MATERIALS AND METHODS

Animals

Male Wistar albino rats (weight 150–200 g; 10–12 weeks of age) were obtained from the Central Animal House facility of University College of Medical Sciences and GTB Hospital and housed in polyacrylic cages (four rats per cage) with controlled temperature ($22 \pm 2^\circ\text{C}$) and humidity ($55\% \pm 5\%$) under standard laboratory conditions with 12 h light:dark cycle. The rats were allowed free excess to a standard pellet diet (Durga Brothers Pvt. Ltd.) and tap water *ad libitum*. The study protocol was reviewed and approved by the Institutional Animal ethics committee (MC/IAEC/121/07) and conformed to the Indian National Science Academy guidelines for the use and care of experimental animals in research.

Drugs and chemicals

ISP was obtained from Sigma Chemical Company. Other chemicals were of analytical grade. Creatine kinase-myocardial band (CK-MB) and serum glutamate oxaloacetate transaminase (SGOT) assay kits were procured from Spinreact SA. Troponin I and interleukin-6 (IL-6) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Calbiotech, C-reactive protein (CRP) ELISA kit was purchased from Biovender Czech Republic, and α -tocopherol was from Merck. Immunohistostaining detection kit based on horseradish peroxidase (HRP) polymer detection system was purchased from Thermo Fisher Scientific and primary antibodies (Bax mouse monoclonal IgG2 and Bcl-2 mouse monoclonal IgG1) were from Santa Cruz Biotechnology. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit was purchased from Roche Diagnostic.

Plant material and extraction

E. jambolana was purchased from local market of Azadpur Mandi and identified by a botanist (voucher specimen no. P-96/6) in the botanical garden. Fresh fruits of

E. jambolana were washed thoroughly, and seeds were separated from fruit pulp. The pulp was ground for 10 min in a mixer and mixed with 10 volumes of ethanol (50% v/v), which was kept at room temperature for 24 h with occasional shaking and sonicated for 30 min before filtration using 5–6 layers of muslin cloth. The whole procedure was repeated twice for complete extraction using residue. All three filtrates were pooled, centrifuged at 8000 g, and then lyophilized. They were protected from light and kept in a tightly closed bottle in the refrigerator ($2\text{--}8^\circ\text{C}$) to be used throughout the experiment. The yield of hydroalcoholic extract was 4.7% w/w of fruit pulp of *E. jambolana*.

Quantitative analysis of the HEEJ by thin layer chromatography and high performance thin layer chromatography

The sample was prepared by taking 1 g of HEEJ in 10 mL methanol by sonication and centrifuging at 1154 g. The supernatant was filtered with $0.22\ \mu\text{m}$ membrane filter and used for high performance thin layer chromatography (HPTLC) analysis. The stock solution of standard gallic acid (purity 98%) was prepared in high-performance liquid chromatography grade methanol to get 1.0 mg/mL solution. The samples were spotted in the form of bands of width 4 mm using a micro liter syringe on pre-coated silica aluminum sheet 60F₂₅₄ ($5.0\text{ cm} \times 10\text{ cm}$, $0.2\ \mu\text{m}$ thickness) using Camag Linomat V sample applicator. A constant application rate of 120 nL/s was employed, and space between two bands was 15 mm. The slit dimension was kept at $4\text{ mm} \times 0.30\text{ mm}$, and scanning speed was maintained at 20 mm/s. The mobile phase consisted of Toluene:Acetone:Glacial acetic acid:Formic acid (35:50:15:5 v/v/v/v). Linear ascending development was carried out in a $10\text{ cm} \times 10\text{ cm}$ twin trough glass chamber, which was previously saturated with mobile phase for 15 min. The length of the chromatogram run was 80 mm. After the development, thin layer chromatography (TLC) plates were dried in a current of air with the help of an air dryer. The densitometric scanning was performed on Camag TLC scanner III that was operated by Win Cats software using wavelength 254 nm.

Induction of myocardial ischemia

ISP was freshly prepared in normal saline and injected subcutaneously at a dose of 85 mg/kg bw to the rats for two consecutive days (28th and 29th day) at an interval of 24 h.^{27–29}

Experimental protocol

The rats were divided into seven groups of eight rats each ($n=8$).

- Group I: healthy rats were orally administered normal saline-normal (sham) control
- Group II: normal saline + ISP (85 mg/kg bw) (ischemic control)
- Group III: HEEJ 100 mg/kg bw + ISP (85 mg/kg bw)
- Group IV: HEEJ 200 mg/kg bw + ISP (85 mg/kg bw)
- Group V: HEEJ 400 mg/kg bw + ISP (85 mg/kg bw)
- Group VI: HEEJ 400 mg/kg bw without ISP
- Group VII: vitamin E 100 mg/kg bw + ISP (85 mg/kg bw)

The pre-treatment was given for 30 consecutive days once a day using an orogastric cannula. On the 28th and 29th days, rats were subjected to ISP (85 mg/kg bw) at an interval of 24 h. The healthy control group was not subjected to ISP insult. Vitamin E was dissolved in 5% gum acacia.

Sample collection

Blood samples were collected from retro-orbital plexus of the animals on days 0, 21, and 30 to perform biochemical studies. After blood sampling on day 30, rats were anesthetized with thiopentone sodium (30 mg/kg bw) intraperitoneally. To carry out immunohistochemical and histological studies, each heart was immediately dissected out, washed in ice-cold saline, and stored in 10% buffered neutral formalin solution.

Biochemical Estimations

Estimation of malondialdehyde. Serum malondialdehyde (MDA) levels were measured as an index of lipid peroxidation using the colorimetric method as described by Satoh.³⁰ Lipid peroxides were precipitated from serum with trichloroacetic acid and heated with thiobarbituric acid. The reaction results in formation of a pink-colored chromogen, which was extracted with *n*-butyl alcohol. Absorbance of organic phase was determined at 530 nm.

Estimation of reduced glutathione. Erythrocyte-reduced glutathione (GSH) content was estimated by the method described by Beutler *et al.*³¹ The method is based on the development of yellow color when 5,5'-dithiobis-2-nitrobenzoic acid is added to sulfhydryl compounds. The absorbance was measured at a wavelength of 412 nm, and results were expressed as mg/dL.

Estimation of superoxide dismutase. Activity of superoxide dismutase (SOD) in erythrocytes was assayed by the method of Marklund and Marklund³² and as modified by Nandi and Chatterjee.³³ The method is based on the ability of the enzyme SOD to inhibit the auto-oxidation of pyrogallol. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation per 3 mL of assay mixture. Fifty-percent inhibition was considered when a change in OD/min from 1 to 3 min at 420 nm was 0.010–0.012. Finally, the results were expressed as unit per gram of hemolysate Hb (U/g Hb).

Assay of cardiac markers

The cardiac markers such as SGOT³⁴ and CK-MB³⁵ in serum were estimated spectrophotometrically using commercially available kits. Cardiac troponin I (cTnI) in serum was measured by standard ELISA method.

Determination of markers of inflammation

CRP and IL-6 in serum were determined by using standard ELISA kits. Tumor necrosis factor alpha (TNF- α) level was determined in cardiac tissue using standard Western blot method as described by Maheshwari *et al.*^{36–38}

Apoptotic studies

Myocardial tissue samples preserved in 10% buffered formalin were carefully embedded in molten paraffin with the help of metallic blocks, covered with flexible plastic molds, and kept under freezing plates to allow the paraffin to solidify. Cross sections (5 μ m thick) of the fixed myocardial tissues were cut from paraffin-embedded blocks on a microtome, mounted onto poly-lysine-coated microscope slides, and dried completely to proceed for immunohistostaining and TUNEL assay.³⁹

Localization of Bax and Bcl-2 proteins by Western blot

Western blotting was performed as described by Maheshwari *et al.*^{36–38} Tissue lysate was prepared in 200 μ L lysis buffer containing 20 mM HEPES (pH 7.4), 2 mM ethylenediaminetetraacetic acid, 50 mM β -glycerophosphate, 1% Triton X-100, 150 mM NaCl, 10% glycerol, and protease inhibitor cocktail (Roche). Lysates were clarified by centrifugation at 12,000 g at 4°C for 20 min, and the protein concentration of the supernatant was determined with the Bradford assay. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis, protein lysate was mixed with Laemmli sample buffer (Bio-Rad Laboratories) and boiled for 10 min. Total protein (50–100 μ g) was separated on a 12% gel and transferred to a nitrocellulose membrane (Millipore) using wet-blotting apparatus (Bio-Rad Laboratories). After blocking in tris buffered saline and tween-20 (TBST) (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween-20, and pH 7.6) with 5% skimmed milk, membranes were incubated with the primary antibodies (1:500–2000) diluted in TBST for 2 h at room temperature. Membranes were then washed thrice with TBST and incubated for an additional 2 h with HRP-linked secondary antibody (1:2000) diluted in TBST. Again, membranes were washed thrice with TBS and labeled protein bands were visualized with the diaminobenzidine (DAB) system (Bangalore Genei). β -Actin was used to monitor an equal loading of protein. Densitometric analysis was performed with the help of Image analysis software (Lab Works Image analysis software 4.0; UVP).

TUNEL assay

Myocardial apoptosis was quantified by detection of DNA fragmentation using the TUNEL technique.³⁹ Briefly, the enzyme terminal deoxynucleotidyl transferase was used to incorporate residues of digoxigenin nucleotide into 3' OH ends of DNA fragments. The free end of cellular DNA was labeled by incubating the specimens in streptavidin conjugated to HRP enzyme and peroxidase substrate. TUNEL signals were used to identify apoptotic cells using secondary reaction with antibodies and DAB chromogen. The slides were counterstained in methyl green. Total cell counts and TUNEL-positive cells in the specimens were determined by light microscopy. The cells with clear nuclear labeling were defined as TUNEL-positive cells.

Histomorphological studies

Myocardial tissue fixed in buffered formalin was processed for paraffin embedding, sectioned at 5 μ m, and

mounted on to microscopic slides. These sections were stained with haematoxylin and eosin, and visualized under a light microscope to study the histoarchitectural changes of the myocardium. The degrees of damage to heart tissues were graded according to their severity.

Statistical analysis

The results were expressed as mean $\pm$ standard error of the mean, and statistical differences between mean values were determined by analysis of covariance taking baseline as a co-variant. Multiple comparisons among the groups were done using Bonferroni adjustment method. A value of $P < .05$ was considered statistically significant. In the present study, out of three time points (days 0, 21, and 30), only two

time point (days 0 and 30) were used; 21st days data were excluded from the study, because ischemia was induced on 28th and 29th days. Twenty-first day rats were normal. There was no significant difference between day 0 and 21 values, therefore they were excluded from the study.

RESULTS

Phytochemical analysis of HEEJ

Phytochemical analysis of HEEJ shows the presence of a significant amount of tannins. Gallic acid represents the major component of tannins. Therefore, the concentration of gallic acid was determined in HEEJ. HEEJ showed the presence of various constituents at different R_f in short

FIG. 1 High-performance thin layer chromatography (HPTLC) images: (A) gallic acid standard; (B) hydroalcoholic extract of *Eugenia jambolana*.

wavelengths (254 nm). The constituent with R_f 0.51 was confirmed as gallic acid on co chromatography with a standard. Quantitative determination of gallic acid was further carried out by HPTLC densitometric analysis using the same solvent at 254 nm. The R_f value was 0.51 at 254 nm. Figure 1A and B shows the HPTLC chromatograms of standard gallic acid and HEEJ. The content of gallic acid was determined to be 0.674% w/w in the HEEJ.

Effect of HEEJ on antioxidant status/oxidative stress parameters

As shown in Table 1, ISP control rats exhibited significantly higher serum MDA levels; however, lower SOD activity and GSH content was observed as compared with the healthy control group. Pre-treatment with different doses of HEEJ (100, 200, and 400 mg/kg bw) and vitamin E (100 mg/kg bw) produced a dose-dependent response on oxidative stress markers. MDA levels were significantly lower, whereas GSH and SOD levels were significantly higher as compared with ISP control. HEEJ 400 mg/kg bw pre-treatment produced significantly greater effects as compared with ISP control group.

Effect of HEEJ on cardiac markers

Figure 2 shows that due to ISP administration, cardiac markers (CK-MB, SGOT, and Troponin I) were significantly elevated in serum, as compared with sham control group. Pre-treatment with different doses of HEEJ (100, 200, and 400 mg/kg bw) and vitamin E (100 mg/kg bw) resulted in significantly lower levels of the elevated cardiac markers, with the lowest levels in HEEJ 400 mg/kg bw as compared with ISP control group.

Effect of HEEJ on IL-6 and CRP

As shown in Figure 3, the levels of IL-6 and CRP in the serum of ISP control rats were significantly higher. Pre-

treatment with HEEJ (100, 200, and 400 mg/kg bw) and vitamin E (100 mg/kg bw) had produced dose-dependent improvements in IL-6 and CRP levels. HEEJ 400 mg/kg bw dose showed significantly suppressed IL-6 and CRP levels as compared with ISP control.

Effect of HEEJ on histomorphological changes

Table 2 depicted the histomorphological changes during ischemic episode. The photomicrographs were used to evaluate the degree of damage in the heart tissues, and they were graded and summarized: (A) No differences were observed in normal control. (+) Focal differences were observed in HEEJ 200, 400, and vitamin E groups; (++) mild differences were observed in HEEJ100 group; and (+++) marked differences were observed in ISP control.

As shown in Figure 4, the microscopic examination of heart sections of the healthy control showed normal architecture of the myocardium (Fig. 4A). ISP control rats showed marked focal myonecrosis, hyper-contracted myofibrils, vacuolar degeneration, and lymphocytic infiltration (Fig. 4B). HEEJ pre-treated rats (100, 200, and 400 mg/kg bw) exhibited structural improvement with regard to a decreased degree of myonecrosis and contraction in myofibrils (Fig. 4C–E). In addition, lesser vacuolization and inflammation in the myocytes was observed. Vitamin E-treated rats also had fewer myocardial abnormalities (Fig. 4F).

Effect of HEEJ on apoptotic markers

Apoptotic markers were quantified by Western blotting. Figure 5 shows the expression of Bcl-2, Bax proteins, and pro-inflammatory cytokine TNF- α in response to ISP and HEEJ. ISP up-regulated pro-apoptotic (Bax) and TNF- α protein and down-regulated anti-apoptotic (Bcl-2) proteins in cardiomyocytes, whereas HEEJ pre-treatment suppressed these apoptosis-provoking alterations by ISP in Bcl-2, Bax, and TNF- α protein expression.

Effect of HEEJ on TUNEL positivity

Figure 6 shows the determination of apoptosis by TUNEL assay. No TUNEL-positive cells were observed in the healthy controls (Fig. 6A). In healthy controls, the cells took up methyl green staining indicative of normal cells. However, TUNEL-positive cells were indicated by arrow, takes brown to black staining in the ischemic control (Fig. 6B). Pre-treatment with HEEJ (100 and 200 mg/kg bw) resulted in moderate TUNEL staining (Fig. 6C, D). Pre-treatment with HEEJ 400 mg/kg bw and vitamin E (100 mg/kg bw) resulted in significantly fewer TUNEL-positive cells as compared with ischemic control group (Fig. 6E, F).

DISCUSSION

The current study entails the cardioprotective potential of the HEEJ against ISP-induced myocardial damage in rats that has not been reported thus far. In the present study, we evaluate the efficacy of HEEJ as a future intervention as an adjunct therapy against ISP-induced MI by establishing its

TABLE 1. EFFECT OF HYDROALCOHOLIC EXTRACT OF *EUGENIA JAMBOLANA* FRUIT PULP PER SE ON OXIDATIVE STRESS PARAMETERS IN ISOPROTERENOL-INDUCED MYOCARDIAL INFARCTED RATS

Experimental groups	MDA (nmol/mL)	GSH (mg/dL)	SOD (U/g Hb)
Healthy control	1.5 $\pm$ 0.05	35.01 $\pm$ 1.86	1250 $\pm$ 25
ISP control, ISP (85)	4.08 $\pm$ 0.09*	22.76 $\pm$ 0.47*	855 $\pm$ 27*
HEEJ 100 + ISP (85)	2.60 $\pm$ 0.20	30.57 $\pm$ 0.50	1006 $\pm$ 26
HEEJ 200 + ISP (85)	2.04 $\pm$ 0.17 [#]	30.84 $\pm$ 1.12 [#]	1101 $\pm$ 28 [#]
HEEJ 400 + ISP (85)	1.95 $\pm$ 0.02 [#]	31.65 $\pm$ 1.08 [#]	1182 $\pm$ 20 [#]
HEEJ 400	1.63 $\pm$ 0.06	33.24 $\pm$ 1.77	1249 $\pm$ 25
Vitamin E 100 + ISP (85)	1.83 $\pm$ 0.10 [#]	31.80 $\pm$ 1.19 [#]	1121 $\pm$ 27 [#]

Values are expressed as mean $\pm$ SEM (mg/kg bw).

* P < .05 as compared with healthy control; [#] P < .05 as compared with ISP control (n = 8).

HEEJ, hydroalcoholic extract of fruit pulp of *Eugenia jambolana*; ISP, isoproterenol; MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase; SEM, standard error of the mean.

FIG. 2. Effect of hydroalcoholic extract of fruit pulp of *E. jambolana* (HEEJ) on cardiac markers (serum glutamate oxaloacetate transaminase [SGOT], creatine kinase-myocardial band [CK-MB], and troponin I) in control, drug-treated, and ischemic rats. Values are expressed as mean $\pm$ standard error of the mean (SEM), * $P < .05$ as compared with healthy control; # $P < .05$ as compared with isoproterenol (ISP) control ($n = 8$).

antioxidant, anti-inflammatory, anti-apoptotic, and cardioprotective potential in rats. Experimental and clinical evaluation of *E. jambolana* suggests that it may be beneficial in the treatment of diabetes, coronary artery diseases, and hypercholesterolemia.^{7,22,40} Synthetic catecholamine ISP is a well-known β 1-adrenergic receptor and cardiotoxic agent that produces severe myocardial injury in rats. The existing experimental evidence suggested that ISP-induced MI leads to acute β -adrenergic receptor stimulation, which conse-

quently generates ROS, decreases total cellular antioxidant capacity, down-regulates SOD activity and GSH levels, induces myocyte toxicity, and, finally, leads to myocardial necrosis.^{41,42} In the present study, ISP administration caused a significant elevation in lipid peroxidation and suppression of antioxidant defenses as evidenced by significantly suppressed SOD activity and GSH levels. Pre-treatment with HEEJ at 200 and 400 mg/kg doses decreased MDA levels and improved the SOD and GSH. Since oxidative stress is

FIG. 3. Effect of HEEJ on markers of inflammation (interleukin [IL]-6 and C-reactive protein [CRP]) in control, drug-treated, and ischemic rats. Values are expressed as mean $\pm$ SEM, * $P < .05$ as compared with healthy control; # $P < .05$ as compared with ISP control ($n = 8$).

TABLE 2. EFFECT OF HYDROALCOHOLIC EXTRACT OF *EUGENIA JAMBOLANA* FRUIT PULP ON THE DEGREE OF DAMAGE IN HEART TISSUE

Experimental groups	Myonecrosis	Inflammation	Edema
Healthy control	A	A	A
ISP control (ISP, 85)	+++	+++	+++
HEEJ 100+ISP 85	+++	+++	+++
HEEJ 200+ISP 85	++	++	+++
HEEJ 400+ISP 85	+	+	+
HEEJ 400	A	A	A
Vitamin E 100+ISP 85	+	+	+

Photomicrographs were used to evaluate the degree of damage in the heart tissues: (A) no changes; (+) focal changes; (++) mild changes; (+++) marked changes.

All doses were taken as mg/kg bw.

the major deleterious factor, it can be suggested that the antioxidant effect of HEEJ abates the development of MI produced by ISP. Due to the ability of ISP to initiate the destruction of myocardial cells, cytosolic enzymes are released into the blood stream and thus serve as the diagnostic markers of myocardial tissue damage.^{43,44} The amount of these cellular enzymes present in blood reflects the alterations in plasma membrane integrity and/or permeability. In

the present study, ISP-treated rats showed significant elevations in the serum levels of cardiac markers (SGOT, CK-MB, and cTnI). Pre-treatment with HEEJ (100, 200, and 400 mg/kg) significantly prevented ISP-induced elevation of cardiac markers. Lower levels of cardiac markers could be due to its action on maintaining membrane integrity, thereby restricting their leakage. Moreover, the significant rise observed in the levels of diagnostic markers in the serum after ISP administration is an indication of the severity of the necrotic damage to the myocardial membrane.⁴⁵ These markers appear in serum in proportion to the number of necrotic cells.⁴⁶ Thus, a decrease in cardiac markers reflects reduced extent of myocardial damage in rats pre-treated with *E. jambolana*.

Markers of inflammation play an important role in MI. Inflammatory responses are integral components of the host immune response to cardiomyocyte injury and play an active role after MI. The important determinant of the host's outcome is the degree of the inflammatory response, because it can lead to healing and restoration of function after acute cardiac rupture.⁴⁷ Markers of inflammation, such as TNF- α , CRP, IL1 β and IL-6, are usually unexpressed in the normal heart, while they are up-regulated during myocardial damage and are clearly present in both animal and human models during MI.⁴⁷⁻⁵⁰ In this study, inflammatory markers

FIG. 4. Representative photomicrographs of myocardial section stained with hematoxylin and eosin (H&E, 200 $\times$) from different experimental groups. (A) Healthy control showing normal architecture of myocardium. (B) ISP control showing myocardial necrosis, edema, and inflammatory cell infiltration (indicated by the arrows). (C, D) HEEJ 100, 200 pre-treatment showing improvement in myocardial architecture. (E, F) HEEJ 400 and vitamin E treated rats, showing significant improvement in myocardial architecture with less inflammation. Color images available online at www.liebertpub.com/jmf

FIG. 5. Immunoblotting analysis of Bcl-2, Bax, and pro-inflammatory cytokine tumor necrosis factor alpha (TNF- α) in response to ISP and HEEJ treatment. 1, healthy control; 2, ISP control; 3, HEEJ 100 mg/kg bw + ISP; 4, HEEJ 200 mg/kg bw + ISP; 5, HEEJ 400 mg/kg bw + ISP; 6, HEEJ 400 mg/kg bw; 7, vitamin E 100 mg/kg bw + ISP.

were up-regulated during ischemic episode, while they were down regulated in HEEJ pre-treated groups, validating their protective effect. There are two distinct types of cell death in myocardium, necrosis, and apoptosis, which have been linked with cardiac damage. Cardiomyocyte apoptosis plays an important role in the initiation and progression of cardiac diseases. Drugs that effectively and specifically inhibit apoptosis might be useful therapeutic agents for attenuating myocardial injury.⁵¹ Hence, screening of HEEJ for anti-apoptotic activity is of clinical importance. In the present study, anti-apoptotic marker Bcl-2 (an inhibitor of apoptosis) and pro-apoptotic Bax (an inducer of apoptosis) were evaluated and a further TUNEL assay was performed to see the involvement of apoptosis in ISP-induced injury.

Results of the present study demonstrated that ISP administration triggers apoptotic cell death as evidenced by increased TUNEL positivity and Bax expression and

FIG. 6. Representative photomicrographs of ventricular tissue stained for nick-end labeling [terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)] for DNA breaks (200 $\times$) from different experimental groups. TUNEL-positive nuclei were stained as brown to black. (A) Healthy control showing no TUNEL-positive cells. (B) ISP control showing prominent TUNEL-positive nuclei, indicated by arrow, takes brown to black staining. (C) HEEJ 100 showing no improvement. (D) HEEJ 200-pretreated rats showing improvement in reduction of TUNEL positivity. (E, F) HEEJ 400 and vitamin E pre-treated rats, significantly reduced TUNEL positivity. Values are expressed as mean $\pm$ SEM. * $P < .05$ as compared with healthy control; # $P < .05$ as compared with ISP control ($n = 8$). Color images available online at www.liebertpub.com/jmf

reduction in Bcl-2 expression in the ISP control group as compared with healthy control group. This observation receives support from earlier studies.^{52,53} Pre-treatment with HEEJ decreased apoptotic cell numbers, which were further confirmed by increased Bcl-2 and decreased Bax expression as compared with ISP control rats. Various studies have demonstrated that not only ROS *per se*, but also their oxidation products and other secondary messenger molecules generated by ROS can trigger the programmed cell death.⁵⁴ It has been reported that programmed cell death pathways can be inhibited by antioxidants.^{55,56}

Therefore, it can be suggested that HEEJ exhibits anti-apoptotic potential due to its strong antioxidant property. Histomorphologic evaluation further confirms the cardioprotective/anti-apoptotic potential of HEEJ. Rats pre-treated with HEEJ demonstrated improvement in structural myocardial morphology in contrast to ischemic control group. Hence, HEEJ may have salvaged myocytes and prevented cell loss induced by apoptosis and necrosis.

In conclusion, the present study shows that chronic administration of HEEJ has strong antioxidant, cardioprotective, and anti-apoptotic potential against myocardial damage induced by ISP. Our study demonstrated that HEEJ pre-treatment has better antioxidant, anti-apoptotic, and anti-inflammatory activity. Hence, it may be useful as adjunct therapy along with conventional drugs and may be useful as a future intervention for various cardiovascular ailments.

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AUTHOR DISCLOSURE STATEMENT

The authors declare that there is no conflict of interest.

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