

Isorhamnetin protects against isoproterenol-induced myocardial infarction via modulation of oxidative stress, apoptosis, and membrane-bound ATPase activities in rats

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Conflict of interest

The authors declare that they have no conflicts of interest.

CRedit authorship contribution statement

Dheshika Ramaraj: Formal analysis, Investigation, Writing - review & editing, Writing original draft, Visualization, Methodology,

Subashini Ragunathan: Resources, Project administration, Validation, Supervision, Investigation, Formal analysis, Data curation, Conceptualization.

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Ethical approval

The Institutional Animal Ethics Committee (IAEC) of Annamalai University in Tamil Nadu, India, approved this study (IAEC Proposal No. AU-IAEC-1356/09/23).

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Declaration of Competing Interest

The authors affirm that the work described in this publication was not influenced by any known competing financial interests or personal relationships.

Data availability

The data that support the findings of this study are available within the article.

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Abstract

Background: Myocardial infarction (MI) is a leading cause of death worldwide, primarily associated with oxidative stress, inflammation, apoptosis, and ionic imbalance. Isoproterenol (ISO)-induced MI in rats mimics human cardiac injury. Flavonoids have gained attention for their antioxidant and cardioprotective effects. Isorhamnetin, a natural flavonoid, possesses potent antioxidant and anti-inflammatory properties, but its cardioprotective mechanisms in MI remain unclear. This study aimed to evaluate the protective role of isorhamnetin against ISO-induced MI in rats.

Methods: Male Wistar albino rats were divided into four groups: normal control, isorhamnetin alone (10 mg/kg), ISO-induced MI (100 mg/kg, s.c., 2 days), and ISO + isorhamnetin-treated. Cardiac injury biomarkers (CK-MB, cTn-T, cTn-I), lipid peroxidation, antioxidant status (enzymatic and non-enzymatic), membrane-bound ATPase activities, and electrolyte levels were assessed. Apoptosis-related proteins were measured by ELISA and gene expression by RT-PCR. Histopathology was performed using H&E and Masson's trichrome staining. In vitro antioxidant activity was assessed via DPPH and ABTS⁺ assays.

Results: ISO administration significantly increased cardiac biomarkers, lipid peroxidation, apoptotic markers, electrolyte imbalance, and histological damage, while reducing antioxidant defenses and Na⁺/K⁺-ATPase activity (P<0.05). Isorhamnetin treatment mitigated these changes by restoring antioxidant enzymes, reducing oxidative stress, normalizing ATPase function and ionic balance, and inhibiting Fas-receptor and caspase-mediated apoptosis (P<0.05). Histology revealed reduced necrosis, inflammation, edema, and fibrosis. In vitro assays confirmed strong free radical scavenging activity of isorhamnetin.

Conclusion: Isorhamnetin protects against ISO-induced MI by reducing oxidative stress, preserving membrane integrity, maintaining ionic homeostasis, and suppressing apoptosis. These findings highlight isorhamnetin as a promising natural cardioprotective agent for MI.

Keywords: Isoproterenol, isorhamnetin, myocardial infarction, antioxidant activity, anti-apoptotic activity, cardiac biomarkers, histopathology

Introduction

Cardiovascular diseases (CVDs) such as atherosclerosis, coronary artery disease (CAD), and stroke are the major causes of death worldwide. The World Health Organization predicts that CVD deaths may reach 23.6 million by 2030, with developing countries affected more due to limited healthcare and lifestyle changes.^{1,2} Obesity, diabetes, high blood pressure, and sedentary lifestyles are the primary causes.¹ In India, CVDs cause 30-42% of deaths, with around 54.5 million cases reported in 2016.³

Myocardial infarction (MI), which is the most severe form of CAD, occurs due to reduced blood flow, consequently causing permanent myocardial damage. It is typically precipitated by plaque rupture, thrombosis, or full artery blockage, which in turn removes the oxygen supply.^{5,6} This in turn causes the production of large amounts of reactive oxygen species (ROS), changes in cardiac enzymes, and initiation of apoptotic pathways.⁷

A dependable rat model for cardiac injury research is isoproterenol (ISO)-induced MI. ISO promotes ROS, lipid peroxidation, oxidative stress, hypoxia, and calcium overload. These elements lead to membrane injury, and then to apoptosis, necrosis, and fibrosis, along with impaired electrical conduction.^{7,8} Because this

model closely resembles human MI, it is frequently used to study potential cardioprotective agents, especially phytochemicals.^{9,10}

The cardiac adenosine triphosphates (ATPases) play an essential role in ensuring sodium, potassium, and calcium homeostasis of the cardiomyocytes through contraction and relaxation of the heart and transmission of intracellular signals. Disruption in ATPase activity causes impaired ion gradients and disturbed mineral homeostasis, and ultimately, dysfunction of the heart following MI. A recent investigation indicates that ATPase can be of interest in therapeutic studies in MI and other heart related diseases.^{11,12}

Although MI is widely studied, its molecular mechanisms are still not fully understood, so new treatments are needed.¹³ Strong antioxidants, cardio-protective, and anti-inflammatory properties have been developed by flavonoids. Studies show that higher flavonoid intake lowers CVD risk.^{14,15} Isorhamnetin, found in berries, wine, onions, almonds, and some medicinal plants,¹⁶ has many biological effects, including antibacterial, neuroprotective, anticancer, anti-hypoxic, antiviral, and antihyperuricemic actions.¹⁷

This study, therefore, investigated the cardioprotective effects of isorhamnetin in ISO-induced MI rats by measuring oxidative stress, antioxidant enzymes, lipid peroxidation, histopathological changes, apoptotic gene expression (RT-PCR), and apoptosis-related proteins (ELISA). ATPase activity, electrolyte imbalance, and in vitro antioxidant activity were also evaluated.

Materials and methods

Animals

Male albino Wistar rats weighing 180–200g were procured from the Biogen Laboratory Animal Facility, Bengaluru, India. Animals are purchased from licensed

breeders and maintained in a disease-free state to guarantee accurate data gathering. The animals were housed in polypropylene cages measuring $47 \times 34 \times 20$ cm under a 12-hour light/dark cycle at a controlled temperature of approximately 22°C in the Central Animal House of Annamalai University. A typical pellet meal and unlimited tap water were given to the rats. The study was conducted in accordance with the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA), New Delhi, India. This study was approved by the Institutional Animal Ethics Committee (IAEC), Annamalai University, Tamil Nadu, India (IAEC Proposal No. AU-IAEC-1356/09/23).

Experimental design

Phase I: Dose Selection Study

Following a 7-day acclimatization period, healthy rats were randomly divided into eight groups ($n = 6$ per group) to evaluate the dose-dependent effects of isorhamnetin (2.5, 5, and 10 mg/kg b.w.) in ISO-induced MI rats. Serum CK-MB activity was assessed to determine the optimal dose of isorhamnetin for subsequent investigations.

Group A: Normal control rats received 2 ml of normal saline orally once daily via an intragastric tube throughout the experimental period (days 1–18; $n = 6$).

Group B: Rats received normal saline during days 1–4 of the experimental period and subsequently received isorhamnetin (2.5 mg/kg b.w.) orally once daily for 14 consecutive days from day 5 to day 18 ($n = 6$).

Group C: Rats received normal saline during days 1–4 of the experimental period and subsequently received isorhamnetin (5 mg/kg b.w.) orally once daily for 14 consecutive days from day 5 to day 18 ($n = 6$).

Group D: Rats received normal saline during days 1-4 of the experimental period and subsequently received isorhamnetin (10 mg/kg b.w.) orally once daily for 14 consecutive days from day 5 to day 18 (n = 6).

Group E: MI was induced by subcutaneous administration of ISO (100 mg/kg b.w.) at 24-h intervals on days 1 and 2 of the experimental period (n = 6). Following ISO administration, animals were maintained without further treatment for a 2-day myocardial injury development period (days 3 and 4) and thereafter continued under standard experimental conditions until the common study endpoint (day 18).

Group F: MI was induced by subcutaneous administration of ISO (100 mg/kg b.w.) at 24-h intervals on days 1 and 2 of the experimental period. Following a 2-day myocardial injury development period (days 3 and 4), rats received isorhamnetin (2.5 mg/kg b.w.) orally in 2 ml normal saline once daily for 14 consecutive days from day 5 to day 18 (n = 6).

Group G: MI was induced by subcutaneous administration of ISO (100 mg/kg b.w.) at 24-h intervals on days 1 and 2 of the experimental period. Following a 2-day myocardial injury development period (days 3 and 4), rats received isorhamnetin (5 mg/kg b.w.) orally in 2 ml normal saline once daily for 14 consecutive days from day 5 to day 18 (n = 6).

Group H: MI was induced by subcutaneous administration of ISO (100 mg/kg b.w.) at 24-h intervals on days 1 and 2 of the experimental period. Following a 2-day myocardial injury development period (days 3 and 4), rats received isorhamnetin (10 mg/kg b.w.) orally in 2 ml normal saline once daily for 14 consecutive days from day 5 to day 18 (n = 6).

Phase II: Main Experimental Study

Based on the dose-selection study, groups 1, 4, 5, and 8 were selected for further biochemical, antioxidant, molecular, and histopathological analyses and redesignated as follows:

Group 1: Normal control rats received 2 ml of normal saline orally once daily from day 1 to 18 of the experimental period (n = 6).

Group 2: Rats received 2 ml of normal saline orally once daily from day 1 to 4, followed by isorhamnetin (10 mg/kg b.w.) orally in 2 ml normal saline once daily from day 5 to 18 (n = 6).

Group 3: MI was induced by subcutaneous administration of ISO (100 mg/kg b.w.) at 24 h intervals on days 1 and 2 of the experiment, followed by oral administration of 2 ml normal saline once daily from day 3 to 18 (n = 6).

Group 4: MI was induced by subcutaneous administration of ISO (100 mg/kg b.w.) at 24 h intervals on days 1 and 2 of the experiment. Following a 2-day MI development period (days 3 and 4), rats received isorhamnetin (10 mg/kg b.w.) orally in 2 ml normal saline once daily from day 5 to 18 (n = 6).

At the end of the experimental period, all animals were euthanized on day 18 of the experiment. In the treatment group, isorhamnetin administration was initiated on day 5 following ISO-induced MI and continued for 14 consecutive days (days 5-18). To ensure uniformity in sample collection and facilitate valid intergroup comparisons at a common endpoint, all experimental groups, including the ISO-alone control group, were maintained until day 18 prior to euthanasia. Rats were euthanized using pentobarbital sodium (60 mg/kg b.w.) and sacrificed by cervical decapitation. Two tubes were filled with blood, one containing an anticoagulant and one non-anticoagulant. The non-anticoagulated blood was allowed to clot for 30 minutes at

room temperature, after which serum was separated by centrifugation at 3000 rpm for 15 minutes. Plasma was obtained by cold centrifugation of the anticoagulated blood at $1500 \times g$ for 15 minutes.¹⁸ Serum and plasma samples were used for biochemical analyses. The hearts were excised, rinsed with ice-cold physiological saline, stored at -80°C , and utilised for various biochemical, molecular, and histochemical investigations.

The establishment of an experimental myocardial infarction model

A simple, widely used, conventional, and non-invasive experimental model for assessing cardioprotective agents is ISO-induced MI. In this study, myocardial infarction was induced in rats through subcutaneous administration of commercially available isoproterenol-hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 100 mg/kg body weight, administered twice on days 1 and 2 with a 24-hour interval.^{19,20} Myocardial infarction was indicated by elevated levels of cardiac troponin-T (cTn-T), cardiac troponin-I (cTn-I), and creatine kinase-MB (CK-MB).

Homogenate preparation

The entire heart was weighed after the tissues were quickly removed, washed in ice-cold saline, and dried in tissue paper. A known weight of cardiac tissues was homogenised using 5ml of 0.1M Tris-HCl buffer (pH 7.4). After centrifugating the homogenate, different biochemical parameters were assayed using clear supernatants.²¹

Cardiac enzyme biomarkers, heart weight index and biochemical parameters

Creatine kinase-MB (dose-dependent) activity was assessed using a commercial kit following the manufacturer's protocol, employing the serum immunoinhibition method (Product No. 11405001, Agappe Diagnostics, Ernakulam, Kerala, India). Serum levels of cardiac troponin-T and troponin-I were measured

using an electrochemiluminescence immunoassay kit (Elecsys Troponin-T and -I STAT reagent kit, Product Code 04660307190, Roche Diagnostics, Mannheim, Germany)

The body weights of the euthanised rats were recorded. After dissecting away the aorta and adipose tissue, the hearts were excised, cleaned, and weighed. The heart weight index (HWI) was calculated by the ratio of heart weight to body weight [18]. Heart tissues were then processed for histological analysis and preparation of tissue homogenates.

The levels of thiobarbituric acid reactive substances (TBARS) in plasma and heart tissues were determined according to the methods described by references 22 and 23. The levels of Lipid Hydroperoxides (LOOH) in the plasma and heart tissues were estimated using the method of reference 24. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST) in plasma and heart tissues were determined using standard methods.²⁵⁻²⁸ The levels of reduced glutathione (GSH), vitamin C, and vitamin E were measured using the methods described in references 29-31, respectively. The cardiac homogenate's protein content was determined using the Lowry method.³²

The activities of Na^+/K^+ -ATPase, Ca^{2+} -ATPase, and Mg^{2+} -ATPase were measured using the methods described in references 33-35, respectively.

The levels of cardiac Na^+ and K^+ were measured using commercial kits from Monozyme India (Secunderabad, India). Using a reagent kit purchased from Span Diagnostic (Surat, India), the o-Cresolphthalein complexone method was used to measure the levels of cardiac Ca^{2+} .

Heart apoptotic markers by Enzyme-Linked Immunosorbent Assay (ELISA)

The Bcl-2, Bax, Bcl-xL, Bad, cytochrome-C, Fas receptor, caspase-3, caspase-8, and caspase-9 concentrations in the heart homogenates were determined using the rat ELISA Kits, Cat. No.: MBS2515143; Cat. No.: MBS935667 (My Biosource Co, USA); Cat. No.: CSB-E19604r (Cusabio Co., China); Cat. No.: MBS2507517; Cat. No.: MBS9304546; Cat. No.: MBS451586; Cat. No.: MBS018987; Cat. No.: MBS260539; Cat. No.: MBS264306 (My Biosource Co, USA), respectively.

Gene expression study

RT-PCR analysis was performed to evaluate the differential expression of cardiac genes, including Bcl-2, Bcl-xL, Bax, Fas receptor, cytochrome c, and caspases-8, -9, and -3, in the myocardium. A TRISOL reagent was used to extract the total RNA from the cardiac tissues, and a kit (Aura Biotechnologies Private Limited, Chennai) was used to synthesise cDNA. Next, PCR amplification was carried out. Table 1 lists the rat forward and reverse primers used in RT-PCR.

Histopathological staining of the heart

The hearts were cleansed with xylene, dehydrated in successive alcohol solutions (100%–70%), and then preserved in paraffin during the autopsy. After being mounted on glass slides and deparaffinized on a hot plate, the 4µm specimen sections were stained with H&E and trichrome. Using ImageJ (NIH, Version 1.44p), photomicrographs of the histological alterations in cardiac tissues were taken under a light microscope.

In-vitro studies

Isorhamnetin's ability to scavenge the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was evaluated in vitro by the method.³⁶ Isorhamnetin's total antioxidant potential was assessed in vitro using the (2,2'-azinobis-(3-

ethylbenzothiazoline-6-sulfonic acid)) (ABTS⁺) radical assay was evaluated by the method.³⁷

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) using SPSS software version 31.0. Data are presented as mean $\pm$ standard deviation (SD) for six rats in each group. Differences were considered statistically significant at $P < 0.05$.

RT-PCR amplified products were quantified by densitometric analysis using ImageJ software (NIH, Bethesda, MD, USA). The intensity of each band was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and gene expression levels were expressed as relative arbitrary units.

RESULTS

Serum CK-MB (Dose-dependent study)

To determine the dose-dependent effects of isorhamnetin, serum CK-MB activity was evaluated at three different doses (2.5, 5, and 10 mg/kg body weight). Rats induced with ISO (Group 5) had significantly ($p < 0.05$) increased serum CK-MB activity than normal control rats (Group 1). Compared with ISO-induced rats (Group 5), myocardial infarcted rats administered isorhamnetin orally (2.5, 5, and 10 mg/kg body weight) for 14 days exhibited significantly lower ($p < 0.05$) serum CK-MB activity in groups 6, 7, and 8. Additionally, isorhamnetin (2.5, 5, and 10 mg/kg body weight) did not affect the serum CK-MB levels in normal rats (Groups 2, 3, and 4). From the findings, it was revealed that isorhamnetin (10mg/kg body weight) had the most significant effect. Therefore, we used this dosage in further research (Figure 1).

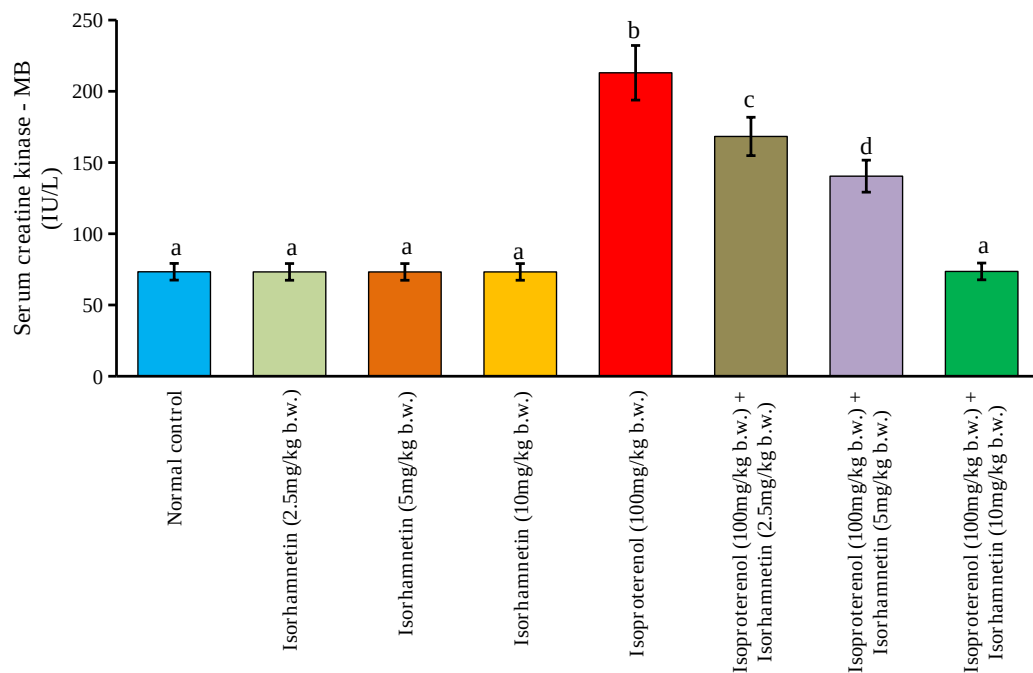


Figure 1.

Serum cardiac biomarkers

Serum levels of cTn-T and cTn-I were significantly ($p < 0.05$) higher in ISO-induced animals than in normal control rats. ISO-induced rats administered with isorhamnetin revealed ($p < 0.05$) reduced serum cTn-T and cTn-I levels compared with ISO- alone induced rats (Figure 2).

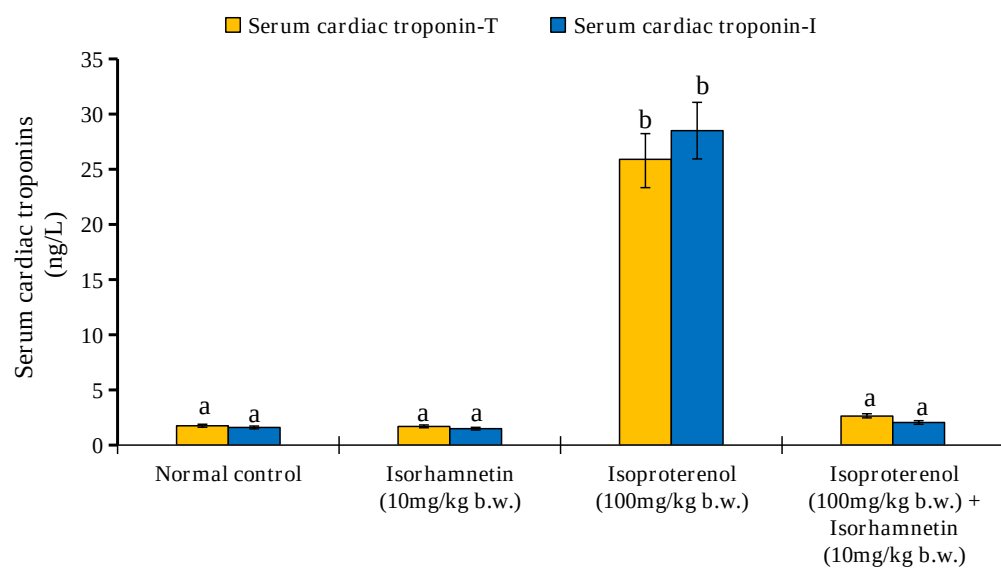


Figure 2.

Body weight, heart weight, and heart weight to body weight ratio

Evaluation of animal body weights pre- and post-euthanasia demonstrated that the ISO-induced myocardial infarcted rats had a significant ($p<0.05$) decrease in body weight as compared to normal control rats. Rats treated with isorhamnetin to ISO-induced myocardial infarcted rats exhibited a significant ($p<0.05$) increase compared to ISO-alone induced myocardial infarcted rats (Figure 3A).

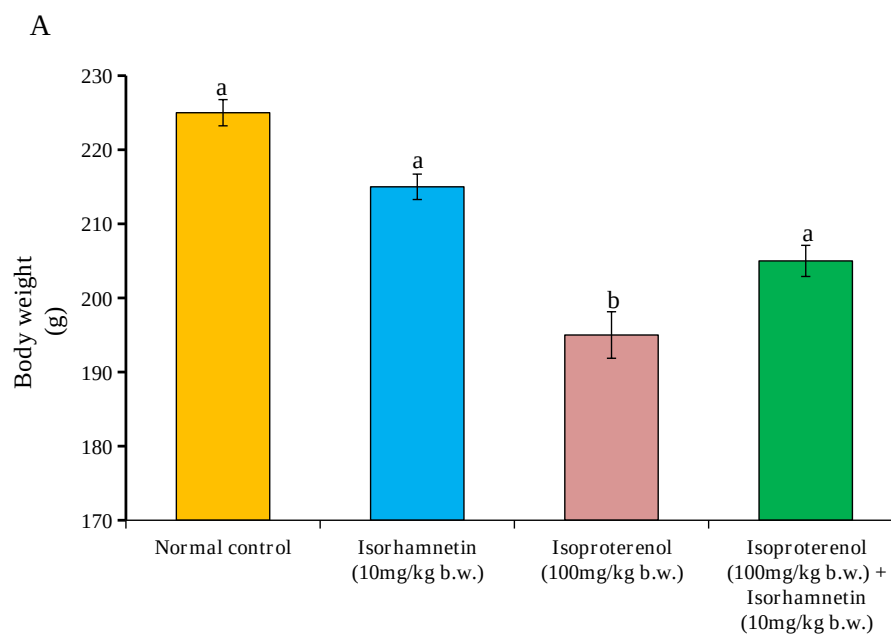


Figure 3A.

The whole heart weight of the ISO-induced myocardial infarcted rats was significantly ($p<0.05$) higher than normal control rats. However, compared to myocardial infarcted rats produced by ISO alone, the isorhamnetin post-treated rats had a considerably ($p<0.05$) lower heart weight (Figure 3B).

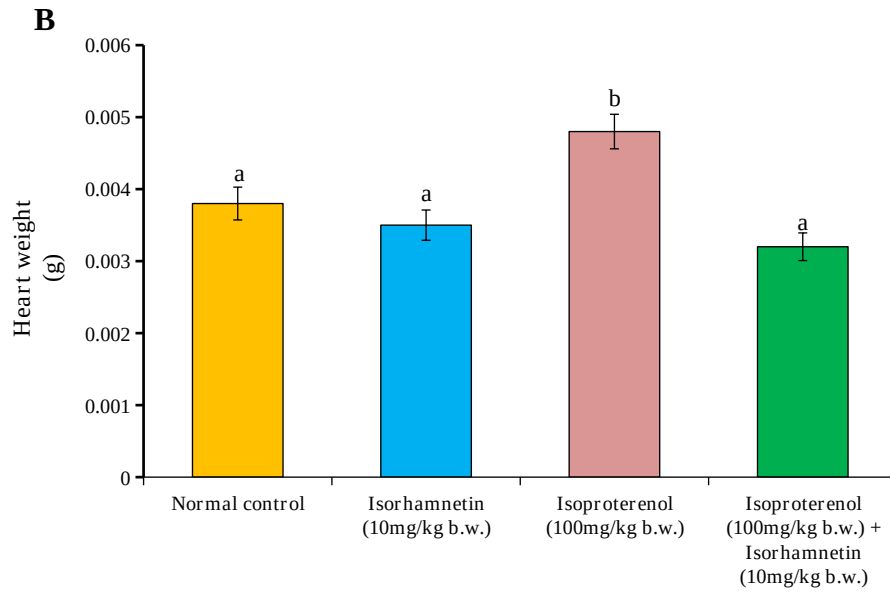


Figure 3B.

The increase in cardiac mass induced by ISO administration was measured using the heart weight to body weight ratio (HW/BW) (Figure 3C). The ratio was significantly ($p<0.05$) greater in ISO-treated rats compared to the normal control group. ISO-induced rats treated with isorhamnetin caused significantly ($p<0.05$) lower in HW/BW as compared to ISO-alone treated rats.

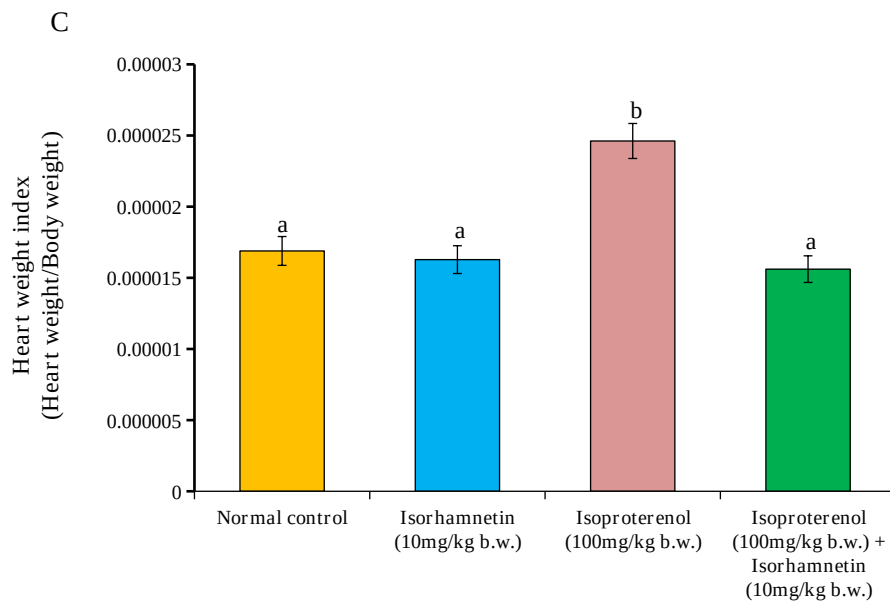


Figure 3C.

Lipid peroxidation products

Figure 4 (A-D) shows the levels of TBARS and LOOH in the plasma and heart of both normal and experimental rats. When compared to normal control rats, ISO-induced rats showed a significant ($p<0.05$) increase in TBARS and LOOH levels in the plasma and heart. Rats treated with isorhamnetin to ISO-induced myocardial infarcted rats significantly ($p<0.05$) reduced when compared to ISO-alone treated rats.

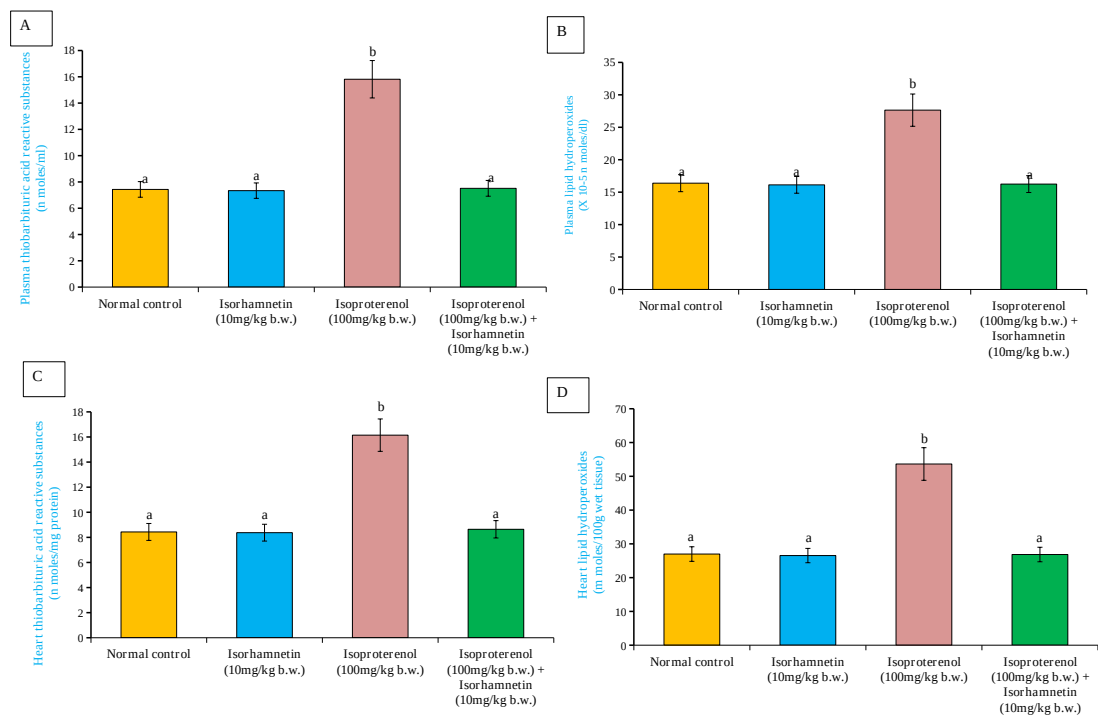


Figure 4.

Enzymatic and non-enzymatic antioxidants

The activities of SOD, catalase, and GPx in the hearts of both normal and experimental rats were shown in Figure 5 (A-C). When compared to normal control rats, the activities of these enzymes in the hearts of ISO-induced rats was significantly reduced ($p<0.05$). Rats treated with isorhamnetin in the ISO-induced group significantly ($p<0.05$) increased compared to the ISO-alone induced group.

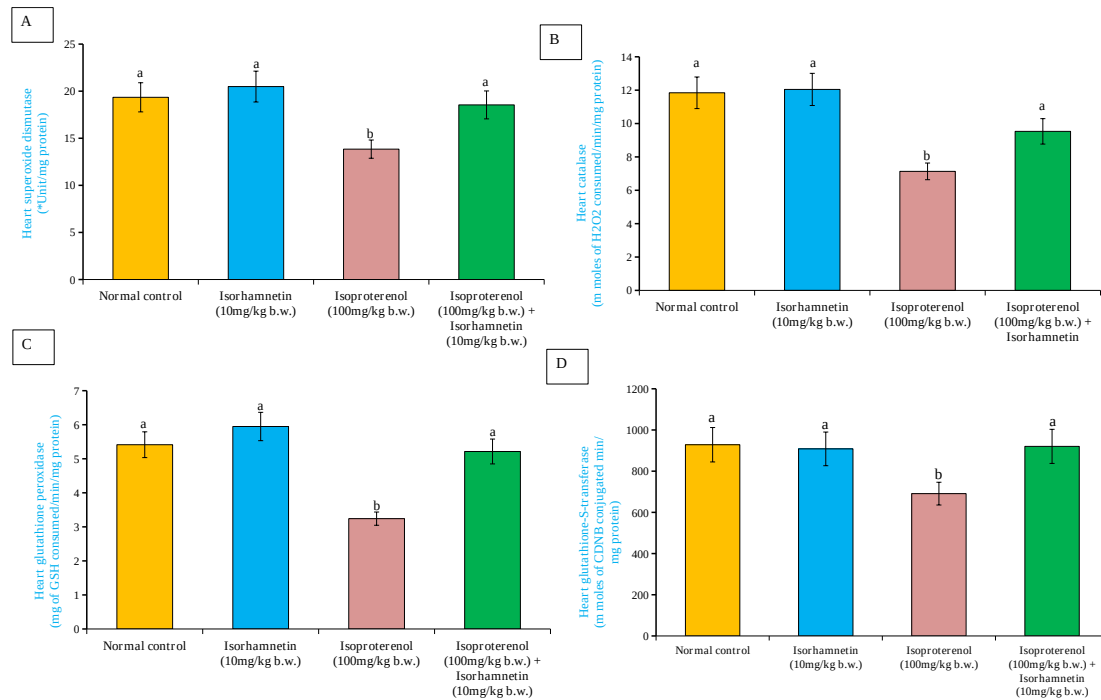


Figure 5 (A-D).

The activity of GST in the hearts of both normal and ISO-induced rats was shown in Figure 5D. Compared to normal control rats, ISO-induced rats had considerably ($p < 0.05$) reduced GST activity. GST activity was significantly ($p < 0.05$) higher in ISO-induced myocardial infarcted rats treated with isorhamnetin than in the ISO-alone treated group.

Figures 6 (A-F) display the amounts of GSH, vitamin C, and vitamin E in the plasma and heart of normal and ISO-induced rats, respectively. GSH, vitamin C, and E levels in the plasma and heart of ISO-induced rats were significantly ($p < 0.05$) lower than the normal control rats. When isorhamnetin was administered to rats with MI caused by ISO, the levels of GSH, vitamin C, and E in the heart and plasma were considerably ($p < 0.05$) higher than in rats with ISO alone induced rats.

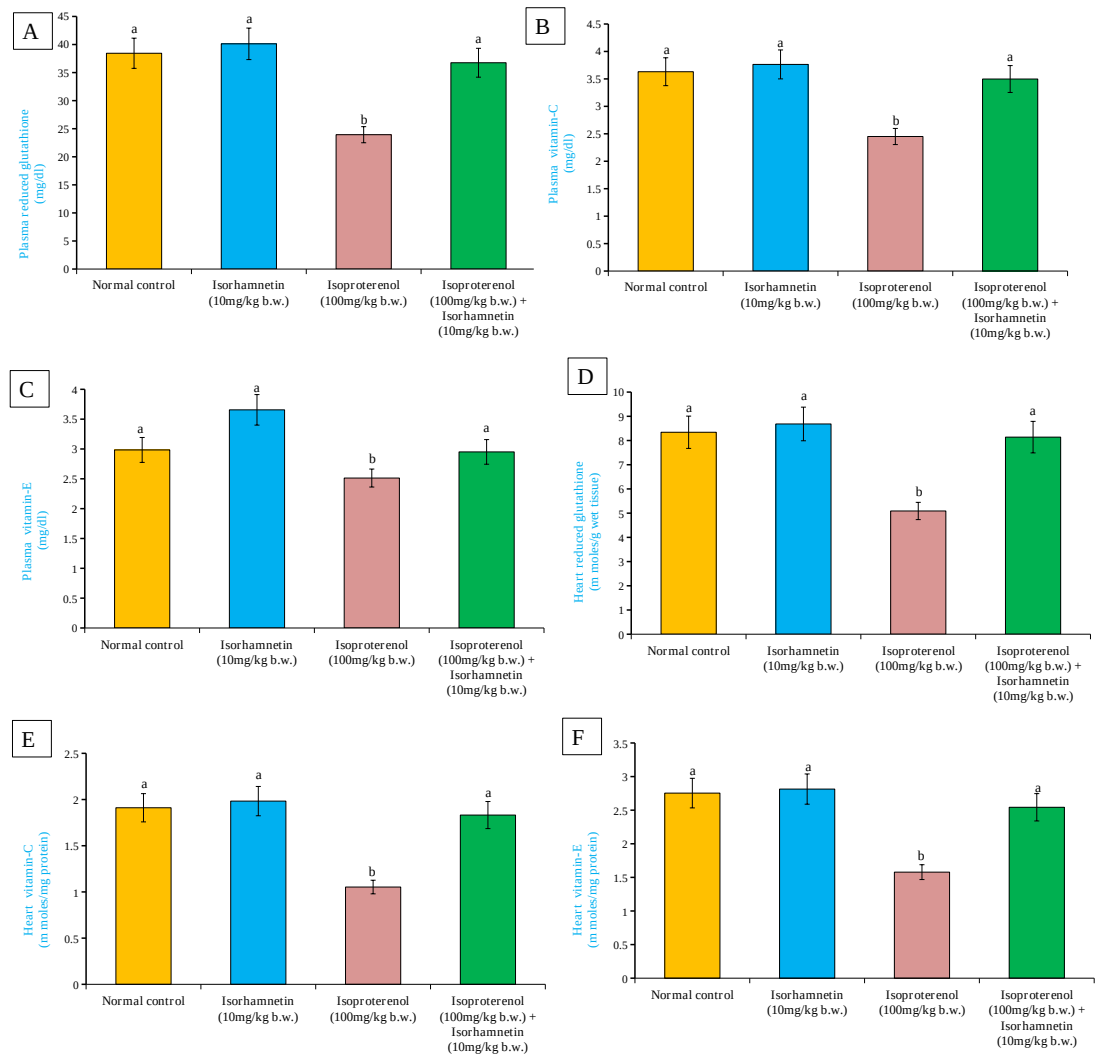


Figure 6 (A-F).

Heart membrane-bound ATPase and electrolyte imbalance

In ISO-induced myocardial infarcted rats, Figures 7A and 7B show a considerable ($p < 0.05$) decrease in Na^+/K^+ ATPase activity and a significant ($p < 0.05$) increase in Ca^{2+} and Mg^{2+} ATPase activity. Additionally, the hearts of ISO-induced rats showed considerably ($p < 0.05$) lower K^+ concentrations and significantly ($p < 0.05$) greater Na^+ and Ca^{2+} concentrations compared to normal control rats. After

isorhamnetin was administered to ISO-induced rats, the concentrations of Na^+/K^+ ATPase and K^+ were considerably ($p < 0.05$) increased, whereas the concentrations of Ca^{2+} and Mg^{2+} ATPases, Na^+ and Ca^{2+} were dramatically ($p < 0.05$) decreased compared to the ISO-alone treated group.

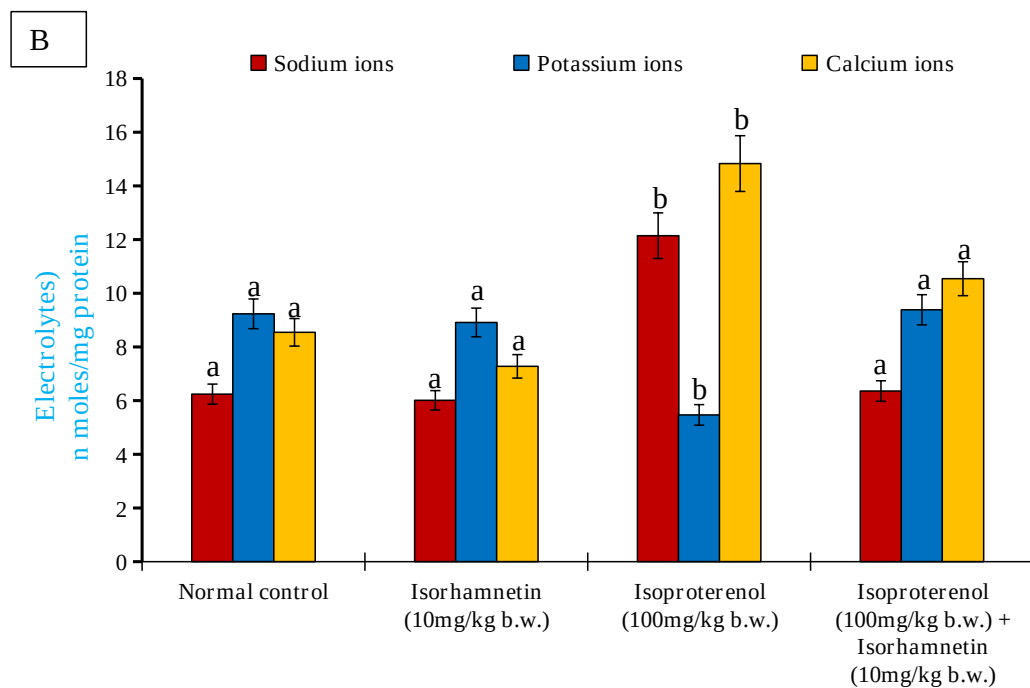
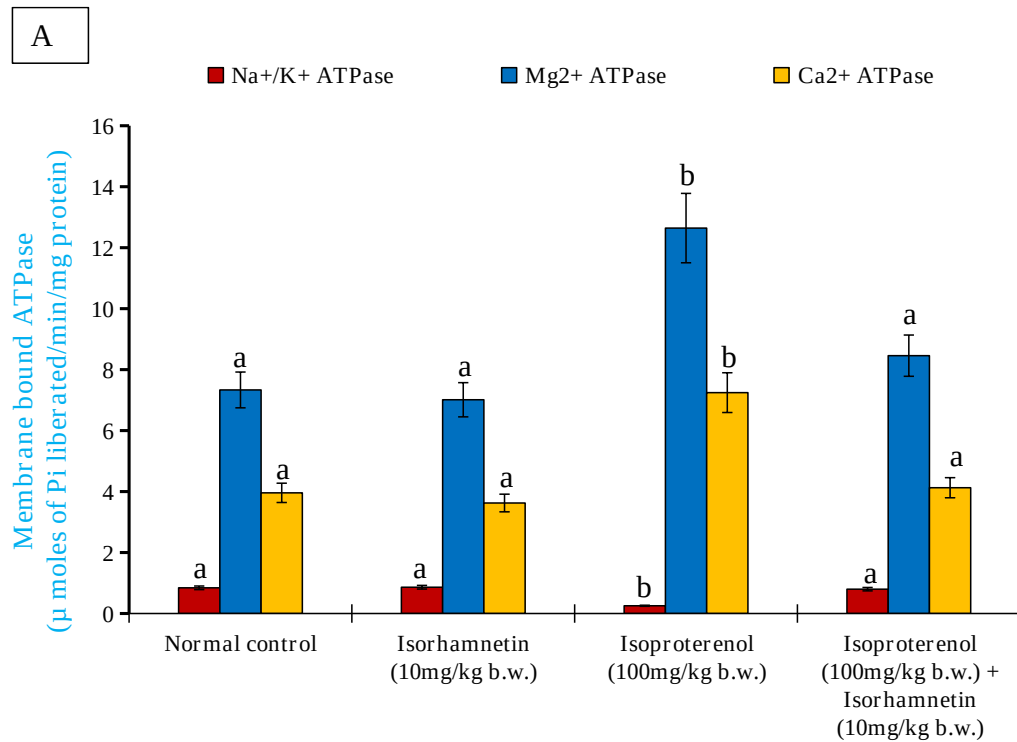
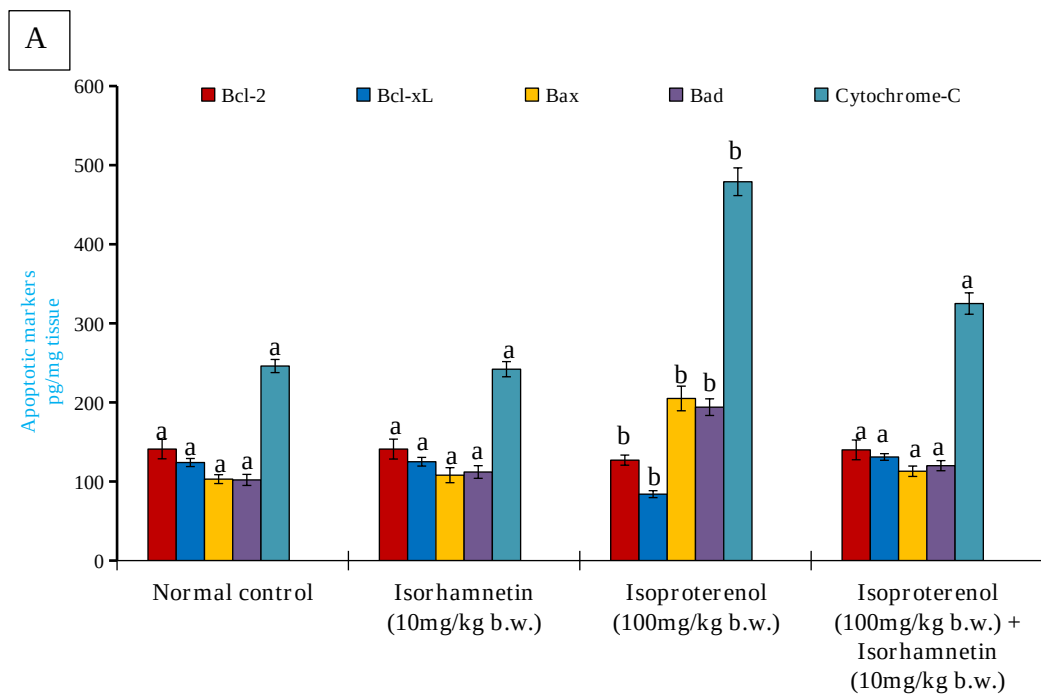


Figure 7A and 7B.

Apoptosis-related biomarkers were quantified using ELISA in the heart

When comparing ISO-induced myocardial infarcted rats to normal control rats, the ELISA study revealed significant ($p<0.05$) downregulation of Bcl-2 and Bcl-XL (Figure 8A) and significant ($p<0.05$) upregulation of Bad, Bax, cytochrome-C, Fas receptor, caspase-8, caspase-9, and caspase-3 compared to normal control rats (Figure 8B). In comparison to ISO-alone induced myocardial rats, rats treated with isorhamnetin after ISO-induction shows significant ($p<0.05$) increase in Bcl-2 and Bcl-XL biomarkers (Figure 8A) and significant ($p<0.05$) decrease in Bad and Bax, cytochrome-C, fas receptor, caspase-8, caspase-9, and caspase-3 biomarkers (Figure 8B).



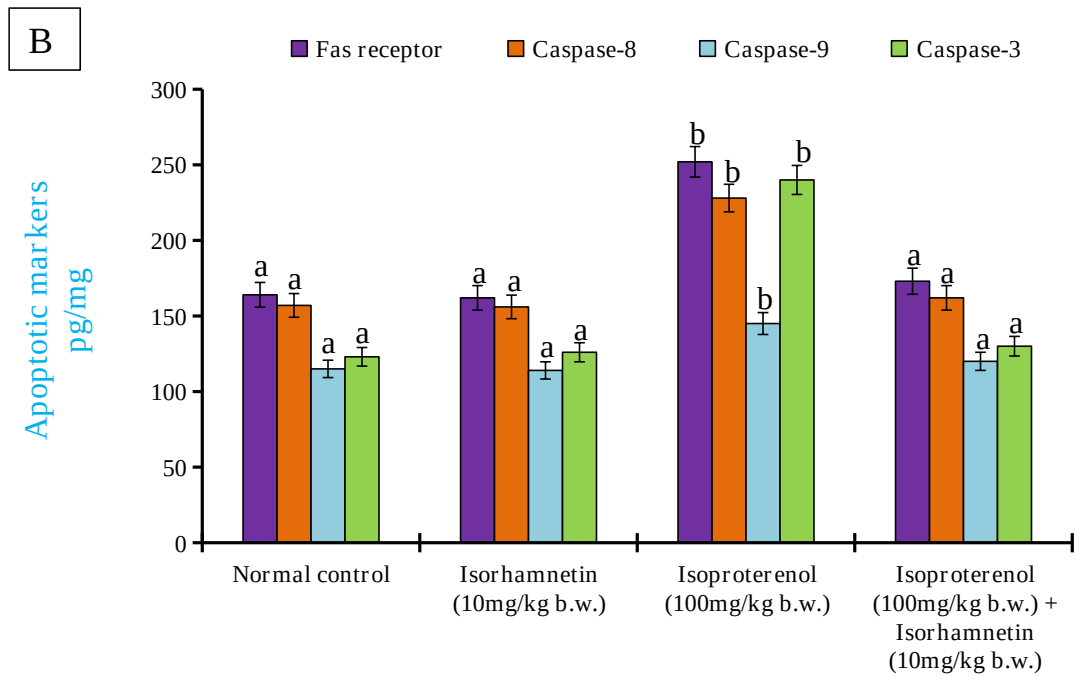


Figure 8A and 8B.

Myocardial expression of Bcl-2 family genes such as Bcl-2, Bcl-xL, Bax, and cytochrome-C by RT-PCR

Figure 9A exhibits RT-PCR analysis of the cardiac expression of Bcl2 family genes, whereas Figure 9B illustrates densitometric analysis of the same genes. When compared to the normal control rats, the ISO-induced myocardial infarcted rats showed considerable ($p < 0.05$) decreased myocardial expression of Bcl-2 and Bcl-xL genes and significant ($p < 0.05$) enhanced myocardial expression of Bax and cytochrome-C genes. Rats treated with isorhamnetin showed a substantial ($p < 0.05$) increase in the expression of Bcl-2 and Bcl-xL genes and a substantial ($p < 0.05$) decrease in the expression of Bax and cytochrome-C genes when compared to rats with ISO-alone-induced cardiomyopathy.

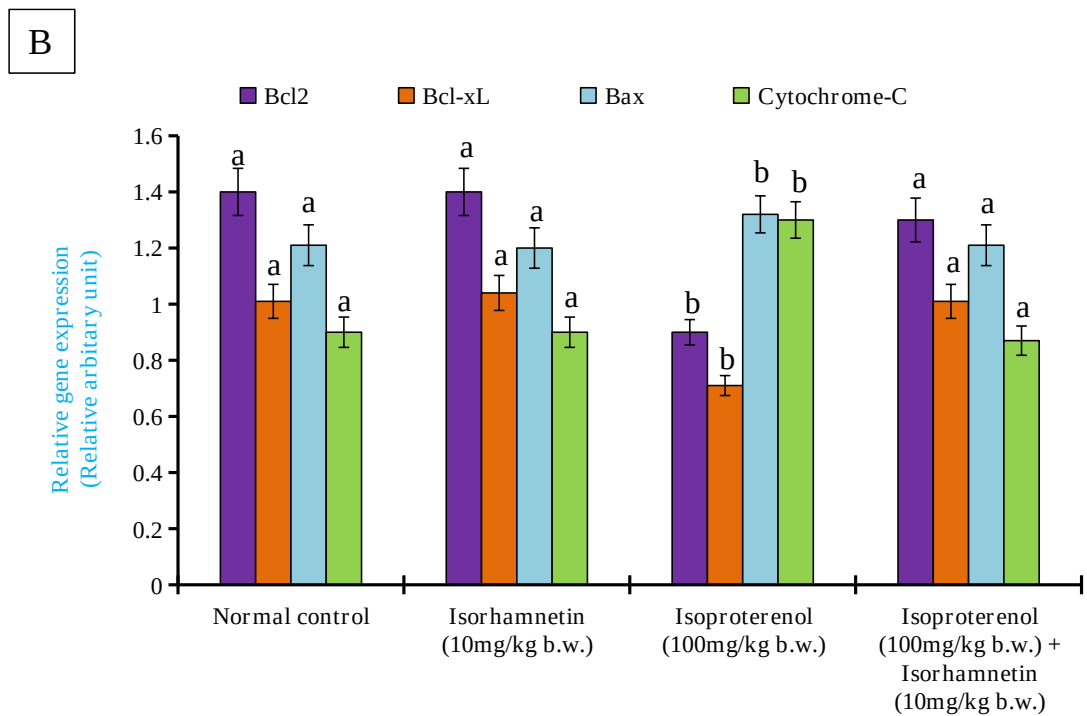
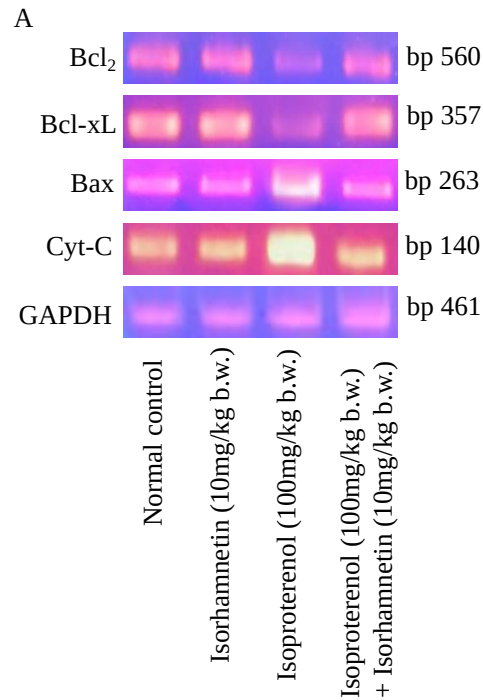
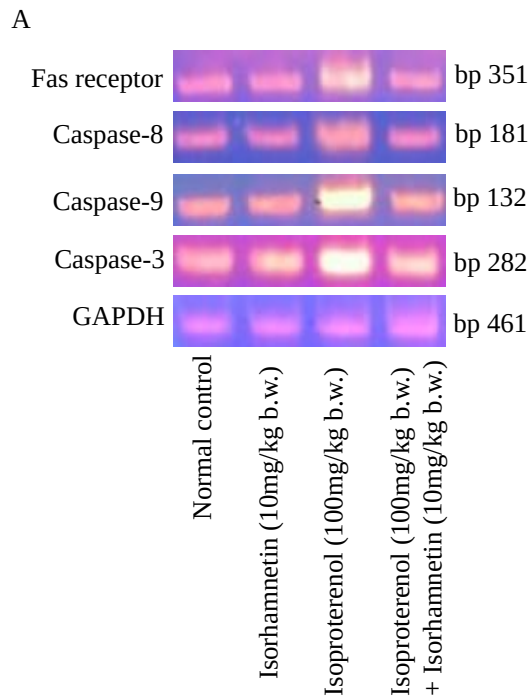


Figure 9A and 9B.

Myocardial expression of fas-receptor, caspase-8, caspase-9 and caspase-3 genes by RT-PCR

An RT-PCR examines the expression of the fas-receptor, caspases-8, -9, and -3 genes in the heart was shown in Figure 10A. The densitometric analysis of these genes was shown in Figure 10B. In comparison to the normal control rats, the ISO-induced myocardial infarcted rats had significant ($p < 0.05$) increase in myocardial expression of the Fas-receptor, caspase-8, -9 and -3 genes. The expression levels of Fas receptor, caspase-8, caspase-9, and caspase-3 genes in ISO-induced rats were significantly ($p < 0.05$) reduced following treatment with isorhamnetin compared with ISO-alone-treated rats.



B

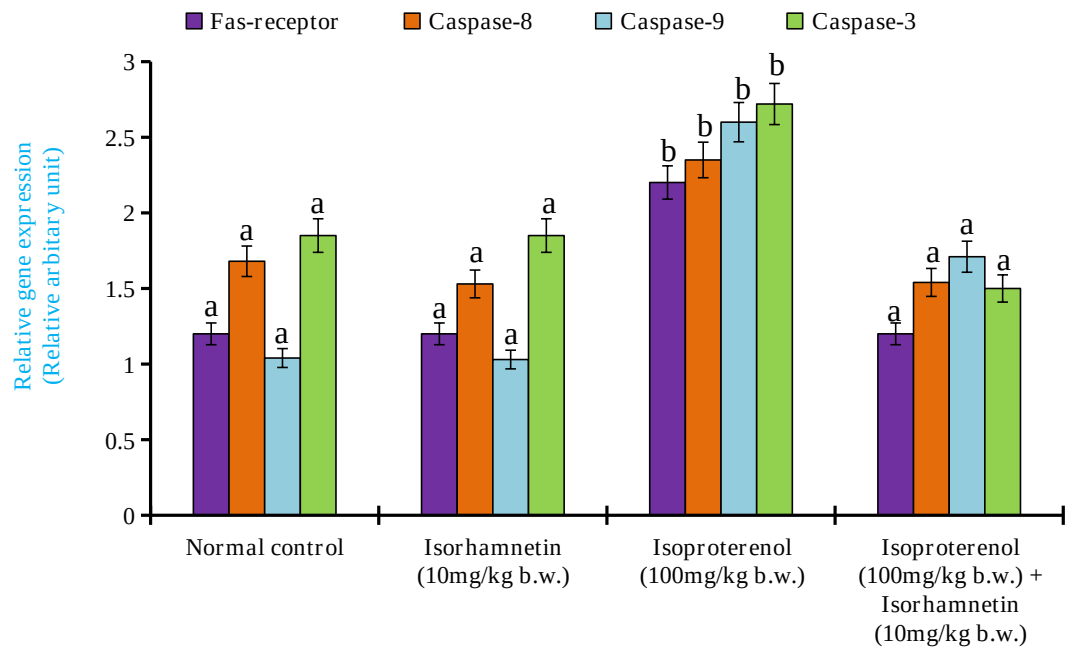


Figure 10A and 10B.

Histopathological changes

H&E staining (Figure 11A) showed that in the heart tissue of the control group, the myocardium, tissue myocytes, and fibroblasts were arranged in order, with clear nuclei, and regular myocardial striations without atrophy or hypertrophy, indicating normal myocardial structure. Myocardial cells in the ISO group showed obvious inflammatory cell infiltration, cell necrosis, and obvious disorder of the myocardial cells. The degree of myocardial tissue injury was improved in the isorhamnetin-treated group.

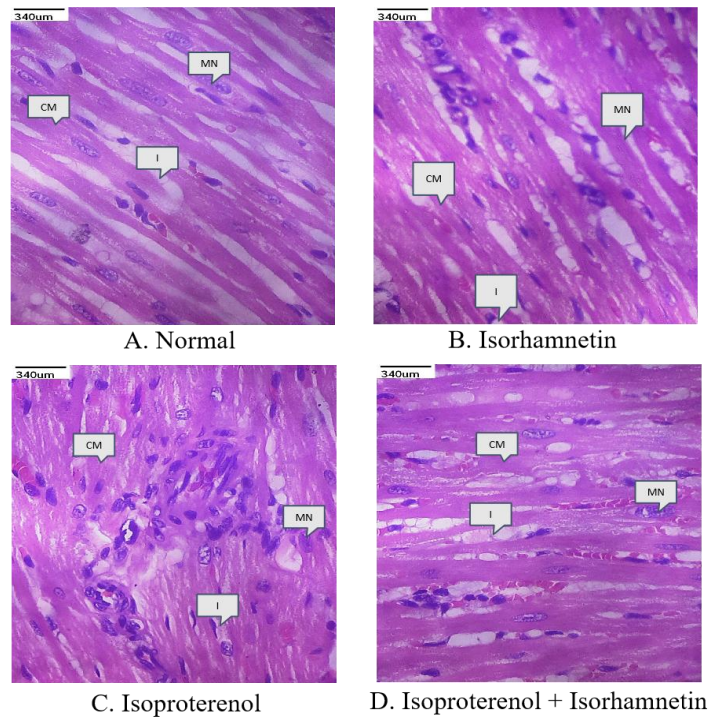


Figure 11A.

Using Masson trichome staining, the severity of myocardial injury was evaluated (Figure 11B). In the rats of the ISO-induced group in which isorhamnetin was administered, the rest part of the myocardial tissue and the thickness of the heart muscle in the ischemic region were high as compared to those of the ISO-induced MI group. Additionally, the fibrotic area was dramatically smaller in the isorhamnetin-treated group than in the ISO-induced MI group.

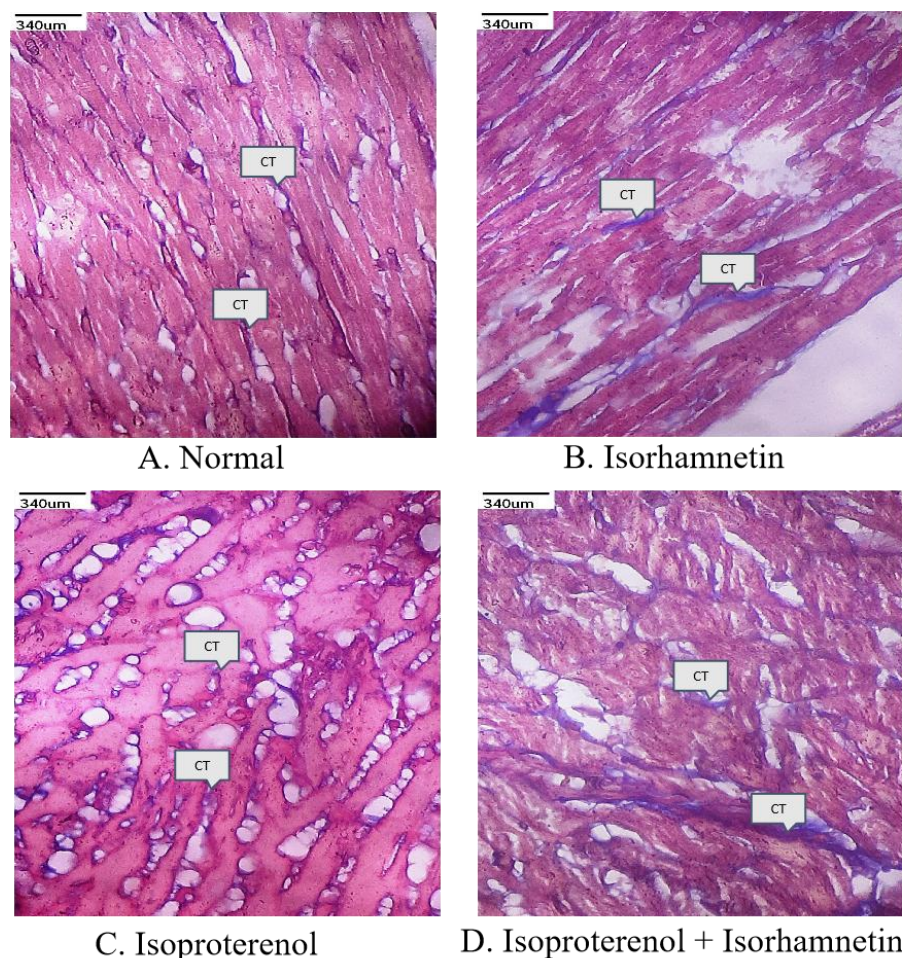


Figure 11B.

The in-vitro DPPH and ABTS⁺ scavenging effects

Figures 12 illustrate the percentage in vitro scavenging activity of isorhamnetin against DPPH and ABTS⁺ radicals, respectively. In vitro, isorhamnetin scavenged these radicals at concentrations of 6, 12, 18, 24, and 30 µM. The percentage scavenging activity of isorhamnetin increased with increasing concentration. The percentage of DPPH radical scavenging at various isorhamnetin concentrations was 13.18, 27.56, 48.86, 58.94, and 73.03%. The percentage of ABTS⁺ scavenging was determined to be 18.84, 36.41, 52.12, 67.55, and 82.83%. In contrast to DPPH radical scavenging activity (73.03%), isorhamnetin exhibited the highest percentage of ABTS⁺ scavenging activity (82.83%) at a concentration of 30 µM.

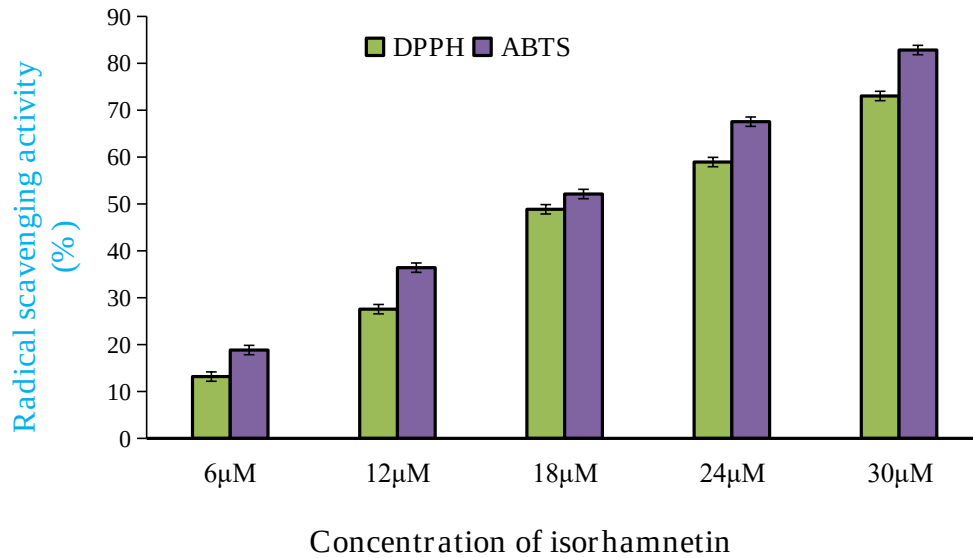


Figure 12.

Discussion

An imbalance between oxygen supply and demand causes MI, which leads to cardiomyocyte necrosis and structural damage. At high dosages, ISO, a synthetic β -adrenergic agonist, causes MI by producing excessive ROS and oxidative stress. Its ease of administration and reproducible pathological changes make it a standard model for MI research.^{10,38}

Due to a rise in heart weight with minimal change in body weight, ISO significantly raised the heart weight to body weight (Hw/Bw) ratio. The Hw/Bw ratio is a reliable indicator of myocardial edema and cardiac enlargement.³⁹ Increased heart weight results from elevated water content, intramuscular edema, protein accumulation, and inflammatory infiltration.¹⁸ Even a 1% rise in myocardial water content can reduce heart function by 10%.⁴⁰ ISO-induced membrane permeability changes promote tissue edema, raising the Hw/Bw ratio. This aligns with histopathology showing edematous muscle tissue and high protein content.⁴¹ Post-treatment with isorhamnetin restored the Hw/Bw ratio toward normal, suggesting

reduced myocardial edema and prevention of cardiac hypertrophy, likely through improved membrane stability and decreased water retention.

Serum CK-MB is present in high levels in cardiac tissue, which is a very specific and sensitive marker for MI.⁴² Although CK-MB levels are known to rise rapidly during the acute phase of MI, the measurements obtained in the present study were performed at the terminal experimental endpoint (day 18) and therefore reflect the persistence of myocardial injury following ISO administration.⁴³ CK-MB makes up for 20% of myocardial tissue and, upon ischemic or oxidative damage, leaks into the bloodstream.^{18,44} Cardiac damage, which is induced by ISO, greatly increases CK-MB due to membrane breakdown and necrosis. Isorhamnetin treatment reduced CK-MB leakage by stabilising cell membranes and lessening oxidative stress.⁴⁵

Cardiac troponins (cTnT, cTnI) are markers that have been identified for myocardial necrosis. They are undetectable in healthy individuals but rise greatly at the site of cardiac injury because of membrane rupture and insufficient oxygen delivery. ISO raised cTnT and cTnI, thus we can report that the heart damage is of a serious nature.⁴⁶ Persistent increases in these markers report the extent of the infarct and also the associated risk.^{40,47} In our study, rats treated with isorhamnetin had lower troponin levels, which in turn proves its role in preserving contractile and structural heart health.⁴⁵

ISO raised levels of lipid peroxidation (TBARS, LOOH), which in turn means the heart tissue is more prone to oxidative injury. ISO causes tissue damage and myocyte death by increasing lipid hydroperoxide levels, which in turn also causes more free radical production.⁴⁷ Isorhamnetin reduced levels of TBARS and LOOH and thus proved its ability to scavenge free radicals and reduce lipid peroxidation.

It was also noted that SOD and catalase activities of the rats induced with ISO would be reduced, which could be because of the excessive production of hydrogen peroxide and superoxide anions. Nonetheless, our results distinctly indicated that isorhamnetin post-treatment augmented the activities of these enzymes, indicating its cardioprotective action. The ISO reduced the activity of GSH-dependent antioxidant enzymes (GST and GPx), stalling the process of lipid peroxide detoxification.⁴⁸ Isorhamnetin minimized the oxidative injury to the cells by recuperating GPx and GST functions.^{2,11} The rats treated with ISO exhibited reduced vitamin E and vitamin C levels, as these antioxidants were utilized to counteract the excessive free radicals generated by ISO. Isorhamnetin treatment significantly restored the levels of these vitamins, possibly through its antioxidant activity, which reduced oxidative stress and helped preserve endogenous antioxidant defenses.

ISO decreased Na^+/K^+ -ATPase and increased Ca^{2+} - and Mg^{2+} -ATPase activities, leading to elevated Na^+ and Ca^{2+} and reduced K^+ in cardiac tissue. This results from oxidative damage affecting membrane lipid-dependent ATPases.^{12,40} Isorhamnetin significantly improved the altered electrolyte levels and restored ATPase activities toward values comparable to those observed in the normal control group, possibly through its antioxidant and membrane-stabilizing properties, thereby helping to maintain ionic homeostasis in myocardial tissue.

ISO-induced myocardial infarcted rats showed reduced expression of the anti-apoptotic genes Bcl-2 and Bcl-xL and increased expression of the pro-apoptotic genes Bax and cytochrome-C, indicating diminished cardiomyocyte survival and enhanced apoptosis linked to ISO-induced oxidative stress.^{41,45} Isorhamnetin reduced oxidative stress, increased Bcl-2 and Bcl-xL expression, and decreased Bax and cytochrome-C expression, thereby protecting cardiomyocytes from apoptosis in ISO-induced rats.

ISO also elevated Fas receptor and caspase-8, -9, and -3 expression, demonstrating activation of Fas-receptor and caspase-dependent apoptotic pathways.⁴⁹ Oxidative stress is known to trigger caspase activation and accelerate cardiomyocyte death in ISO-induced MI, as reported in previous studies.⁴⁵ Post-treatment with isorhamnetin downregulated Fas receptor and caspases-8, -9, and -3, indicating inhibition of the Fas/caspase-mediated apoptotic pathway. In line with earlier research, the present study confirms that isorhamnetin reduces oxidative stress and myocardial apoptosis in MI-induced male Wistar rats.⁴⁵ These findings suggest that isorhamnetin protects against ISO-induced myocardial damage and support further experimental investigation.

Normal control rats' hearts stained with H&E in histopathology demonstrated an apparent homogeneity and membrane integrity of the heart with intact myocardial architecture devoid of edema, inflammation, and necrosis. In contrast, the rats subjected to ISO exhibited dramatic histological alterations, which were similar to acute MI, such as coagulative necrosis, inflammatory cell invasion (lymphocytes, macrophages), interstitial edema, myofibril dissociation, and vascular congestion. Also, there was vacuolization, nuclear shrinkage (pyknosis), and muscle striations loss as a result of ISO injection, which are signs of damage caused by oxidative stress.^{1,50} The raised HWI in the ISO group might be due to interstitial edema observed in the present study that manifested as a broader spacing among myocardial fibers. Due to the potent antioxidant properties of isorhamnetin, post-treatment significantly attenuated these histopathological alterations and almost normal cardiac morphology was noticed with reduced necrosis, edema as well and inflammation.

Masson's trichrome staining reported that in ISO-treated rats, there was marked interstitial fibrosis, which is indicated by the blue stained collagen fibers. This

results in heart stiffness and poor function. Fibrosis at the same time brought about myocyte growth as well as extracellular matrix disruption, which is a sign of poor cardiac remodeling.^{42,50} In the current study, isorhamnetin treatment lessened fibrosis, reduced collagen accumulation, and improved tissue architecture, suggesting inhibition of the fibrogenic pathway. Also in agreement with past reports, isorhamnetin preserved myofibril structure, reduced white blood cell invasion, and prevented overproduction of collagen, which supports its heart-protective role.⁴⁵

The DPPH• method was used in this investigation to measure isorhamnetin's in vitro free radical scavenging activity. The capacity of antioxidant substances to scavenge free radicals is often evaluated using DPPH•.¹⁰ At a concentration of 30 µM, isorhamnetin scavenged 73.03% of DPPH• in a dose-dependent manner. Increased free radical generation is a key mechanism behind ISO-induced MI since ISO metabolism produces an excess of ROS, such as superoxide anion and hydroxyl radicals.⁴⁸ These radicals are neutralized by isorhamnetin's strong free radical scavenging action, preserving the myocardium in ISO-induced rats. This implies that isorhamnetin is an effective scavenger of free radicals. ABTS⁺ radical cation scavenging was assessed in order to better understand isorhamnetin's antioxidant mechanism. A popular spectrophotometric technique for assessing total antioxidant activity in solutions of pure substances is based on the production of ABTS⁺.¹⁰ Additionally, isorhamnetin scavenged ABTS⁺ in a dose-dependent manner, reaching 82.83% at 30 µM. All of these results show that isorhamnetin has strong antioxidant activity, which helps explain why it protects the cardiac myocardium against ISO-induced myocardial infarction.

Conclusion

To the best of our knowledge, this study is the first to show that in isoproterenol-induced myocardial infarction (100 mg/kg body weight), isorhamnetin (10 mg/kg body weight) exhibits potent antioxidant and anti-apoptotic effects. By altering Bcl-2 family genes and blocking Fas-receptor/caspase-dependent signaling, isorhamnetin decreased oxidative stress, maintained antioxidant defenses, and prevented cardiomyocyte apoptosis. It improved ionic homeostasis, regulated lipid peroxidation and ATPase activity, decreased CK-MB and cardiac troponin leakage, and restored key myocardial biochemical markers, indicating membrane stabilization and improved cardiac function. H&E and Masson's trichrome staining analyses confirmed reductions in necrosis, inflammation, edema, and fibrosis, with preservation of tissue structure. In vitro studies demonstrated high free radical scavenging activity, proving the antioxidant basis of its cardioprotection. Importantly, isorhamnetin alone produced no significant biochemical, molecular, or histological alterations compared with controls, indicating its non-toxic nature at 10 mg/kg body weight. Overall, these findings show that isorhamnetin has potent antioxidant and anti-apoptotic properties in MI, supports its potential as a therapeutic candidate, and suggests it as an alternative agent for healing human MI.

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TABLE

Table-1: Primer sequences used for the amplification of Bcl-2, Bcl-XL, Bax, ytc-C, Fas receptor, Caspase-8, Caspase-9 and Caspase-3 genes and internal control GAPDH gene and the base pairs of PCR amplified products.

Gene	Primers	Base Pair
Bcl-2	Forward primer: CTG GTG GAC AAC ATC GCT CTG Reverse primer: GGT CTG CTG ACC TCA CTT GTG	bp 560
Bcl-XL	Forward primer: AGG CTG GCG ATG AGT TTG AA Reverse primer: TGA AAC GCT CCT GGC CTT TC	bp 357
Bax	Forward primer: TTC ATC CAG GAT CGA GCA GA Reverse primer: GCA AAG TAG AAG GCA ACG	bp 263
Cytc-C	Forward primer: CCT TTG TGG TGT TGA CCA GC Reverse primer: CCA TGG AGG TTT GGT CCA GT	bp 140
Fas - receptor	Forward primer: GCA ATG CTT CTC TCT GTG ACC ACT Reverse primer: GCT GTT GTG CTC GAT CTC ATC G	bp 351
Caspase-8	Forward primer: CTG GGA AGG ATC GAC GAT TA Reverse primer: CAT GTC CTG CAT TTT GAT GG	bp 181
Caspase-9	Forward primer: AGC CAG ATG CTG TCC CAT AC Reverse primer: CAG GAG ACA AAA CCT GGG AA	bp 132
Caspase-3	Forward primer: ACG GTA CGC GAA GAA A-A GTG AC Reverse primer: TCC TGA CTT CGT ATT TCA GGG C	bp 282
GAPDH	Forward primer: TTC TTG TGC AGT GCC AGC CTC GTC Reverse primer: TAG GAA CAC GGA AGG CCA TGC CAG	bp 461