Pharmacogn. Mag.
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ORIGINAL ARTICLE

Cardioprotective Effect of Plumbagin and Amelioration of Pro-Inflammatory Cytokines through Suppression of Na^+/K^+-ATPase on Myocardial Ischemia

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Submitted: 19-Dec-2020 Revised: 29-May-2021 Accepted: 26-Jul-2021 Published: 11-Nov-2021

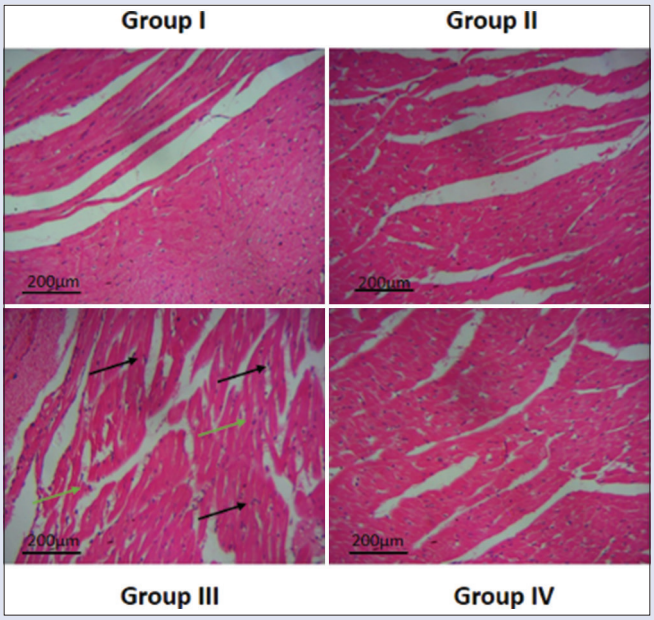
ABSTRACT

Background: Ischemia heart disease in acute phase has formed Myocardial Infarction (MI) due to imbalance between the vascular supply of oxygen and nutrients and myocardial remands leads necrosis. **Objective:** Herein, we investigated the cardioprotective effect of plumbagin in isoprenaline hydrochloride (ISO)-induced inflammatory response and increase in antioxidant enzymes and Na^+/K^+-ATPase activity in MI rats. **Materials and Methods:** The animals were divided into four groups: Vehicle control group (0.1%), plumbagin group (25 mg/kg/b.w.), ISO group (85 mg/kg/b.w.) injected subcutaneously for 2 days at 24 h interval after a week to induce MI, and plumbagin (25 mg/kg/b.w.) by oral administration for 1 month. The inflammatory and cardiac markers were examined using ELISA kits. The oxidative markers, antioxidant markers, and Na^+/K^+-ATPase activity was detected using standard methods. **Results:** According to our results, plumbagin significantly increased the activities of creatine kinase and cardiac troponin T (cTnT). It significantly restored the levels of lipid peroxidation markers (thiobarbituric acid reactive substances [TBARS] and lipid hydroperoxide), increased the antioxidative enzymes (superoxide dismutase, catalase, glutathione [GSH] peroxidase, and the content of GSH), and decreased the levels of pro-inflammatory cytokines (tumor necrosis factor-α, interleukin-6, and nuclear factor kappa B). Histological studies also reveal the decreased inflammatory signs and tissue damages. **Conclusion:** Our results indicate that plumbagin increased antioxidant enzymes, reduced inflammatory response, and reduced Na^+/K^+-ATPase activity in rats with MI. Hence, we recommended that plumbagin confers effective cardioprotective activity against ISO-induced MI heart injury.

Key words: Anti-inflammatory, isoprenaline, myocardial infarction, oxidative stress, plumbagin

SUMMARY

- Plumbagin demonstrates potent biological activities such as antioxidant, anti-inflammatory, neuroprotective, and anticancer activities
- The results of this study reveal that plumbagin administered to isoprenaline hydrochloride-induced rats produced cardioprotective effects against myocardial infarction-induced heart injury.



Abbreviations used: MI: Myocardial infarction; ROS: Reactive oxygen species; ISO: Isoprenaline hydrochloride; TNF: Tumor necrosis factor; IL: Interleukin; ASK1: Apoptosis signal regulated kinase; cTnT-cardiac troponin T.

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DOI: 10.4103/pm.pm_565_20

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INTRODUCTION

The World Health Organization were diagnosed the heart disease (early stage of myocardial infarction [MI]) cause of mortality worldwide till date 2020. Myocardial damage found, when suppresses a clinical threshold in cardiac ischemia. Among cardiac disorders, MI is common and serious consequence leading to increased rate of morbidity and mortality worldwide.[1] Acute ischemic heart disease leads to the formation of MI due to a reduced vascular supply thereby reduced oxygen and nutrients and myocardial remands leads necrosis. In the heart, greater production of reactive oxygen species (ROS) leads to the formation of MI, which creates toxic environment and further cardiac apoptosis. Cardiomyocytes in rats induced with hydrogen peroxide show cellular dysfunction due to the increased production of ROS, which is identical to the oxidative stress in heart.[2]

Cite this article as: Zhang G, Ni X, Zhou Y. Cardioprotective effect of plumbagin and amelioration of pro-inflammatory cytokines through suppression of Na^+/K^+-ATPase on myocardial ischemia. Phcog Mag 2021;17:643-8.

Oxidative stress-induced production of free radicals increases lipid peroxidation, depletion in the level of antioxidant enzymes, and structural changes to the proteins leads loss of cardiomyocytes degradation by apoptosis.[3] Rat cardiomyocytes treated with isoprenaline hydrochloride (ISO) undergoes cellular dysfunction where in different steps similar cause the MIs.[4] ISO, a β-adrenoreceptor agonist, activates mitochondrial membrane potential leading to oxidative stress. ISO induces ischemic MI in ISO rat model.[5,6] Therefore, the protective effect of phytocompounds against ISO-mediated oxidative stress in myocardial rat models have been greatly studied.

The cardiac myocytes transdifferentiate when activated, and during oxidative stress in the heart, the extracellular matrix deposited subsequently with key cellular events that moved to exhibit fibrotic response. Tumor necrosis factor (TNF)-α induces inflammatory cells, which is a crucial role in this mechanism. A previous study has shown that macrophages stimulate the production of cytokines (interleukins [ILs]-1 β and 6).[7] Furthermore, Na^+/K^+-ATPase is an important ion pump and is essential to balance the optimal membrane potential. It exports three sodium ions and imports two potassium ions into the cell and concurrently transports as concentration dependent. It breakdowns adenosine triphosphate (ATP) molecules to provide the energy for transport machinery.[8] The changes in the activity of cardiac Na^+/K^+-ATPase enzyme during an ischemic episode and reperfusion damage has been well established, and it was identified before the occurrence of cell death.[9,10]

Phytochemicals derived from plants play a major role in various medicinal systems. Secondary metabolites from plants demonstrate various pharmacological properties including cardioprotective role.[11] Plumbagin (5-hydroxy-2-methyl-1,4-napthoquinone) exhibits various biological activities, for example, the quinine structure is responsible for its cytotoxicity effect. Compounds containing quinine group exhibit radical scavenging activity, anti-inflammatory activity, neuroprotective, and anticancer activities.[12] Plumbagin induced apoptosis signal-regulated kinase/TNF receptor-associated factor in colon cancer. Yuan *et al.*[13] reported that plumbagin at very low concentration induced cytotoxicity against human cancer than the chemotherapeutic drug system. The anticancer activities of plumbagin has been well-established.[14] For example, plumbagin exhibited cardioprotective activity against doxorubicin-induced cardiotoxicity in rats.[15] Therefore, in this study, we analyzed the cardioprotective effects of plumbagin in ISO-induced MI in rats. To achieve this, we evaluated cardiac marker enzymes, lipid peroxidation products, activity of Na^+/K^+-ATPase, and inflammatory protein markers, as well as performed histological analysis.

MATERIALS AND METHODS

Chemicals

Plumbagin (CAS Number: 481-42-5; purity: >98.0%) and ISO (purity: 98%) were purchased from Sigma Aldrich (USA). Rabbit polyclonal antibodies against TNF-α, IL-6, and nuclear factor kappa B (NF-κB) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals used were of analytical grade and were procured from HiMedia (USA).

Animals

Wistar Albino rats (6–8 weeks old) weighing above 120–150 g were procured from the institutional animal house. All study protocols involving animals were approved by the Institutional Animal Ethical Committee (approval number: NCU2019087115G) and all experiments were performed based on the guidelines therein. The rats were housed under controlled conditions with 12 h dark/light cycles at 25°C ± 2°C and 35°C–60°C relative humidity.

Experimental design

- Group I: Control normal rats administered with vehicle (0.1% NaCl)
- Group II: The rats were orally administered with plumbagin (25 mg/kg/b.w.) alone for 1 month
- Group III: ISO (85 mg/kg b.w.) administered for 2 days' consecutive days last 2 days to induce myocardial injury
- Group IV: The rats were orally administered with plumbagin (25 mg/kg/b.w.) for 1 month and ISO (85 mg/kg/b.w.) on the last 2 days to induce MI.

After the injection of ISO for 12 h completion the rats serum prepared for analysis, and the heart was harvested surgically and washed with 0.9% cold saline. The rats were anesthetized by 125 mg/100 g of urethane through intraperitoneal.[16] The blood was collected through retro-orbital plexus using capillary tube for the serum preparation. Then, the heart was excised from the rats, washed with 0.9% cold saline and weighed using digital balance to analyze the changes in heart weight. The heart was homogenized prior to biochemical analysis.

Preparation of tissue homogenate

The excised heart tissues were suddenly cleaned with the chilled buffer and homogenized using tissue homogenizer. Then, suspension was centrifuged for 5 min at 6000 rpm and the supernatant was used for the further assays.

Evaluation of antioxidant and lipid peroxidation marker

The tissue homogenate was used to analyze by the Naehius and Samuelssol 1968[17] methods the lipid peroxidation products such as lipid hydroperoxide (LOOH), thiobarbituric acid reactive species (TBARS), and glutathione (GSH).[18] The activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) was based on the previously described methods.[19-21]

Evaluation of cardiac markers

The serum levels cardiac troponin T (cTnT) and creatine kinase-MB (CK-MB) were analyzed using commercially available ELISA Kit.[22]

Evaluation of Na^+/K^+-ATPase activity

The cardiac tissue homogenate (0.2 mL) was mixed with 10 mL of phosphate buffer followed by the addition of 0.2 mL of $MgSO_4$, 0.2 mL of NaCl, and 0.2 mL of KCl. Then, 0.2 m ethylenediaminetetraacetic acid and 0.2 mL ATP and incubated for 15 min at room temperature. The reaction was stopped by adding 1 mL of ice cold 10% trichloroacetic acid finally added the reaction for startup reaction at 15 min termination period. Phosphate was determined by the method of Fiske and Subbarow.[23] Sample was mixed with 0.4 mL ANS reagent and incubated for 10 min at 37°C. The color developed was quantified at 640 nm.[24]

Histological examination

After the experimental period, the heart tissue sample was washed with saline and fixed in 10% formalin. Then, the samples were embedding in paraffin blocks and 5 mm thick sections were made. Then histological analysis was done under a light microscope.[5]

Measurement of inflammatory cytokines tumor necrosis factor-α, interleukin-6, and nuclear factor kappa B

The cytosolic or nuclear fractions were isolated using separation kits obtained from BioVision Inc., (USA). The level of TNF-α, IL-6, and

NF-κB was measured in cytosolic fraction using ELISA Kits (Ray Biotechnology, USA).

Statistical analysis

The data were analyzed using one-way analysis of variance, followed by Duncan's multiple range test using SPSS software 17.0 (SPSS Inc., Chicago, IL, USA). Results are presented as mean ± standard deviation and $P \leq 0.05$ was considered as statistically significant. Non-parametric Kruskal–Wallis test was conducted for Western blot analysis (significant $P < 0.05$).

RESULTS

Effect of plumbagin on modified in heart weight

Figure 1 shows the heart weight of experimental rats. It shows that the ISO-induced rats demonstrated significant increase in the heart weight ($P \leq 0.05$) compared to the vehicle control rats. Plumbagin (25 mg/kg/b.w.) protected the improvement of the heart weight in group IV rats. Vehicle control and plumbagin-alone treated rats revealed normal heart weight compared to ISO-induced rats.

Effect of plumbagin on cardiac marker enzyme

Figure 2 shows the level of CK-MB and cTnT in tissue homogenate. These enzymes were significantly leaked ($P \leq 0.05$) in MI rats induced by ISO than that of normal rats. In contrast, plumbagin significantly reduced their levels at a dosage of 25 mg/kg/b.w. compared to ISO-induced rats.

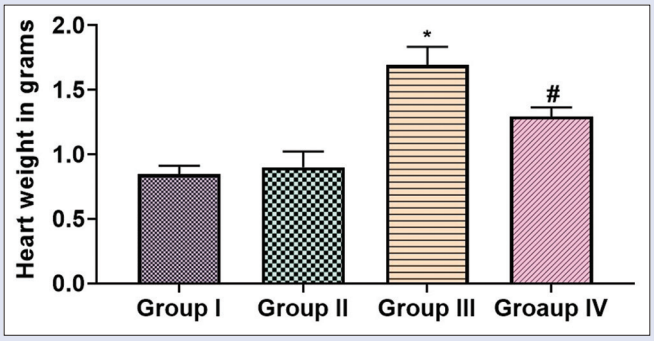

Figure 1: Effect of Plumbagin on the heart weight of myocardiac infarction induced rats. Data are of mean ± standard deviation by triplicates ($P < 0.05$) comparison between control group and treated groups

Effect of plumbagin on the level of lipid peroxidation and the antioxidant enzymes

Figure 3 shows the status of TBARS and LOOH in experimental and control group. The level of TBARS and LOOH was significantly increased ($P \leq 0.01$ and $P \leq 0.001$, respectively). Figure 4 shows the level of different cardiac antioxidants enzymes such as SOD, CAT, GPx, and GST, which were significantly lowered ($P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and $P \leq 0.1$, respectively) in ISO-induced MI rats. However, plumbagin (25 mg/kg/b.w. administered for 30 days) showed (TBARS and LOOH) significantly ($P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and $P \leq 0.1$) diminished respectively along with cardiac antioxidants were improved such as SOD, CAT, GSH, and GST in ISO-induced rats than ISO alone rats.

Effect of plumbagin on Na^+ K^+-ATPase

Figure 5 shows the effect of plumbagin on the activity of Na^+/K^+-ATPase. In ISO-induced rats, there was a significant reduction in the activity of Na^+/K^+-ATPase ($P \leq 0.05$) when compared to the control rats. However, plumbagin (25 mg/kg/b.w.) significantly increased the activity of Na^+/K^+-ATPase in ISO-induced rats ($P \leq 0.05$).

Effect of plumbagin on inflammatory cytokines

Figure 6 shows the amount of proinflammatory cytokines (TNF-α, IL-6, and NF-κB) in the heart tissues of the experimental rats. According to the results, the proinflammatory cytokines were significantly increased ($P \leq 0.05$) after induction with ISO than that of the normal vehicle control rats. Plumbagin (25 mg/kg/b.w. for 30 days) administration in ISO-induced rats revealed significant reduction in the levels of TNF-α, IL-6, and NF-κB ($P \leq 0.05$, $P \leq 0.001$, and $P \leq 0.01$, respectively).

The effect of plumbagin on the histology of heart tissues

Figure 7 shows that the histological changes in the heart tissue of rats in experimental and control rats. In ISO-induced rats, there was necrosis of the cardiac muscle fibers and infiltration of the inflammatory cells

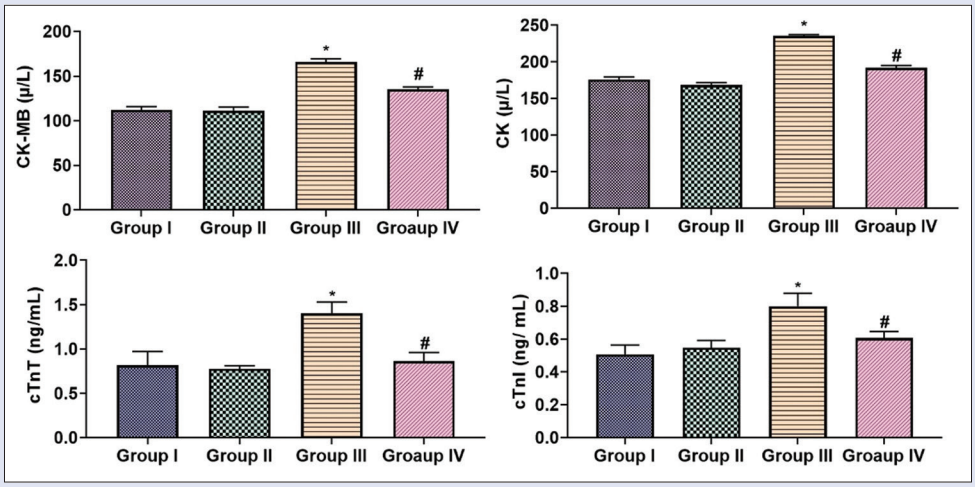

Figure 2: Effect of Plumbagin on serum creatine kinase and cardiotroponins of myocardiac infarction induced rats. Data are of mean ± standard deviation by triplicates ($P < 0.05$) comparison between control group and treated groups

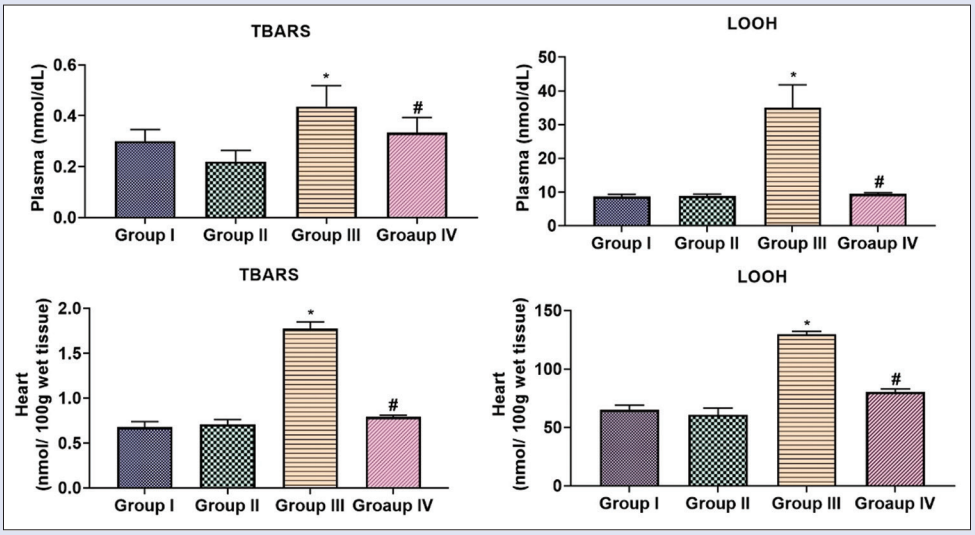

Figure 3: Effect of Plumbagin on the levels of TBARS and lipid hydroperoxide in the plasma and heart of myocardiac infarction-induced rats. Data are of mean ± standard deviation by triplicates ($P < 0.05$) comparison between control group and treated groups

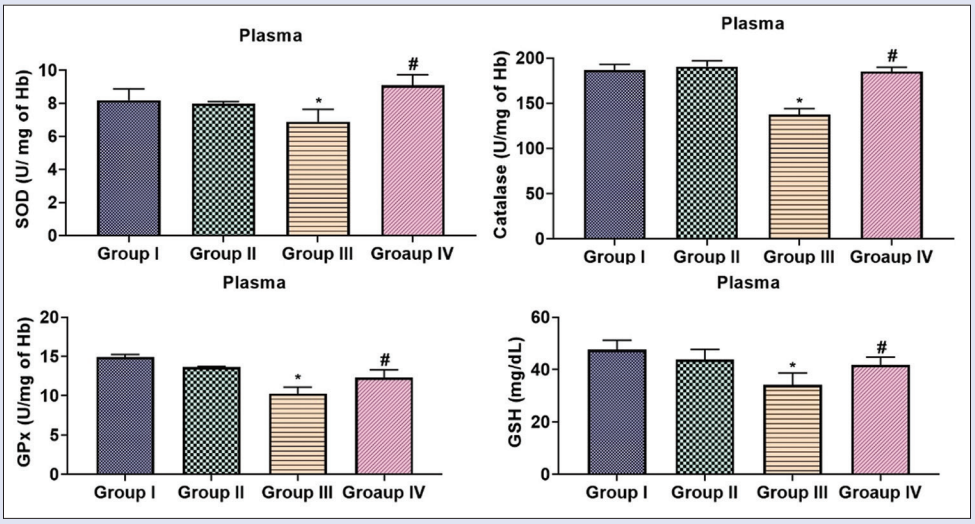

Figure 4: Effect of Plumbagin on the activities of plasma antioxidants of myocardiac infarction induced rats. Data are of mean ± standard deviation by triplicates ($P < 0.05$) comparison between control group and treated groups

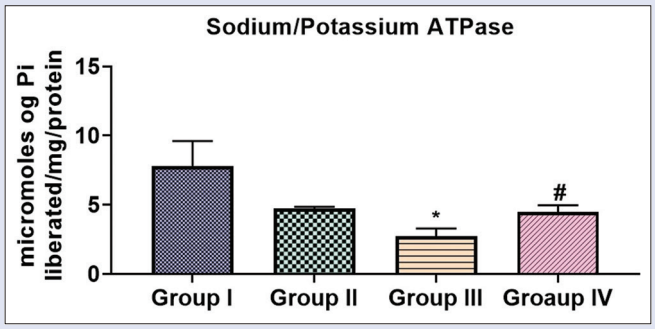

Figure 5: Effect of Plumbagin on the sodium/potassium ATPase level of myocardiac infarction-induced rats. Data are of mean ± standard deviation by triplicates ($P < 0.05$) comparison between control group and treated groups

as compared with vehicle control rats. Plumbagin (25 mg/kg/b.w., administered for 30 days) in ISO-induced rats modified form of necrosis, edema, and inflammatory dysfunction cells as compared to ISO-induced rats.

DISCUSSION

In this study, we hypothesized that plumbagin shows cardioprotective effect against the ISO-induced MI in rat model. Plumbagin significantly suppressed the expression of TNF-α, IL-6, and NF-κB and bring about cardioprotective effect against ISO-induced MI. It reduced the levels of inflammatory cytokines, lipid peroxidation, and improved antioxidant enzyme status. Wexler and Greenberg[25] reported that the ISO-induced necrosis of cardiac cells by increasing the myocardial stress. ISO is a β-adrenergic agonist administered subcutaneously to induce MI in rats compared to normal group that is the symptoms/causes of ISO-induced

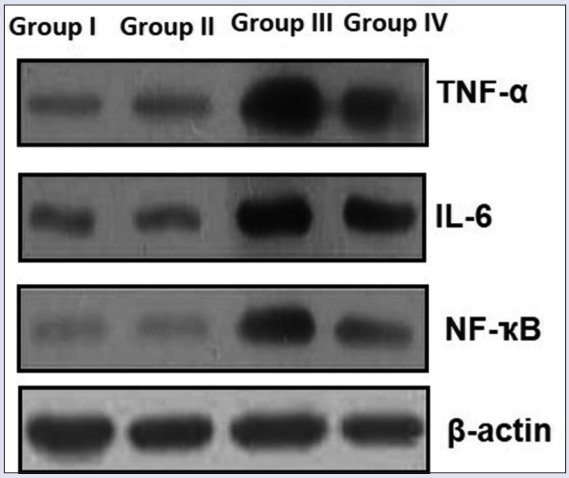

Figure 6: Effect of Plumbagin on inflammatory protein expression in myocardiac infarction induced rats heart tissue

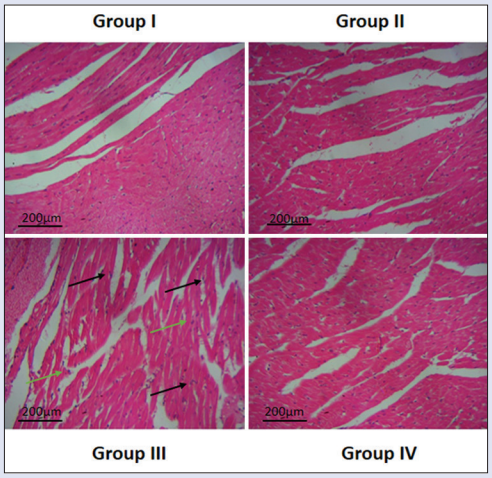

Figure 7: Effect of antioxidant Plumbagin on histoarchitecture of myocardiac infarction induced rat heart tissue. Control and plumbagin alone treated animals exhibited normal histological architectures of heart tissues (Group I and II). In isoprenaline hydrochloride-induced rats found the necrosis of cardiac muscle fibers (green arrows) and inflammatory cells infiltration (Group III). Plumbagin administered isoprenaline hydrochloride-induced rats demonstrated the reduced necrosis and inflammatory cells infiltration (Group IV) (magnification × 40)

MI and infection. Zaafan *et al.*[26] reported that the reduction in heart weight due to anti-hypertrophic potentiality and lowered the anti-hypertrophic potentiality and lowered the reduction of myocardial dysfunction by the administration of plumbagin (25 mg/kg/b.w.) the role of cardioprotective activity and associated with the inflammatory pathways and expressed pattern declines against heart hypertrophy. In this study, we showed that ISO-induced MI modification in heart weight among the treatment groups.

ISO increases muscle contraction, which increases cell membrane permeability. This helps in the release of cardiac marker enzymes such as CK-MB and cTnT into the blood stream.[27] In this study, plumbagin (25 mg/kg/b.w.) administered in ISO-induced rats revealed normal membrane potential. This result is in-line with a previous study,[28] wherein the authors detected the cardiac marker enzymes in serum of rats. The extent of lipid peroxidation in heart tissues was determined by the analysis of TBARS content and LOOH in ISO-stimulated rats.[29,30] ISO stimulates the production of ROS which in turn initiate the formation of peroxides of membrane-bound fatty acids, which leads to cardiac dysfunction.[31,32] In this study, plumbagin (25 mg/kg/b.w., administered for 30 days) significantly reduced the elevated levels of TBARS and LOOH. These findings demonstrate that plumbagin shows cardioprotective effect. Similar findings have been reported by Long *et al.*[33]

Antioxidant activities and free radical scavenging mechanism a vital and effective, safer zone maintained in intracellular organelles. Antioxidant systems are activated during oxidative stress. Enzymes responsible for cellular defense mechanism are SOD, CAT, GSH, GPx, and GST.[34] In this study, ISO-induced rats showed significant reduction in the levels of SOD, CAT, GPX, and GST, which results in MI. In this study, the normalized levels of antioxidants enzymes when plumbagin (25 mg/kg/b.w.)-treated rats. Plumbagin shows cardioprotective effect by reducing the formation of ROS. A previous study reported similar results.[28]

Imbalances of ion levels in cardiac cells could modified in the levels/ability of membrane bound enzymes may affects make changes with SH group are malformed active transport potential in the cell membrane between Na^+/K^+-ATPase levels make chances with SH group are malformed active transports potential in the cell membrane between Na^+ and K^+. These can maintain ionic distribution and may cause ischemia and arrhythmia.[35] In ISO-induced rats, there was reduced NA^+/K^+-ATPase activity, which is improved the free radical with lipid peroxidation.[36] Plumbagin induced the activity Na^+/K^+-ATPase in ISO-induced rats, which shows that plumbagin stabilized the membrane potential. These results are well-corroborated with numerous reports on NA^+/K^+-ATPase activity.[37]

During MI in ISO-induced heart diseases, the histological appearance must evaluate the aforementioned antioxidant, membrane bound enzyme, cardiac marker enzymes, and molecular inflammatory TNF-α, IL-6, and NF-κB showed changes the ISO-induced cardiac segments of rats shows severe neutrophil infiltration, degeneration of myofibril and necrotic changes. Plumbagin (25 mg/kg/b.w.)-treated rats showed neutrophil infiltration with intact myofibrillar structure due to its anti-inflammatory and antioxidant properties. This result shows that plumbagin shows cardioprotective effect against MI-induced heart disease.

Inflammation is responsible for the dysfunction of cardiac muscles in ischemic stages.[38] Plumbagin showed the antiproliferative pathway regulation may suppression of NF-κB in human T-cell signaling pathway in blood cancer cell line model (T-ACC). MoLT-4 cell line. Sandur *et al.*[39] described that plumbagin reduced the activation of IL-6-induced STAT3 (signal transducers and activators of transcription 3 phosphorylation which is inhibits the JAK1/2 and cSrc activation). Furthermore, Hafeez *et al.*, Xu *et al.*, and Zang *et al.*[40-42] revealed that plumbagin suppresses the degradation of IκBα. In this study, ISO-induced elevation of proinflammatory cytokines TNF-α, IL-6, and NF-κB leads to local necrosis and cell damage of heart cells resulted ROS unlimited generation were observed in the heart tissue sections, this findings correlate with the reported Cusack *et al.*; Tawfik *et al.*[43,44]

CONCLUSION

In this study, we demonstrated that plumbagin administered to ISO-induced rats produced cardioprotective effect against MI-induced heart injury. We analyzed the effect of plumbagin through cardiac membrane bound enzyme, antioxidant, anti-inflammatory response, and histological studies. Daily administration of plumbagin protected the rats from high-risk of MI. These data show that plumbagin can be used

as an active pharmaceutical and therapeutic agent against myocardial injury. However, we suggest that additional works should be carried out to support the therapeutic role of plumbagin.

Acknowledgements

Authors are grateful to The Fourth Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, 330003, China, for their support during this study.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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