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Betulin protects against isoproterenol-induced myocardial injury by inhibiting NF- κ B signaling and attenuating cardiac inflammation and oxidative stress in ratsHital Shah¹, Tejal Gandhi²✉¹Gujarat Technological University, Ahmedabad, Gujarat 382424, India²Department of Pharmacology, Anand Pharmacy College, Anand, Gujarat 388001, India

ABSTRACT

Objective: To investigate the cardioprotective potential of betulin in isoproterenol (ISO)-induced myocardial injury in rats.

Methods: Wistar rats were divided into five groups ($n=10$): normal, ISO, nebivolol 5 mg/kg, and betulin (20 & 40 mg/kg). Nebivolol and betulin were administered orally for 29 days. ISO (85 mg/kg) was administered subcutaneously on day 27 and day 28 to induce myocardial injury. On day 29, blood was collected for determination of cardiac markers, and hemodynamic parameters were investigated. The levels of oxidative stress markers and the gene expressions of apoptotic markers and inflammatory mediators were evaluated. Moreover, 2,3,5-triphenyltetrazolium chloride staining and histopathological analysis were also performed.

Results: Betulin reduced the size of myocardial infarction, decreased elevated levels of cardiac enzymes, and maintained hemodynamic functions. It also inhibited ISO-induced upregulation of *Bax*, *caspase-3*, *NF- κ B*, and *IL-6*, enhanced endogenous antioxidant enzymes, and reduced lipid peroxidation. Additionally, pretreatment with betulin alleviated myocardial ischemic damage, as reflected by reduced myonecrosis, edema, and inflammatory changes.

Conclusions: Betulin exhibits strong cardioprotective activity against ISO-induced myocardial injury by anti-inflammatory, anti-apoptotic, and antioxidant activities.

KEYWORDS: NF- κ B; Isoproterenol; Betulin; Myocardial injury; Oxidative stress; Inflammation; Apoptosis

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death worldwide, accounting for 17.9 million deaths in 2019 (which is 32% of all deaths worldwide). Of these deaths, 85% were caused

by heart attacks and strokes. Among the 17 million premature deaths (those under 70) from noncommunicable diseases in 2019, 38% were related to CVDs[1]. The number of cases of CVD nearly doubled from 271 million in 1990 to 523 million in 2019, and the number of CVD deaths increased steadily from 12.1 million in 1990 to 18.6 million in 2019. In India, CVD is the leading cause of death[2]. Coronary artery narrowing and plaque rupture cause myocardial ischemia, which is a common cause of death. This condition results in inadequate oxygen transport to the myocardium, which causes myocardial ischemia damage and infarction[3]. The implementation of several cardiac repair techniques in clinical settings is hampered by difficulties with cell transplantation and scaffolds made of biomaterials[4]. The criteria for immune compatibility and bio-functionality of advanced biomaterial-based

Significance

The widely distributed *Betula* species contain betulin, a pentacyclic triterpene that is beneficial for the treatment of cancer and diabetes. However, its cardioprotective properties remain unexplored. The current study demonstrated that betulin has cardioprotective effects against isoproterenol-induced myocardial damage by reducing cardiac oxidative stress, necrosis, and inflammation via the NF- κ B/IL-6 signaling pathway.

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scaffolds used in tissue engineering are limiting[3]. Treatment for myocardial ischemia followed by infarction is still a complex challenge despite significant advancements in medical knowledge and technology. This is because the condition is multifactorial, involving genetic predisposition, lifestyle factors, cardio-metabolic syndrome, and the intricate interplay of molecular pathways[5]. Numerous studies show that myocardial ischemia injury causes oxidative stress and cellular inflammation[6], which in turn causes tissue damage[7]. New therapeutic strategies that focus on the different molecular pathways linked to oxidative stress, apoptosis, and inflammation are therefore imperative. Numerous cardiovascular disorders, such as atherosclerosis, cardiac hypertrophy, and heart failure, have been linked to the NF- κ B. Consequently, inhibiting NF- κ B may be a useful tactic to prevent heart diseases, including ischemia, from worsening. Natural compounds are currently receiving attention because of their low side effects, affordability, and ability to target multiple components at once. Including naturally occurring biologically active substances in diets could be a successful treatment strategy[8]. Natural triterpene betulin is present in birch trees and is thought to play a part in a number of diseases, including inflammation, cancer, oxidative stress, diabetes, and liver disorders[9]. The molecular mechanisms of betulin, as a multi-targeted anti-inflammatory, antioxidative, and anti-apoptotic compound in myocardial ischemia, remain unclear despite its diverse biological and therapeutic effects. Therefore, in this study, we aimed to explore the potential beneficial efficacy of betulin in mitigating myocardial injury, particularly focusing on its modulation of the NF- κ B signaling pathway.

2. Materials and methods

2.1. Chemicals

The analytical grade chemicals utilized in this study were sourced from SD Fine Chemicals, Mumbai, India, and Astron, Ahmedabad, India. Betulin (B9757-5G, $\geq 98\%$, Lot # MKCG3208) and isoproterenol hydrochloride (ISO) were procured from Sigma-Aldrich (St. Louis, MO, USA). The standard reagent kits were obtained from CPC Diagnostics (Chennai, India) and used for biochemical investigations. Troponin- I levels were assessed with a Cal-Bio ELISA kit. Blood pressure, electrocardiogram (ECG), and left ventricular function were measured using the NIBP 200A small animal tail noninvasive blood pressure system, which was outfitted with a computerized MP35 data acquisition system (BIOPAC, Santa Barbara, California, USA). Thermo Scientific's first-strand cDNA synthesis, Qiagen's DNase I, Sigma's TRI, Qiagen's RNeasy, and KAPA's SYBR Green PCR kit were all utilized for the gene expression analysis. The NCBI BLAST primer tool was used

to develop the primers, which were subsequently commercially synthesized by Eurofins Genomics.

2.2. Animals

In this study, male Wistar rats weighing (250 ± 20) g were procured from Zydus Research Center, Ahmedabad. The rats were kept in an environment with carefully regulated humidity ($55 \pm 5\%$), temperature (22 ± 2) °C, and light/dark cycles (12 h/12 h). Rats had free access to water, along with standard laboratory diets provided by Pranav Argo Pvt. Ltd.

2.3. Experimental protocol

Animals were randomly allocated into five groups ($n=10$) based on their creatine kinase-MB (CK-MB) levels as follows: Group I (normal control), Group II model control [ISO 85 mg/kg, *s.c.*, (2 injections at the interval of 24 h on days 27 and 28)], Group III standard control [nebivolol 5 mg/kg, *p.o.*/day for 29 d, along with ISO 85 mg/kg, *s.c.*, (2 injections at the interval of 24 h on days 27 and 28)], Group IV–V treatment groups [betulin 20 mg/kg and 40 mg/kg, *p.o.*/day for 29 d, along with ISO 85 mg/kg, *s.c.*, (2 injections at the interval of 24 h on days 27 and 28)]. An ISO 85 mg/kg subcutaneous (*s.c.*) dose was selected based on the pilot study. Nebivolol and test drugs were administered orally using an 18G oral feeding needle. The doses of betulin were selected based on the previous study on diabetic rats[10]. Twenty-four hours following the final ISO injection, mean arterial blood pressure (MAP) and ECG were recorded. Under anesthesia, blood samples were obtained retro-orbitally using ketamine (50 mg/kg) & xylazine (10 mg/kg), and were kept for 30 min. The serum was extracted using a high-speed, high G-force refrigerated centrifuge (Plasto Crafts, Rota 4RV/FA) for 15 min at $1000 \times g$. The extracted serum was then used to estimate cardiac-specific parameters: CK-MB, lactate dehydrogenase (LDH), calcium, and troponin- I. The fully automated biochemistry analyzer (Turbochem 100) was utilized for investigation of biochemical parameters. The heart was isolated, used for Langendorff's study, rinsed with ice-cold physiological saline, and weighed. They were then kept for additional analysis at -80°C (Cryo Scientific, India). RNeasy was used to store the heart samples for quantitative polymerase chain reaction (RT-qPCR). Antioxidants [catalase, glutathione (GSH) and superoxide dismutase (SOD)], as well as malondialdehyde (MDA) levels were measured in the heart homogenate (Figure 1).

2.4. MAP, ECG, and heart rate (HR)

The MP35 data acquisition system (BIOPAC, Santa Barbara, California) was utilized to record mean blood pressure (using tail-

cuff sensor, NIBP200A), ECG, and HR. QT prolongation, ST-segment elevation or depression, and T-wave inversion or elevation were analyzed by using Acknowledge software.

2.5. Langendorff isolated perfused heart preparation

An intraperitoneal injection of 50–80 mg/kg pentobarbital was used to anesthetize the animals. In order to avoid thrombus formation in the removed heart, the rats received an intraperitoneal injection of 500 IU heparin prior to excision. The animals were rendered unconscious and placed in the supine position so that a midsternal thoracotomy could be performed to open the chest cavity. The heart was then excised and placed into an oxygenated, ice-cold, modified Krebs-Henseleit buffer [KHB; comprising of the following components: glucose (5.55 mM), Na-pyruvate (2 mM), KH_2PO_4 (1.18 mM), MgSO_4 (1.66 mM), NaCl (118 mM), NaHCO_3 (24.88 mM), and CaCl_2 (1.5 mM)]. Before use, the buffer was passed through a 0.45 μm membrane filter. The aorta was connected to the oxygenated KHB-buffered cannula. Subsequently, the heart-attached cannula was connected to the Langendorff perfusion apparatus [buffer flow rate: (9.7 ± 0.5) mL/min; carbogen: 95% O_2 and 5% CO_2 ; temperature: 37°C]. A latex balloon attached to the end of a polyethylene tube that was connected to a pressure transducer was placed into the isolated heart's left ventricle in order to measure the pharmacodynamic response. Then 50% of methanol was used to inflate the balloon to a diastolic pressure of 4–6 mmHg. The Langendorff assembly was allowed to stabilize for 5 min before measuring pharmacodynamics including left ventricular end-diastolic pressure (LVEDP), $+\text{dp}/\text{dt}$ max (rate of maximum LV pressure rise), and $-\text{dp}/\text{dt}$ max (rate of maximum LV pressure fall).

2.6. Oxidative stress parameters

The isolated heart tissues were homogenized in chilled Tris-HCL buffer (0.1 M, pH 7.4). The homogenate was placed in a refrigerated centrifuge (4°C) and centrifuged for 10 min at $1000 \times g$. The amount of MDA in the supernatant was determined using Slater and Sawyer's method[11] as a lipid peroxidation indicator. The following antioxidant enzyme levels were determined using the methods of Moron *et al.*[12], Misra and Fridovich[13], and Aebi *et al.*[14] respectively for GSH, SOD, and catalase. Protein concentrations were measured using Lowry's method[15].

2.7. RT-qPCR

The total RNA from heart tissues was extracted using the TRIzol reagent. Simultaneously, PCR and cDNA synthesis were performed using the F416 L SYBR Green qPCR Kit. qPCR was carried out using the QuantStudioTM 5 RT-qPCR system (Applied Biosystems, USA). The housekeeping gene β -actin was used as an internal control. The relative expression was calculated using the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta\text{Ct}}$). Table 1 lists the sequences of forward and reverse primers.

2.8. Determination of the size of myocardial infarction by 2,3,5-triphenyltetrazolium chloride (TTC) staining

The heart tissues were cleaned with saline and wiped dry three times before being frozen in a refrigerator at -20°C for 25 min. The tissues were then sliced into 3–6 mm pieces. These slices were then kept in a 2% TTC (Solarbio, China) solution dissolved in phosphate-

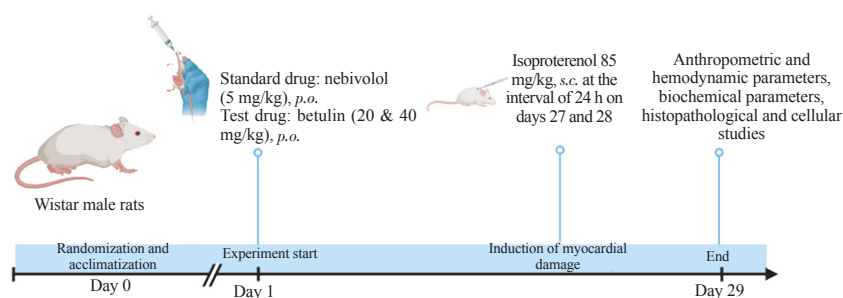


Figure 1. Experimental design (created in BioRender).

Table 1. Primer sequences used for RTq-PCR analysis.

Genes	Forward 5'-3'	Reverse 5'-3'
β -actin	CCCGCGAGTACAACCTTCTTG	GTCATCCATGGCGAACTGGTG
IL-6	GCTCTGGTCTTCTGGAGTTCC	GGAGAGCATTGGAAGTTGGG
Caspase-3	GAGCTTGAACGCGAAGAAA	TAACCGGGTGCGGTAGAGTA
NF- κ B	GAGGCCATTGAAGTGATCCAG	TGAGTTTGCGGAAGGATGTCT
Bax	AGGACGCATCCACCAAGAAG	CAGTTGAAGTTGCCGTCTGC

buffer saline (pH 7.4) for 30 min at 37 °C. The size and extent of the infarction in the heart tissue were examined using ImageJ software.

2.9. Histopathological evaluation by hematoxylin and eosin (H & E) staining and Masson's trichrome staining

A formalin-fixed paraffin-embedded tissue embedding system was used to fix the heart tissue in 4% paraformaldehyde and embed it in paraffin wax. After cutting the myocardium into 3–5 μ m thin slices using a microtome (ERMA type manual rotary-ASD 202A) and mounting it on histology slides, the tissue was deparaffinized by heating it to 60 °C. H & E and Masson's trichrome were used to stain the slides. The extent of myocardial infarction was evaluated by Masson's trichrome staining. An attached digital camera (Nikon, Japan) connected to a microscope (Nikon, Japan) was used to acquire and analyze the histopathological images.

At the end of the study protocol, there were four, two, three, and two cases of mortality found in ISO 85 mg/kg, nebivolol 5 mg/kg, betulin 20 mg/kg, and betulin 40 mg/kg respectively.

2.10. Statistical analysis

All the data are expressed as mean $\pm$ SEM. A computer program (Prism, GraphPad version 8.01, GraphPad Software, Inc.) was used to compare the results. One-way ANOVA was used to analyze the data followed by Dunnett's test. *P* values less than 0.05 indicate a

statistically significant difference.

2.11. Ethical statement

The experimental protocol (APC/2021-IAEC/2103) was approved by the Institutional Animal Ethical Committee. All the experiments were performed as per the guidelines of the Committee for Control and Supervision of Experiments on Animals.

3. Results

3.1. Effect of betulin on biochemical parameters in rats with ISO-induced myocardial injury

The ISO group had significantly ($P < 0.01$) higher levels of CK-MB, LDH, calcium, and troponin- I as compared to the normal control group. Figure 2 illustrates that betulin (20 mg/kg and 40 mg/kg) significantly inhibited ISO-mediated elevation of CK-MB, LDH, calcium, and troponin- I levels in the serum.

3.2. Effect of betulin on anthropometric and hemodynamic parameters in rats with ISO-induced myocardial injury

Table 2 displays the effects of ISO (85 mg/kg *s.c.*) and betulin (20 and 40 mg/kg *p.o.*, respectively) on heart weight, HR, and

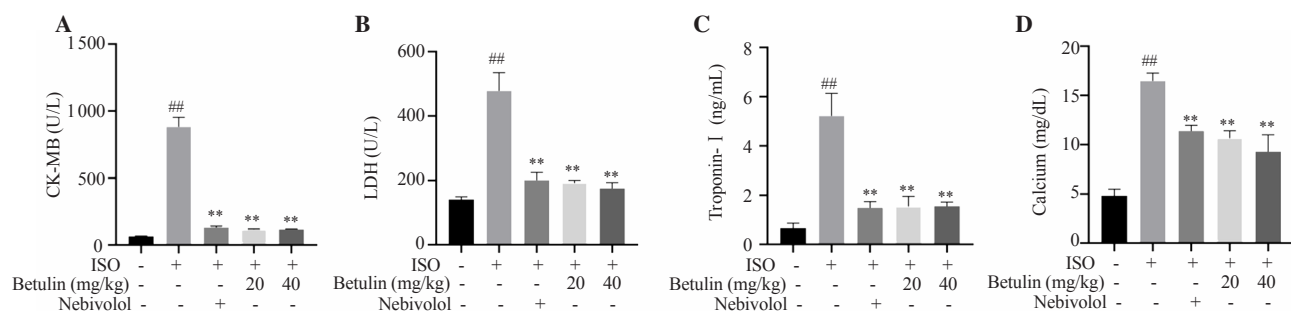


Figure 2. Effect of betulin on cardiac markers in rats with isoproterenol (ISO)-induced myocardial injury. Values are presented as mean $\pm$ SEM. Statistical analysis is carried out using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. ^{##} $P < 0.01$ compared to the normal control group. ^{**} $P < 0.01$ compared to the ISO control group. CK-MB: creatine kinase MB; LDH: lactate dehydrogenase.

Table 2. Effect of betulin on anthropometric and hemodynamic parameters in rats with isoproterenol-induced myocardial injury.

Parameters	Normal control	ISO (85 mg/kg)	Nebivolol (5 mg/kg)	Betulin (20 mg/kg)	Betulin (40 mg/kg)
HW (g)	1.01 $\pm$ 0.02	1.49 $\pm$ 0.03 ^{##}	1.12 $\pm$ 0.05 ^{**}	1.19 $\pm$ 0.02 ^{**}	1.15 $\pm$ 0.04 ^{**}
% Infarct area	0.00 $\pm$ 0.00	57.98 $\pm$ 2.02 ^{##}	44.50 $\pm$ 1.74 ^{**}	45.67 $\pm$ 1.97 ^{**}	40.50 $\pm$ 1.87 ^{**}
HR	361.00 $\pm$ 13.52	453.20 $\pm$ 14.22 ^{##}	379.30 $\pm$ 21.17 [*]	374.60 $\pm$ 11.27 [*]	387.00 $\pm$ 21.74 [*]
MAP (mmHg)	114.40 $\pm$ 3.57	86.98 $\pm$ 4.99 ^{##}	113.00 $\pm$ 3.25 ^{**}	106.10 $\pm$ 3.26 ^{**}	109.90 $\pm$ 2.59 ^{**}
ST elevation (mV)	0.13 $\pm$ 0.01	0.33 $\pm$ 0.02 ^{##}	0.22 $\pm$ 0.02 ^{**}	0.23 $\pm$ 0.01 ^{**}	0.22 $\pm$ 0.01 ^{**}
LVEDP (mmHg)	8.83 $\pm$ 0.56	22.81 $\pm$ 1.31 ^{##}	13.96 $\pm$ 0.45 ^{**}	15.20 $\pm$ 0.85 ^{**}	13.40 $\pm$ 1.18 ^{**}
+dp/dt max (mmHg/s)	3612.00 $\pm$ 220.20	1698.00 $\pm$ 270.80 ^{##}	2659.00 $\pm$ 82.44 ^{**}	2759.00 $\pm$ 139.60 ^{**}	2756.00 $\pm$ 154.40 ^{**}
-dp/dt max (mmHg/s)	3012.00 $\pm$ 116.90	1132.00 $\pm$ 151.20 ^{##}	1727.00 $\pm$ 216.80 [*]	2014.00 $\pm$ 145.30 ^{**}	2075.00 $\pm$ 126.30 ^{**}

Values are presented as mean $\pm$ SEM. Statistical analysis is carried out using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. ^{##} $P < 0.01$ compared with the normal control group. ^{*} $P < 0.05$, and ^{**} $P < 0.01$ compared to the ISO control group. ISO: isoproterenol; HW: heart weight; HR: heart rate; MAP: mean arterial pressure; LVEDP: left ventricular end-diastolic pressure.

hemodynamic functions. In comparison to the normal control group of rats, the heart weight of ISO-administered rats increased significantly. Rats treated with betulin had a significantly lower heart weight ($P<0.01$) than the rats treated with ISO. A considerable increase in HR and LVEDP was observed in rats treated with ISO (85 mg/kg/day) in comparison to the normal control group, suggesting LV hemodynamic overload. Conversely, when compared to the normal control, ISO significantly reduced MAP ($P<0.01$) and $\pm dp/dt$ max ($P<0.01$). Betulin improved HR, MAP, $\pm dp/dt$ max, and LVEDP, reflecting the restoration of heart function ($P<0.05$).

3.3. Effect of betulin on ECG in rats with ISO-induced myocardial injury

The ECG revealed that the ISO group's ST segment was noticeably higher than that of the normal control group. Pretreatment with betulin (20 and 40 mg/kg *p.o.*, respectively) (Supplementary Figure 1) considerably lowered the ST segment.

3.4. Effect of betulin on antioxidant and lipid peroxidation parameters in rats with ISO-induced myocardial injury

As shown in Table 3, ISO administration caused a significant rise in MDA levels and a marked reduction in antioxidant levels ($P<0.01$). Pretreatment with betulin (20 and 40 mg/kg) reversed ISO-induced alternations in GSH, SOD, and catalase activities, as well as MDA level ($P<0.05$), suggesting betulin could considerably mitigate myocardial injury and oxidative stress damage caused by ISO in rats.

3.5. Effect of betulin on inflammatory markers in rats with ISO-induced myocardial injury

Compared to the normal control rats, ISO administration dramatically increased the gene expression of *IL-6* and *NF- κ B* ($P<0.01$) (Figure 3). However, betulin pretreatment significantly inhibited the overexpression of inflammatory markers induced by ISO ($P<0.05$). On the other hand, the rats that received 40 mg/kg betulin pretreatment showed favorable results.

3.6. Effect of betulin on apoptotic markers in rats with ISO-induced myocardial injury

The expression profiles of *Bax* and *caspase-3* were upregulated in the ISO-induced myocardial injury group compared to the normal control group ($P<0.05$) (Figure 3). Pretreatment with betulin significantly downregulated the expression of *Bax* and *caspase-3* ($P<0.05$ and $P<0.01$, respectively).

3.7. Effect of betulin on the size and area of infarction in rats with ISO-induced myocardial injury

As shown in Table 2, rats administered with ISO had a greater infarction area (57.98%, $P<0.01$) than rats in the normal control group. However, ISO rats treated with betulin for 29 d exhibited a substantial reduction in the extent and staining of the infarction in the heart tissue (40.50%, $P<0.01$).

Table 3. Effect of betulin on antioxidative and lipid peroxidation parameters in rats with isoproterenol-induced myocardial injury.

Parameters	Normal control	ISO (85 mg/kg)	Nebivolol (5 mg/kg)	Betulin (20 mg/kg)	Betulin (40 mg/kg)
SOD (U/mg of protein)	223.50 $\pm$ 26.62	37.06 $\pm$ 5.04 ^{##}	146.20 $\pm$ 17.18 ^{**}	180.40 $\pm$ 31.50 ^{**}	179.90 $\pm$ 20.62 ^{**}
GSH (μ mol/mg of protein)	17.65 $\pm$ 1.04	5.52 $\pm$ 0.93 ^{##}	9.99 $\pm$ 0.89 [*]	10.61 $\pm$ 1.34 [*]	11.77 $\pm$ 1.64 ^{**}
Catalase (mmol H ₂ O ₂ /mg of protein)	6.34 $\pm$ 0.39	0.26 $\pm$ 0.09 ^{##}	1.87 $\pm$ 0.36 [*]	1.68 $\pm$ 0.22 [*]	2.44 $\pm$ 0.56 ^{**}
MDA (μ mol/mg of protein)	0.08 $\pm$ 0.02	0.40 $\pm$ 0.01 ^{##}	0.18 $\pm$ 0.01 ^{**}	0.15 $\pm$ 0.03 ^{**}	0.22 $\pm$ 0.04 ^{**}

Values are presented as mean $\pm$ SEM. Statistical analysis is carried out using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test.

^{##} $P<0.01$ compared with the normal control group. ^{*} $P<0.05$, and ^{**} $P<0.01$ compared to the ISO control group. SOD: superoxide dismutase; GSH: glutathione; MDA: malonaldehyde.

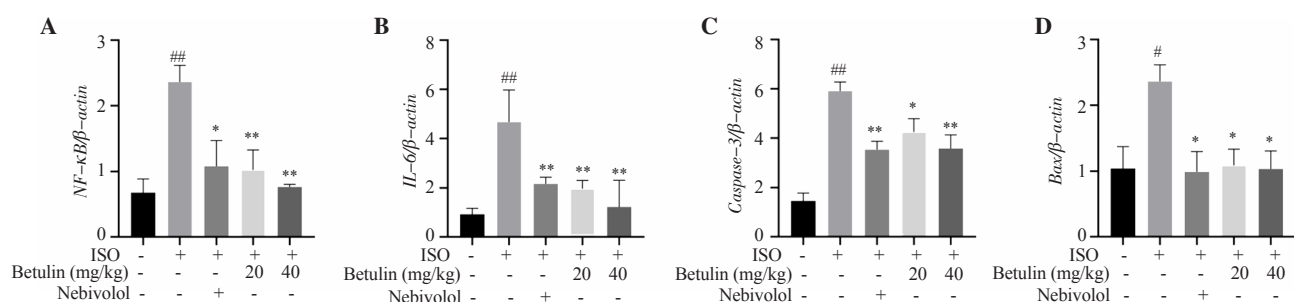


Figure 3. Effect of betulin on inflammatory and apoptotic genes in rats with isoproterenol-induced myocardial injury. Values are presented as mean $\pm$ SEM. Statistical analysis is carried out using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. [#] $P<0.05$, ^{##} $P<0.01$ compared to the normal control group. ^{*} $P<0.05$, ^{**} $P<0.01$ compared to the ISO control group.

3.8. Effect of betulin on histopathological changes in heart tissue of rats with ISO-induced myocardial injury

Figure 4 shows the histopathological architecture of the hearts of rats receiving different treatments. The hearts from normal control rats exhibited a normal histological structural arrangement of myofibers in line with myofibrils, with no evidence of inflammation or necrosis. The heart tissues of the ISO group exhibited myofiber necrosis, inflammatory cell infiltration, damaged and fragmented myofibers, and interstitial edema (Figure 4A and B). However, pretreatment with nebivolol alleviated myocardial damage, as evidenced by a decrease in the inflammatory cells and reduced edema, while treatment with betulin at 20 mg/kg presented mild inflammation and myonecrosis. Remarkably, rats treated with 40 mg/kg betulin exhibited the intact morphological structure of cardiac muscle fibers with a reduced extent of necrosis as indicated by viable cells in red color (Figure 4B).

4. Discussion

Scientific research suggests that natural product-based medications have a history of preventing oxidative damage-mediated cardiac damage[16]. We investigated the preventive impact of betulin against ISO-mediated myocardial damage by evaluating cardiac indicators, lipid peroxidation markers, antioxidant status, as well as inflammatory and apoptotic markers. The symptoms of myocardial ischemia include diastolic and systolic dysfunction. ISO administration in this study markedly raised HR and lowered MAP. Research indicates that terpenes prevent mitochondrial dysfunction and oxidative stress mediated by ISO[17]. Myocardial damage can be specifically identified by abnormally increased levels of CK-MB. Serum levels of LDH and CK-MB rise within 3-6 h

following myocardial ischemia. Cardiac-specific indicators, such as troponin- I , are ideal for diagnosing myocardial injury since they are very sensitive and specific markers for cardiac diseases[18]. One strategy for mitigating myocardial damage caused by ISO is to prevent damage to the heart muscle membrane. In this investigation, the administration of ISO resulted in elevated levels of CK-MB, LDH, and troponin- I as important markers of overall myocardial damage. This finding was corroborated by an increase in the size of myocardial infarctions in the ISO group, as determined by TTC staining. Viable cells are stained brick red when the TTC stain combines with the dehydrogenase enzyme in the presence of the cofactor NADH to create formazan pigment. On the other hand, dehydrogenase enzyme is virtually completely absent from damaged cells; as a result, the infarcted area of the myocardium does not stain with TTC[19]. The rise in ISO-induced CK-MB, LDH, and troponin- I levels was inhibited by betulin pretreatment. In rats with acute myocardial injury, betulin has been demonstrated to stabilize membrane damage, reducing the infarct size and myocardial enzymes such as CK-MB, LDH, and troponin- I .

Changes in hemodynamics result in left ventricular dysfunction, which is a crucial factor in predicting myocardial ischemia and infarction. The administration of ISO causes calcium overload as a result of decreased myocardial contractility and increased oxidative stress-induced membrane permeability. The current study found that, in comparison to ISO-treated rats, pretreatment with betulin improved LV functions by maintaining cardiac contractility as evidenced by improved minimum and maximum pressure (dp/dt), calcium level, and LVEDP.

Rats given ISO showed notable alterations in their ECG patterns, such as pathological Q waves, ST-segment elevation, and T-wave inversion, all of which are indicative of myocardial ischemia injury. The loss of membrane in the ruptured myocardium could account for these events. Betulin pretreatment effectively prevented the elevation

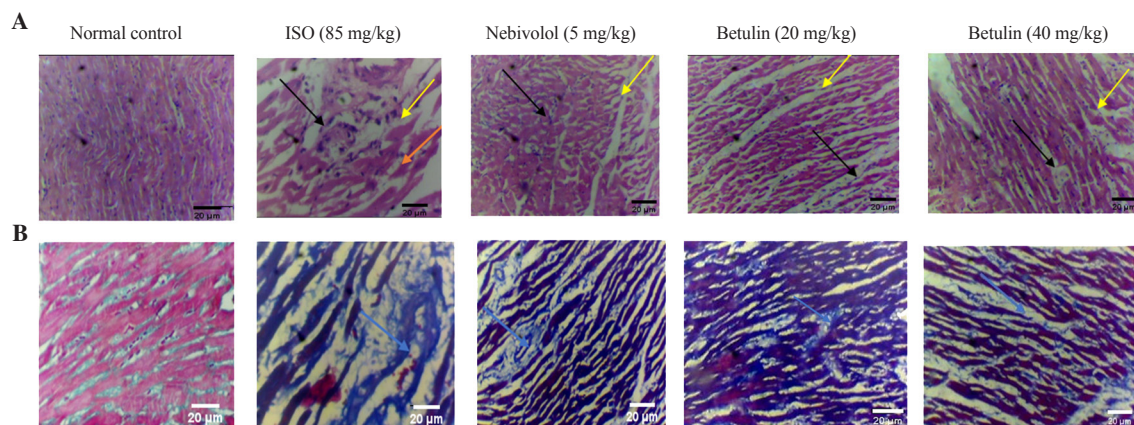


Figure 4. Effect of betulin on histopathological changes in ISO-induced myocardial injury in rats. (A) H&E stain, (B) Masson's trichrome staining (magnification: 45 $\times$). Black arrow: Accumulation of inflammatory cells, yellow arrow: breakdown of cardiac muscle fibers, pink arrow: edematous intramuscular space, blue arrow: necrotic myocardium.

of ST by preserving the integrity of the cell membrane.

Under conditions of cardiac injury, lipid peroxidation is a dynamic pathogenic process. It is commonly known how ISO affects lipid peroxidation in mouse heart tissue[20]. The coordinated action of the cardiac enzymatic antioxidant system preserves cellular redox balance. The myocardial tissue's endogenous antioxidant systems have been depleted, and oxidative damages linked to increased lipid peroxidation, and mitochondrial impairment are all signs of ISO-mediated myocardial injury, which has disturbed the redox balance[17,21]. It has been extensively documented that myocardial tissue undergoes alterations in redox balance upon ISO injection. SOD, catalase, and GSH levels in the heart tissue were significantly decreased in the current study after injection of ISO. This may be because free radicals produced by ISO are scavenged by cellular enzymatic and nonenzymatic antioxidants[22]. However, betulin pretreatment prevented ISO-induced loss of SOD, catalase, and GSH in the cardiac tissue and restored the antioxidant system. The free radical scavenging activity of betulin may be ascribed to Nrf2/HO-1 pathway stimulation[9]. It has been revealed that betulin treatment has cardioprotective effects, which include preventing the expression of cardiac markers in the heart tissue, oxidative damage indices like lipid peroxidation, and antioxidant loss.

Recent research has revealed that certain cardiac apoptotic and inflammatory genes may be upregulated, leading to histological changes[23,24]. Comprehending the molecular mechanism of myocardial ischemia injury could potentially offer valuable perspectives on the disease's pathogenesis and lay the groundwork for the advancement of preventive and therapeutic approaches. A better understanding of the underlying molecular basis of the disease was attained by assessing changes in *caspase-3* and *Bax* gene expression resulting from myocardial ischemia injury using the qPCR System. By cleaving particular peptide sequences, caspases—a class of proteases that are dependent on cysteines and directed toward aspartates—play a vital role in both the initiation and execution stages of apoptosis. Caspases are activated in a cascade manner during apoptosis by nuclear, metabolic, or exogenous stimuli, which results in nuclear receptor-mediated endocytosis and cell death. Caspases have been found to be highly expressed in the myocardium of experimental animals suffering from heart failure and apoptosis, as well as in patients with right ventricular dysplasia and end-stage heart failure. These findings suggest that caspases may be linked to human heart disease[25]. Nonetheless, research indicates that plant-based components with anti-apoptotic properties can impede the damage caused by ischemic myocardial infarction and be useful in minimizing myocardial reperfusion injury. This effect has been partly ascribed to the reduction of cardiomyocyte apoptosis[26,27]. In the present investigation, rats treated with betulin showed a decrease in the expression levels of *caspase-3* and *Bax* genes, whereas ISO significantly increased these levels in rats.

Furthermore, it has been shown that active components like diallyl trisulfide and S-allyl-cysteine sulfoxide (alliin) reduce caspase-3 expression and shield the heart from ischemic injury in rats by preventing apoptosis[28]. Thus, preventing myocardial damage and infarction by blocking the expression of the apoptotic *caspase-3* and *Bax* genes may be beneficial. Results of the current study showed that pretreatment with betulin in rats with ISO-induced myocardial injury improved cardiac histoarchitecture while also significantly reducing the expression of *Bax* and *caspase-3* genes.

Numerous synthetic drugs have been used as anti-inflammatory agents to lessen myocardial damage because inflammatory responses and signaling play a critical role in CVD. According to a recent study, myocardial injury is caused by an excess of pro-inflammatory cytokines, specifically TNF- α and IL-6[29], and is primarily dependent on NF- κ B activation. Generally, the activation of the inhibitory kappa B (I κ B) family precludes NF- κ B. The I κ B kinase enzymes phosphorylate and degrade I κ B under ischemic conditions. As a result of I κ B's inability to regulate NF- κ B, NF- κ B is further enabled to activate, translocate from the cytosol to the nucleus, bind to target gene promoter sequences, and induce excessive TNF- α production. Additionally, IL-6, an inflammatory factor that is pro-hypertrophic and mediates the action of the JAK2/STAT3 pathway, is promoted[30]. In the current investigation, ISO-administered rats exhibited a marked increase in the expressions of *NF- κ B* and *IL-6*. According to our findings, betulin pretreatment substantially reduced *NF- κ B* and *IL-6* expressions in the heart tissue when compared to ISO control rats. This suggests that betulin has anti-inflammatory activity that could inhibit the JAK2/STAT3 pathway, therefore shielding the heart tissue. JAK2/STAT3 downregulation most likely results from inhibition of IL-6, one of the pro-inflammatory cytokines. This inhibition stops STAT3 from being phosphorylated, which stops the translocation of STAT3 from the cytoplasm to the nucleus. Terpenes found in naturally occurring medicinal plants effectively prevent inflammatory reactions and modify pro- and anti-inflammatory cytokines, which are two important signaling molecules involved in inflammation. Updated scientific research evidence indicates that betulin has been reported to inhibit TNF- α and IL-6 through the suppression of NF- κ B pathways in mouse and rat models of diabetes[31]. The production of proinflammatory cytokines (IL-6, IL-1 β , TNF- α), HMGB-1, NF- κ B, and MAPK is inhibited by the antioxidant and anti-inflammatory properties of betulin. The results are consistent with the research conducted by Adepoju *et al.*[9], which showed that betulin treatment decreased the degree of liver and lung damage in septic rats[32].

Histopathological analyses revealed that the administration of betulin reduced necrosis, inflammatory cells, and disruption of the cardiac myofibrils. Additionally, it was found that the systematic arrangements of myofibrils and the distribution of nuclei were improved by betulin pretreatment[25,33].

In conclusion, betulin alleviates ISO-induced myocardial injury via its antioxidant and anti-inflammatory properties by modulating the NF- κ B and IL-6 signalings. However further studies are necessary to fully understand the molecular mechanisms of cardioprotective action.

Conflict of interest statement

The authors declared no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

HS and TG developed the project conceptualization, experimental design, and protocol preparation, and obtained ethical approval. HS conducted the experimental work, analyzed the data, prepared the draft manuscript, and revised manuscript. TG monitored and supervised the project, reviewed the draft manuscript, and provided research facilities and resources.

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