Pharmacogn. Mag.
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ORIGINAL ARTICLE

Cardioprotective Effect of Gedunin on Isoproterenol-Induced Cardiotoxicity through the Attenuation of NF-κB-Mediated Inflammatory Pathway in Rats

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Submitted: 20-Jan-2022 Revised: 18-Apr-2022 Accepted: 01-Aug-2022 Published: 23-Nov-2022

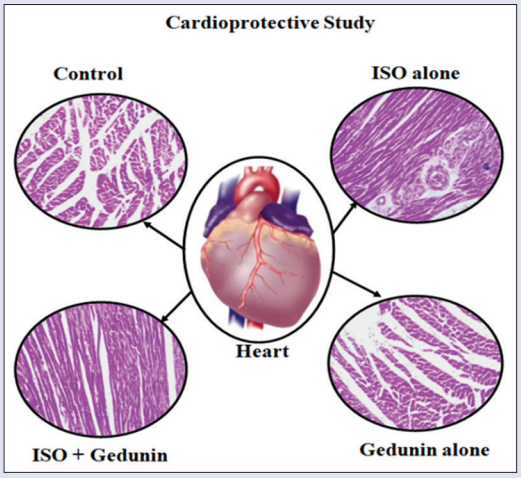
ABSTRACT

Background: There is an increasing interest in new natural compounds for the treatment of myocardial infarction (MI). Gedunin is a tetranortriterpenoid extracted from the Indian neem tree (*Azadirachta indica*). The main objective of this study is to understand the cardioprotective effects of gedunin against MI through the suppression of NF-κB-mediated inflammatory pathways. **Materials and Methods:** Male Wistar rats were categorized into 4 groups of 6 each: control, inducer isoproterenol (ISO) alone, ISO + gedunin-treated, and gedunin (50 mg/kg b.w.)-pretreated rats followed by ISO induction. The infarct size, heart-to-body weight ratio, cardiac enzymes, ATP values, Ca^{2+} levels, and inflammatory and apoptotic markers were studied and compared in all these groups. Data were expressed as mean ± SD. One-way analysis of variance (ANOVA) and *post hoc* Tukey–Kramer test were used for comparing multiple values. **Results:** Gedunin pre-treatment caused a reduction in cardiac size, attenuated the levels of cardiac bio-enzymes, and reduced immune cell infiltration and necrosis. It also enhanced the secretion of antioxidant enzymes glutathione-S-transferase (GST) and superoxide dismutase (SOD) and reduced glutathione (GSH) and glutathione peroxidase (GPx). Gedunin suppressed inflammation by down-regulating the secretion of interleukin 10 (IL-10), interleukin-1β (IL-1β), nuclear factor-kappa B (NF-κB), tumor necrosis factor-α (TNF-α), and apoptotic markers caspase-3, caspase-9, Bax, and Bcl-2. **Conclusion:** Gedunin inhibited apoptosis in cardiotoxicity-induced MI in rats by inhibiting inflammation and apoptosis markers. Thus, gedunin is a potential natural cardioprotective compound.

Key words: Antioxidants, apoptosis, cardiotoxicity, gedunin, inflammation

SUMMARY

- Gedunin pre-treatment suppressed the levels of Ca^{2+} and ATP in ISO-induced cardiotoxicity in rats.
- Gedunin reduces infarct size, cardiac bio-enzymes, necrosis, apoptosis, and immune cell infiltration.
- Gedunin reverses the expressions of Bcl-2, Bax, caspase-3, and -9 induced by ISO.
- Gedunin significantly suppressed the ISO-induced inflammatory markers in the cardiac tissues of rats.



Abbreviations used: mTOR: Mammalian target of rapamycin; AkT: protein kinase B; PI3K: phosphoinositide 3-kinases; AMPK: AMP-activated protein kinase; NF-κB: nuclear factor-kappa-B; Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated X protein; IL-10: interleukin 10; TNF-α: tumor necrosis factor alpha.

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DOI: 10.4103/pm.pm_29_22

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INTRODUCTION

Cardiotoxicity-induced myocardial infarction (MI) is one of the leading causes of heart failure and cardiac dysfunction.[1] Myocardial damage entices inflammatory response by secreting cytokines, oxygen-free radicals, and other proteases. During wound healing, extracellular matrix degradation causes scar formation.[2] Free radical-induced reactive oxygen species (ROS) instigate structural and functional changes in the signaling of cellular hypertrophy, remodeling, and cardiac cell death.[3] In several studies, p53, mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK) (c-Jun N-terminal kinase (JNK), p38 kinase, extracellular signal-regulated kinase (ERK) 1/2), and AkT pathways were found to be regulated by the ROS.[4-6] Among these activated signaling pathways, certain compounds might exhibit a cardioprotective effect. 5'-Adenosine monophosphate-activated protein kinase (AMPK) is one such compound that provides energy and nutrients to cardiac muscles. It has been proven that AMPK subsides ROS levels.[7-9]

It has also been proven that primary reperfusion and ischemia trigger an inflammatory response and stimulate cardiac remodeling.[10,11] Toward the

Cite this article as: Sun Y, You H. Cardioprotective effect of gedunin on isoproterenol-induced cardiotoxicity through the attenuation of NF-κB-Mediated inflammatory pathway in rats. Phcog Mag 2022;18:1177-82.

end of wound healing in heart tissue, the inflammatory cells at the site of injury enable scar formation and worsen the infarction.[1,12-14] An AMPK, mTOR, AkT (protein kinase B), and PI3K (phosphoinositide 3-kinases) pathway is established during the autophagy process and plays an important role in cell growth.[15] Other factors such as Nrf2 (nuclear factor erythroid 2 related factors) and Keap 1 (Kelch-like ECH-associated protein) also have a role. Nrf2 promotes cell survival and inhibits apoptosis by mediating mitochondrial function and stimulating the expression of the antioxidant enzyme.[16] Alterations in inflammatory response could promote a reduction in infarct size and enhance cardiac stability and function. Hence, plant-based anti-inflammatory compounds proven for therapeutic effects in other diseases are being studied for their potential role in MI.

Gedunin is a natural limonoid compound mostly present in the plants of the *Meliaceae* family with proven medicinal values. Gedunin acts as a potent Hsp 90 inhibitor in neuropathological disorders.[17] Few reports have demonstrated the antibacterial and antifungal properties of gedunin.[18,19] Gedunin is also proven to inhibit cellular proliferation and induce apoptosis in several cancers. Both *in vitro* and *in vivo* studies have shown the molecular effects of gedunin against all types of cancers.[20,21] Gedunin was also found to have anti-parasitic and anti-diabetic therapeutic properties.[22,23] In this study, we planned to investigate the action of gedunin on ISO-induced MI by exploring vF-κB-mediated inflammatory signaling pathways.

MATERIALS AND METHODS

Chemicals

Gedunin and isoproterenol (ISO) were commercially obtained from Merck, Sigma. Catalase (CAT), GST, SOD, GSH, glutathione peroxidase (GPx), and ATP assay kits were purchased from Abcam (Boston, MA, USA). Ca^{2+}, apoptotic markers caspase-3 and 9, inflammatory cytokines interleukin 10 (IL-10) and interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), cardiac troponin T (cTNT), creatine kinase-MB fraction (CK-MB), creatine phosphokinase (CPK), and cardiac troponin I (cTnI) were determined using enzyme-linked immunosorbent assay (ELISA) and colorimetric kits purchased from Abcam (Boston, MA, USA).

Preparation of gedunin

Gedunin was dissolved in 1% carboxymethyl cellulose and administered orally by gastric intubation once a day in the morning for the first 21 days.[24]

Induction of experimental MI

ISO (60 mg/kg b.w.) was dissolved in physiological saline (0.9% in NaCl) and injected subcutaneously (s.c.) in the right thigh of the rat for two consecutive days at an interval of 24 h to induce MI.[25]

Experimental animals

Male Wistar rats were maintained under sterile conditions and fed at regular intervals with food pellets and water *ad libitum* based on laboratory standards. This research was approved by Shandong Provincial Hospital Affiliated with Shandong First Medical University's Animal Ethical Committee (approval no. 202138881211D). Animals were split into 4 groups each containing 6 rats: group I: untreated, group II: ISO-treated, group III: ISO-induced gedunin (50 mg/kg b.w.)-treated, and group IV: gedunin (50 mg/kg BW)-treated. Gedunin dissolved in 1% carboxymethyl cellulose was administered orally.

Sample collection

The sacrifice of experimental rats was carried out after 24 h of the second ISO dose. Blood from the experimental rats was collected and subjected to centrifugation for 15 min at 4000g for serum separation.[26] Heart samples were dissected, weighed, and subjected to further analysis.

Measurement of infarct size and heart-to-body weight ratio

Extracted heart samples were sectioned into five portions and incubated with TTC (10% of triphenyl tetrazolium chloride). Tissue samples were fixed in 10% formalin, and the infarct area and area at risk were imaged in each heart slice. JPG imageries of each heart segment were examined using Image J software and areas.

Assessment of cardiac enzymes, antioxidant biomarkers, Ca^{2+}, and ATP

Homogenization of heart tissues was done by mixing with 50 mM Tris-HCl + 0.25M sucrose followed by centrifugation at 800 g for 20 min. This was followed by repeated centrifugation of the supernatant at 9000 g for 15 min. The pellet was finally suspended in a homogenizing buffer. Levels of GSH, mitochondrial ATP and Ca^{2+}, antioxidant biomarkers GPx, GST, SOD, and CAT were quantified using an assay kit as per the manufacturer's protocol (Abcam, Boston, MA, USA).

Histopathological studies

The heart tissues were fixed in 10% formalin and qualitatively analyzed for histological alterations. The tissues were then processed for dehydration and embedded in paraffin wax. Hearts were cut into (3–5 mm thickness) sections and stained with hematoxylin and eosin (H and E) dyes for morphological observation under the light microscope.

Gene expression studies

Quantification of mRNA expressions was analyzed by using real-time polymerase chain reaction (PCR). Specific primers were designed and purchased from Sigma-Aldrich, USA. β-actin was used to normalize the expressions of the target genes.

Apoptotic and inflammatory biomarkers analysis

ELISA kits purchased from Sigma-Aldrich (USA) were used to analyze the inflammatory cytokines TNF-α, IL-6, NF-κB, and IL-1β and apoptotic markers caspase-3 and -9. Analysis was carried out as per the manufacturer's protocol.

Statistical analysis

Data were expressed as mean ± SD. One-way analysis of variance (ANOVA) and *post hoc* Tukey–Kramer test were used for comparing multiple values. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of gedunin on infarct area size

Minimal infarct regions and standard heart-to-body-weight ratios were observed in the untreated and gedunin-treated groups. Isoproterenol (ISO)-treated rats revealed augmented levels of heart-to-body-weight ratio with more numbers of infarct areas ($P < 0.05$). However, pre-treatment with gedunin caused a reduction in the heart-to-body weight ratio and infarct size as compared to ISO-induced rats. 50 mg/kg BW, of gedunin pre-treatment, showed no significant change in the heart-to-body weight ratio and infarct size [Figure 1a and b].

Effect of gedunin on cardiac enzymes

ISO induction increased the levels of cardiac enzymes, creatine kinase-myocardial bound (CK-MB), cardiac troponin I (cTnI), cardiac

troponin T (cTnT), and creatine phosphokinase (CPK). However, pre-treatment with gedunin (50 mg/kg BW) reduced these enzymes significantly ($P < 0.05$) [Figure 1c–f].

Effect of gedunin on mitochondrial antioxidant enzymes

Mitochondrial antioxidant enzymes are a source of free radical scavenging. Reduced antioxidant enzymes (CAT, SOD, GST, and GPx) and non-enzymatic compound GSH levels in ISO-induced rats were elevated after pre-treatment with gedunin [Figure 2a–e].

Histopathological analysis

Hematoxylin and eosin (H&E) staining sections of myocardial tissues revealed a distinct and intact myocardial tissue structure with no inflammation and edema [Figure 3]. However, ISO-induced heart sections revealed medium-to-high-grade necrosis, immune cell infiltration, and edema. Pre-treatment with gedunin (50 mg/kg BW) considerably suppressed edema, necrosis, and infiltration of immune cells as compared to the ISO-induced rats.

Effect of gedunin on ATP and Ca^{2+} levels

ISO-induced MI rats showed higher levels of Ca^{2+} and decreased ATP levels when compared to untreated rats [Figure 4]. These Ca^{2+} levels were significantly ($P < 0.05$) suppressed by gedunin pre-treatment and substantially increased the ATP levels when compared to ISO-induced rats ($P < 0.05$).

Effect of gedunin on the expression of inflammatory biomarkers

Inflammatory biomarkers IL-6, NF-κB, IL-1β, and TNF-α levels were not significantly changed in the control and gedunin-exposed groups [Figure 5a–d]. ISO induction increased these levels of IL-6, NF-κB,

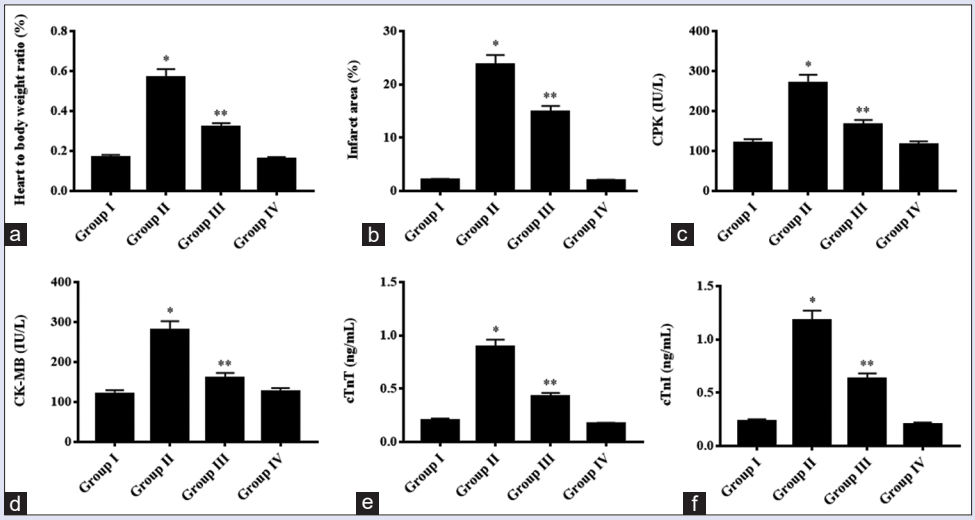

Figure 1: Effect of gedunin on (a) heart-to-body weight ratio, (b) infarct size, (c) creatine phosphokinase (CPK), (d) creatine kinase-myocardial bound (CPK-MB), (e) cardiac troponin T (cTnT), and (f) cardiac troponin (cTnI). Values are expressed as mean ± SD ($n = 6$) and *,**$P < 0.05$ is considered statistically significant

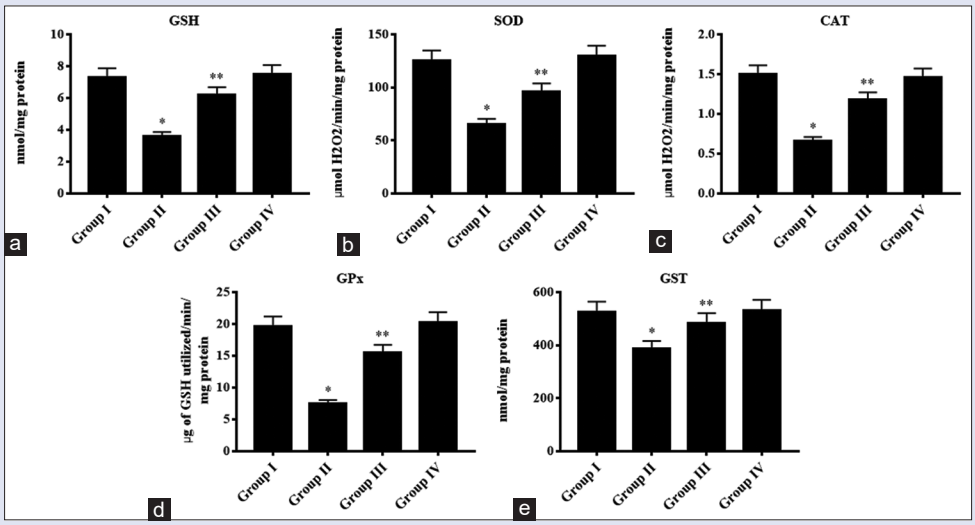

Figure 2: Effect of gedunin on (a) GSH, (b) SOD, (c) CAT, (d) GPx, and (e) GST antioxidant enzymes in ISO-induced MI in rats. Values are expressed as mean ± SD ($n = 6$) and *,**$P < 0.05$ is considered statistically significant

IL-1β, and TNF-α ($P < 0.05$). However, gedunin pre-treatment significantly suppressed the ISO-induced biomarkers in a dose-dependent manner.

Effect of gedunin on apoptotic markers

Expression of caspase-3 and -9, Bcl-2, and Bax markers was not altered in the control and gedunin-treated groups [Figure 6a–d]. However, in ISO-induced rats, the protein expression of cleaved caspase-3 and -9 and mRNA level expression of Bax were up-regulated, and Bcl-2 expression was down-regulated. Gedunin pre-treatment significantly ($P < 0.05$) down-regulates protein levels of caspase-3, -9, and mRNA levels of Bax, followed by up-regulation of mRNA levels of Bcl-2.

DISCUSSION

New therapeutic strategies for curing MI and the associated myocardial damage are being researched. Several plant compounds have been studied for their myocardial protective roles. Extracting these plant compounds and understanding their pharmacological potential has gained popularity among researchers. The current study is to study the curative properties of gedunin in MI and examine the molecular mechanisms in apoptosis and other cell signaling pathways. It has been reported that ISO treatment causes increased cardiac bio-enzymes, apoptosis, mitochondrial alterations, necrosis, and infiltration of inflammatory cells.[27,28] The quantification of cTnI, a contractile protein released during myocardial necrosis and more important in detecting MI at the acute stage, also predicts risk conditions in succeeding infarction.[29] We observed substantial enhancement of the cTnI level of ISO-mediated MI rats might attributable to cTnI leakage on account of ISO-mediated necrosis in the heart. As a result of cTnI leakage, the heart membrane converts porous or else breaks because of O_2 supply scarcity.[30] Gedunin limits enzyme leakage and cTnI and appears to reserve the physical as well as functional integrity of the membrane. C-Reactive protein (CRP) is a deliberately studied marker of inflammation in CVDs.

In the current study, gedunin reduces infarct size, cardiac bio-enzymes, necrosis, apoptosis, and immune cell infiltration. First-line cellular defense enzymes such as SOD, catalase, and GPx scavenge free radicals against oxidative tissue injury.[31] The balance between cellular defense enzymes is a vital role in the removal of intracellular ROS effectively in

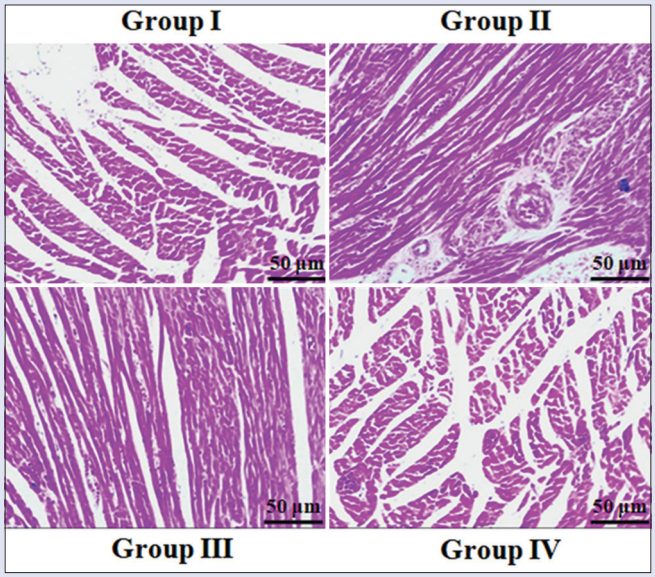

Figure 3: H and E staining of cardiac tissues in untreated and gedunin-treated rats

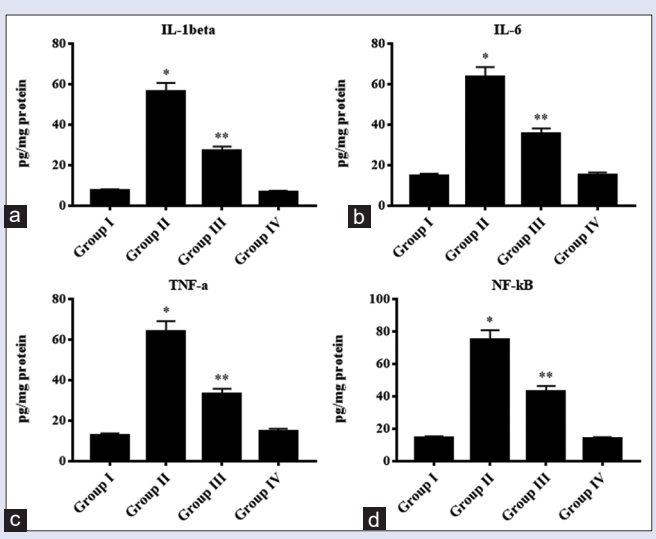

Figure 5: Effect of gedunin pre-treatment on inflammatory biomarkers (a) IL-1β, (b) IL-6, (c) TNF-α, and (d) NF-κB. Values are expressed as mean ± SD ($n = 6$) and *,**$P < 0.05$ is considered statistically significant

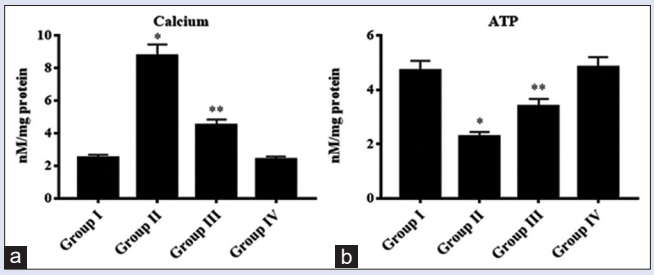

Figure 4: Effect of gedunin pre-treatment on mitochondrial (a) Ca^{2+} and (b) ATP in ISO-induced and gedunin-pre-treated rats. Values are expressed as mean ± SD ($n = 6$) and *,** $P < 0.05$ is considered statistically significant

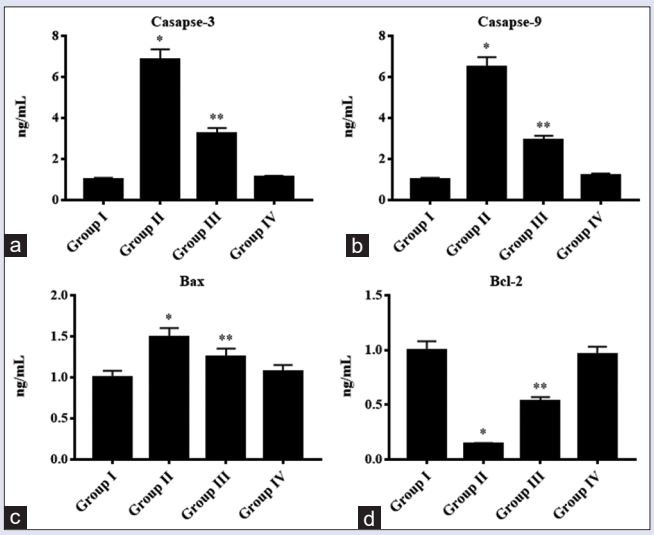

Figure 6: Effect of gedunin pre-treatment on the expression of apoptotic markers, (a) caspase-3, (b) caspase-9, (c) Bax, and (d) Bcl-2. Values are expressed as mean ± SD ($n = 6$) and *,**$P < 0.05$ is considered statistically significant

organelles.[32] The glutathione peroxidase is the front line of guardian against oxidative stress.[33] The researchers noticed decreased GPx activity owing to increased concentration of hydroperoxides or decreased concentration of GSH in ISO-induced MI rats. Antioxidant enzymes CAT, SOD, GST, and GPx and non-enzymatic compound GSH levels were elevated by biochemical analysis. Histopathological investigation of untreated control myocardial tissue demonstrated clear myocardial cell membrane integrity with not any indication of inflammatory cell infiltration and focal necrosis related to ISO-induced MI rat. ISO-induced rats displayed fibers of cardiac muscle separation and wide neutrophil granulocytes infiltration. Gedunin treatment reduced the infarct size, prevented necrosis, and reduced the infiltration of immune cells. ISO causes cardiac mitochondrial oxidative stress, calcium ion (Ca^{2+}) overload, and adenosine triphosphate (ATP) depletion play an important role in the pathogenesis of MI. Gedunin pre-treatment also suppressed the levels of Ca^{2+} and elevated the ATP levels.

High oxidative stress, inflammation, and ROS levels are related to MI. The pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) remain important factors in the MI pathophysiology employing various effects on injury and repair of cardiomyocytes.[34] Associated inflammatory markers; IL-6, NF-κB, IL-1β, and TNF-α levels were found elevated in ISO-induced rats.[35] Bcl-2 inhibits cellular death by binding to the mitochondria, whereas Bax acts as a pro-apoptotic protein present in an inactive form in viable cells. During MI, Bcl-2 is blocked by the conformational changes in Bax.[36,37] In the present study, we studied the molecular level, that is, protein and mRNA expressions of cleaved caspase-3 and -9, Bax, and Bcl-2. The study revealed that Bcl-2 expression was suppressed, whereas Bax, caspase-3, and -9 were up-regulated. However, gedunin pre-treatment reversed the expressions of Bcl-2, Bax, caspase-3, and -9. The suppressed proteins were significantly up-regulated after gedunin pre-treatment ($P < 0.05$). Thus, gedunin demonstrated a cardioprotective effect.

CONCLUSION

The present study demonstrated that gedunin has the potential to protect the myocardium against ISO-induced cardiac injury. From the above results, we conclude that gedunin 50 mg/kg BW exerts an effective cardioprotective role by regulating several cardiac biomarkers of MI such as infarct size, cardiac bio-enzymes, inflammatory and apoptotic markers, Ca^{2+} and ATP levels, and antioxidant enzymes along with molecular factors that inhibit the inflammatory pathway. Histopathological observations are also in correlation with the biochemical parameters. The cardioprotective effect of gedunin could be due to its antioxidant and membrane protection potential. Further pre-trials and clinical trials are recommended before use in humans.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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