Saudi Pharmaceutical Journal 32 (2024) 102103

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Saudi Pharmaceutical Journal

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Original article

Role of diosmin in preventing doxorubicin-induced cardiac oxidative stress, inflammation, and hypertrophy: A mechanistic approach

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ARTICLE INFO

Keywords:
Doxorubicin
Cardiotoxicity
Hypertrophy
Inflammation
Diosmin

ABSTRACT

Chemotherapeutic drugs, such as doxorubicin (Dox), are commonly used to treat a variety of malignancies. However, Dox-induced cardiotoxicity limits the drug's clinical applications. Hence, this study intended to investigate whether diosmin could prevent or limit Dox-induced cardiotoxicity in an animal setting. Thirty-two rats were separated into four distinct groups of controls, those treated with Dox (20 mg/kg, intraperitoneal, i.p.), those treated with diosmin 100 mg plus Dox, and those treated with diosmin 200 mg plus Dox. At the end of the experiment, rats were anesthetized and sacrificed and their blood and hearts were collected. Cardiac toxicity markers were analyzed in the blood, and the heart tissue was analyzed by the biochemical assays MDA, GSH, and CAT, western blot analysis (NF-kB, IL-6, TLR-4, TNF-α, iNOS, and COX-2), and gene expression analysis (β-MHC, BNP). Formalin-fixed tissue was used for histopathological studies. We demonstrated that a Dox insult resulted in increased oxidative stress, inflammation, and hypertrophy as shown by increased MDA levels and reduced GSH content and CAT activity. Furthermore, Dox treatment induced cardiac hypertrophy and damage, as evidenced by the biochemical analysis, ELISA, western blot analysis, and gene expression analysis. However, co-administration of diosmin at both doses, 100 mg and 200 mg, mitigated these alterations. Data derived from the current research revealed that the cardioprotective effect of diosmin was likely due to its ability to mitigate oxidative stress and inflammation. However, further study is required to investigate the protective effects of diosmin against Dox-induced cardiotoxicity.

1. Introduction

Cancer is one of the leading causes of death across the world. Chemotherapy, surgery, immunotherapy, hormone therapy, and radiation therapy are the most common cancer therapies. These therapies can be used alone or in combination, depending on the type and stage of cancer and the particular patient's health. Chemotherapy is the most widely used treatment option, but the side effects of chemotherapeutic drugs limit their use (Naderi et al., 2023; Sung et al., 2021; Falzone et al., 2018; DeVita and Chu, 2008; Guo et al., 2024). Doxorubicin (Dox) is a frequently used anticancer drug with a variety of applications for different types of malignancies (Sritharan and Sivalingam, 2021). However, the utilization of Dox for medical conditions is somewhat restricted because of its prevalent cardiac toxicity (Zhang et al., 2023; Qahtani Abdullah et al., 2024; Sandamali et al., 2021). The exact mechanism responsible for Dox-induced cardiac injury has not been fully investigated, though published studies suggest that oxidative stress, inflammation, and apoptosis are the possible causative factors (Podyacheva et al., 2021; Naderi et al., 2023). Since the heart has relatively low levels of antioxidant enzymes, high mitochondrial density/volume, and elevated oxygen consumption rate, the heart is particularly susceptible to the damage caused by oxidative stress. Oxidative stress, which is produced during the intracellular metabolism of Dox, is assumed to be the primary factor contributing to cardiotoxicity when this drug is used (Rocca et al., 2020; Carvalho et al., 2014; Al-Kuraishy et al., 2022).

Published reports suggest that nuclear factor-kappa B (NF-κB) activation is a significant factor in inflammation caused by a stimulus. This is attributed to the ability of NF-κB to regulate the expression of various inflammatory markers, including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and tumor necrosis factor-α (TNF-α) (Khan et al., 2023).

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https://doi.org/10.1016/j.jsps.2024.102103
Received 25 March 2024; Accepted 14 May 2024
Available online 15 May 2024

Prophylactic measures have been suggested to prevent cardiotoxicity. Based on the current suggested mechanism of cardiotoxicity, a molecule that has the ability to scavenge free radicals would be a promising option for adjuvant treatment (Mohammed et al., 2020). The use of extracts from naturally occurring compounds and their bioactive components in the mitigation of the toxicity caused by drugs and chemicals has been widely acknowledged (Suna et al., 2023; Liao et al., 2023; Mohtadi et al., 2023). Diosmin is a naturally occurring flavonoid glycoside often found in citrus fruits, vegetables, and tea (Ali et al., 2021; AlAsmari et al., 2021). Previously published reports have demonstrated several beneficial properties of diosmin, such as neuroprotective, hepatoprotective, nephroprotective, and anti-inflammatory properties (Okubo Eneni et al., 2020; AlAsmari et al., 2021; Ali et al., 2021; Zhao et al., 2024).

At present, there is no effective therapy for restoring cardiac function that has been damaged by Dox. Therefore, the search for a highly efficient remedy continues to be an urgent concern. Hence, the current study aimed to investigate whether diosmin could mitigate the adverse effects of Dox on the rat heart, particularly targeting the antioxidative and anti-inflammatory pathways.

2. Methodology

2.1. Animals

The research animals were acquired from the Animal Center of Pharmacy College at King Saud University (KSU), KSA. All animals were housed in a standard room with a temperature of 25 ± 1 °C and a 12-hour light/dark cycle. Also, all animals were provided with free access to water and a standard diet. The use of these animals was approved by the ethical committee (KSU-SE-19-121).

2.2. Study design

In the current study, we used male Wistar rats (4–6 weeks) weighing 180 ± 20 g. Thirty-two rats were randomly separated into four distinct groups of eight rats each. The animals had a one-week acclimatization period. After that, the control group received normal saline orally for 18 days, and on the 17th day, they received an intraperitoneal (i.p.) injection of normal saline (this mimicked the treatment with Dox, which was dissolved in normal saline). Dox group received an i.p. injection of Dox (20 mg/kg body weight) on the 17th day. A third group was given diosmin 100 mg (LDios) plus Dox, and a fourth group was given diosmin 200 mg (HDios) plus Dox for a duration of 18 days. On the 17th day, a single i.p. dosage of Dox was given to both groups at a dose of 20 mg/kg body weight. On day 18, all rats were anesthetized (with a combination of ketamine and xylazine in a 10:1 ratio) and euthanized. Blood samples were collected from the hearts of five rats in each group. Subsequently, the hearts were excised and immediately immersed in liquid nitrogen to prepare them for biochemical assays, gene expression, and western blot analysis. Additionally, for histological analysis, three rats from each group were perfused using phosphate-buffered saline (PBS) and then fixed in 4 % paraformaldehyde (PFA). Their hearts were then harvested and preserved in 4 % PFA.

2.3. Determination of serum markers

In order to extract the serum, the blood collected during euthanasia was centrifuged at 1000 g for 10 min in a refrigerated centrifuge. Subsequently, the collected serum was used for the enzyme-linked immunosorbent assay (ELISA) to analyze the creatine kinase MB (CKMB) and cardiac troponin I levels (cTnI) according to the instructions provided by suppliers.

2.4. Total protein measurement

In the cardiac tissue, proteins were quantified through the bicinchoninic acid (BCA) assay, which was obtained from ThermoScientific (Rockford, Illinois, USA).

2.5. Peroxidation of lipids measurement

The peroxidation of lipids in the heart tissue was assessed using a previously described method of Ohkawa et al. (1979) with a slight modified. In brief, 0.8 % thiobarbituric acid (TBA) and 30 % trichloroacetic acid (TCA) containing tissue homogenates were incubated for 30 min at 90 °C in a shaking water bath. After that, samples were centrifuged at 3000 g for 15 min, and the absorbance was measured at 540 nm (Ohkawa,Ohishi,and Yagi, 1979).

2.6. Reduced glutathione measurement

The glutathione (GSH) content in the heart tissue was assessed using a modified version of the protocol of Sedlak and Lindsay. In brief, after treating the reaction mixture with 0.4 % 5,5′-dithiobis(3-nitrobenzoic acid), the absorbance at 412 nm was quickly determined (Sedlak and Lindsay, 1968).

2.7. Catalase activity measurement

The Claiborne method was employed to assess the catalase (CAT) activity using the post-mitochondrial supernatant (PMS) extracted from cardiac tissue. In brief, PBS (pH 7.4), hydrogen peroxide, and PMS were mixed, and the absorbance was measured for 5 min every 60 s at 240 nm (Claiborne, 1985).

2.8. Western blot analysis

We performed the western blot analysis to measure protein concentrations according to the previously described protocol with slight modifications. In summary, protein extracts were obtained from cardiac tissue and a uniform quantity of protein (20–50 μg) was separated using 10 %–12 % SDS-PAGE gels. Then, PVDF membranes were used and proteins were transferred. In the next step, 3 % non-fat dry milk was used as a blocker for 60 min. Subsequently, membranes were kept with NF-kB, IL-6, TLR-4, TNF-α, iNOS, COX-2, and GAPDH primary antibodies at 4 °C for overnight. Overnight incubation was carried out in a laboratory rocker. Following washing, the membranes were incubated for an hour with the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP). All the primary antibodies were diluted as 1:1000 and secondary antibody was diluted as 1:5000 in 3 % BSA solution. Enhanced chemiluminescence (ECL) reagent was used for visualization, and, finally, images were acquired via a Bio-Rad gel imaging machine (Bio-Rad, Hercules, California, USA).

Table 1
Details of primer sequences used in this study.

Gene	Primer Sequences (5′ → 3′)	Product length (bp)	Accession number
BNP	Forward: CAGAAGCTGCTGGAGCTGATAAGReverse: TGTAGGGCCTTGGTCCTTTG	78	NM_031545.1
β-MHC	Forward: AGAACCCTCCCAAGTTCGACAAGATCGReverse: TGTTTCAAAGGCTCCAGGTCTCAGG	5635	NM_017240.2
GAPDH	Forward: TCTGCTCCTCCCTGTTCTAGAGACAReverse: TTGTGAGGGAGATGCTCAGTGTTGG	1183	NM_017008.4

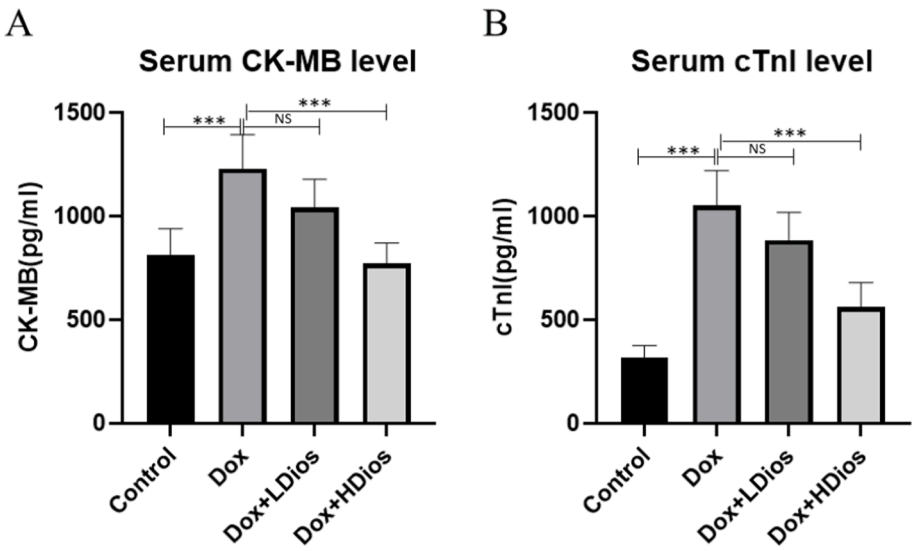

Fig. 1. Effect of diosmin and Dox on CKMB (A) and cTnI (B). NS: Nonsignificant ($p > 0.05$), and ***: $p < 0.001$ ($n = 5$).

2.9. Analysis of gene expression via RT-qPCR

TRIzol reagent was used for the isolation of the total RNA from the cardiac tissue as per the instructions obtained from the company. A NanoDrop spectrophotometer was used to ascertain the purity and concentration of the extracted RNA samples. The isolated RNA (1 μg) was then used to make cDNA utilizing a cDNA synthesis kit. The real-time polymerase chain reaction (RT-PCR) was used to determine the differences in gene expression. GAPDH was used as the normalization housekeeping gene. The ΔΔCt technique was utilized to determine the

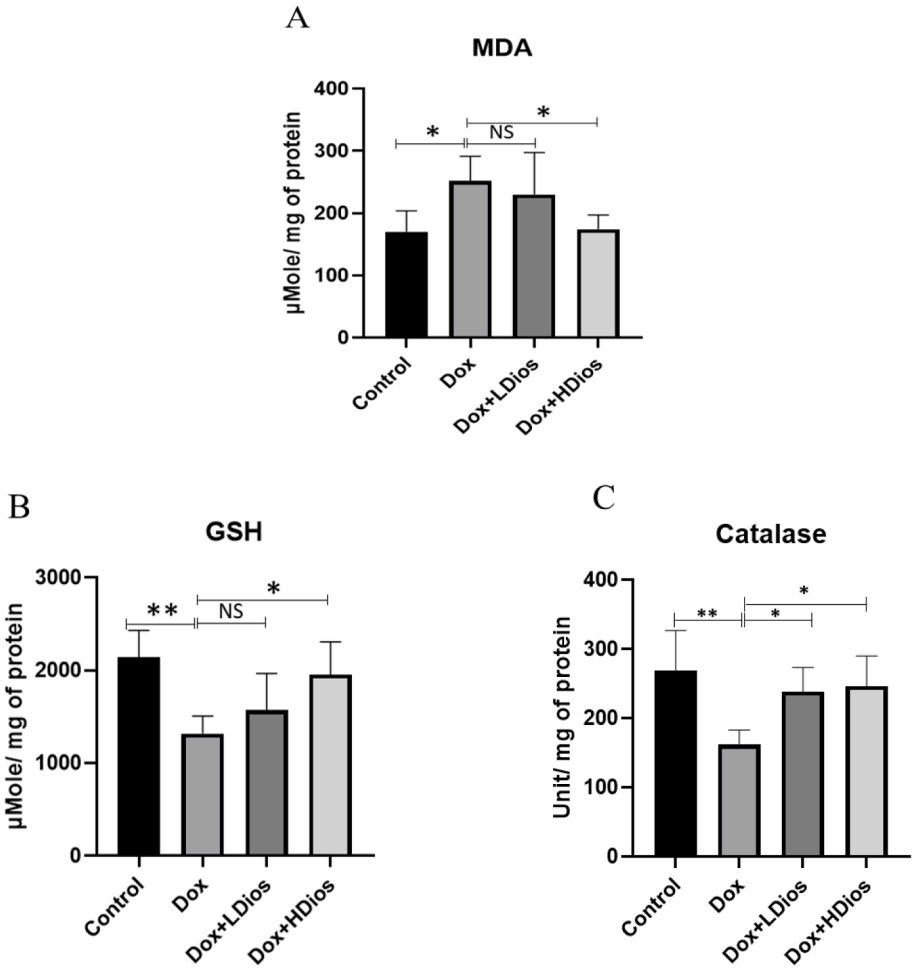

Fig. 2. Effect of diosmin and Dox on oxidative stress markers (A) represents the effects on MDA, 2B represents the effects on GSH, and 2C represents the effects on CAT levels. Where NS: Nonsignificant ($p > 0.05$), *: $p < 0.05$, and **: $p < 0.01$ ($n = 5$).

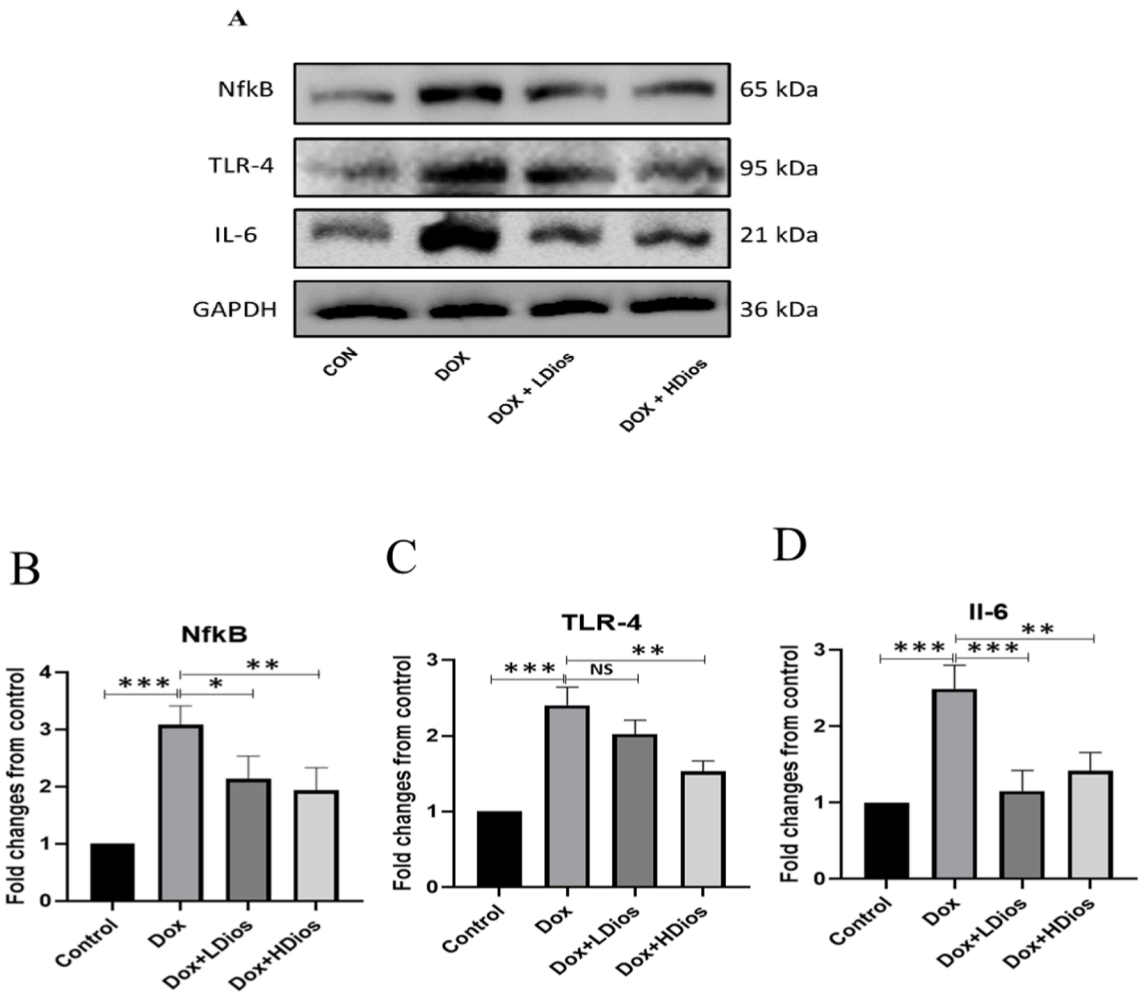

Fig. 3. Effect of diosmin and Dox on cardiac inflammatory protein expression analysis by western blot technique. (A, B, C & D) show immunoblot images and the graphical representation of NF-κB, TLR-4, and IL-6, respectively. Where NS: Nonsignificant ($p > 0.05$), *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ ($n = 3$).

relative gene expression. The sequences of the primers used are shown in Table 1.

2.10. Histological examination of cardiac tissue

Tissue samples from each experimental group were preserved in PFA and then embedded in paraffin to form tissue blocks. Subsequently, the blocks were cut into thin slices measuring 3 μm using a microtome. Afterward, the paraffin was extracted from the sections, and the sections were stained using hematoxylin and eosin (H&E). The tissue was analyzed and histopathological alterations were recorded. Tissues were examined with an Olympus BX microscope.

2.11. Statistical evaluation and analysis

The data were presented as a mean plus or minus the standard deviation (SD) for each group. The analysis of variance (ANOVA) was used to evaluate group differences, followed by post-hoc analysis using the Tukey comparison test. The threshold for statistical significance was set at 0.05.

3. Results

3.1. Diosmin modulates Dox-induced cardiac injury

In order to determine the association between Dox treatment and cardiac damage in the current investigation, blood serum samples were collected and levels of cardiotoxicity markers, such as CK-MB and cTnI, were quantified. The administration of Dox caused a significant increase in the CK-MB and cTnI levels (Fig. 1A and B). In contrast, prophylactic treatment with diosmin caused the increased levels of CK-MB and cTnI to decrease (Fig. 1A and B). These data clearly demonstrated the preventative effect of diosmin in mitigating heart damage induced by Dox.

3.2. Effect of diosmin on oxidative stress

We evaluated the CAT activity, GSH content, and MDA levels in heart tissues to see whether diosmin supplementation would reduce Dox-induced oxidative stress. As expected, Dox injection substantially decreased the CAT activity, reduced the GSH content, and increased the MDA levels (Fig. 2A–C). The administration of diosmin, however, restored these altered parameters to normal (Fig. 2A–C). These findings further validated the protective effect of diosmin against Dox-induced cardiac damage.

3.3. Diosmin modulates Dox-induced inflammation

Previous studies have confirmed the anti-inflammatory properties of diosmin in different tissues (AlAsmari et al., 2021; Ali et al., 2021). Therefore, and in order to confirm the anti-inflammatory effects of diosmin against Dox insult, we evaluated the expression of proteins involved in controlling the inflammation pathway, including NF-kB, IL-

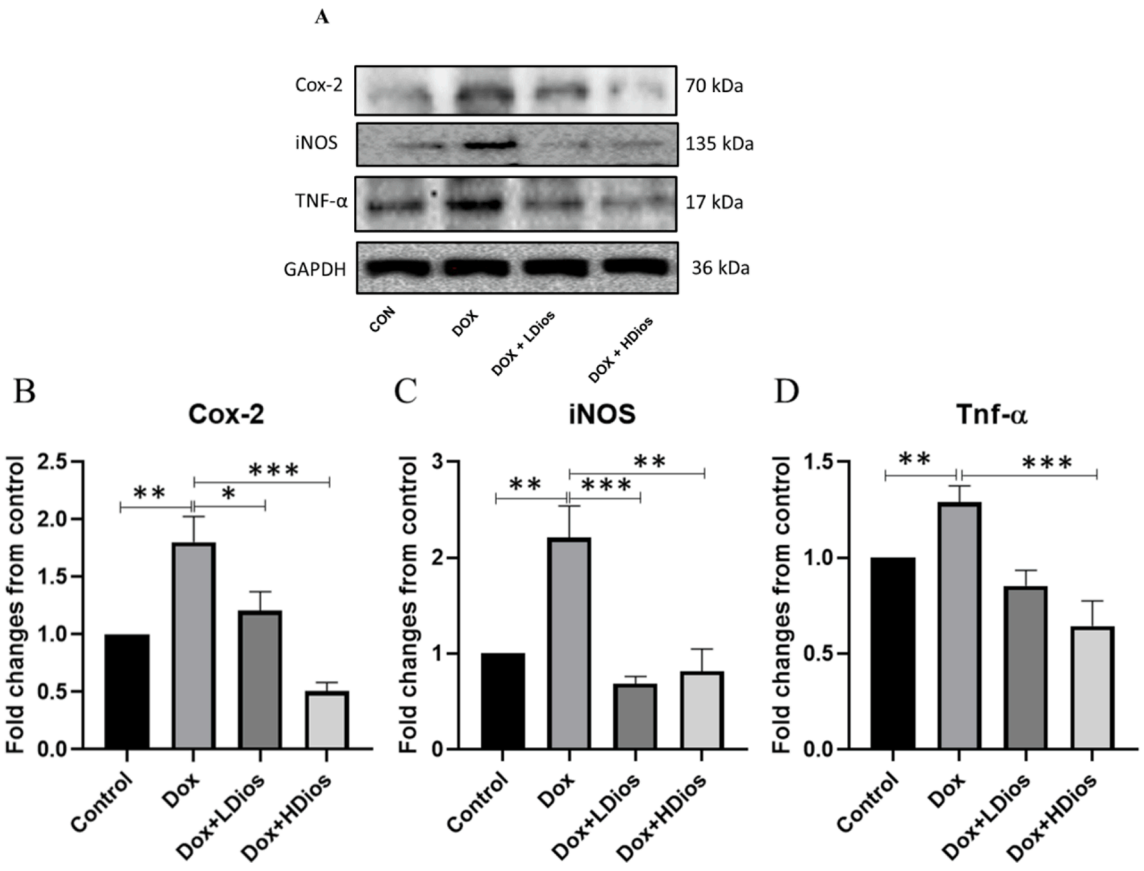

Fig. 4. Effect of diosmin and Dox on cardiac inflammatory protein expression analysis by western blot technique. (A, B, C & D) show immunoblot images and the graphical representation of Cox-2, iNOS, and TNF-α, respectively. Where *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ ($n = 3$).

6, TLR-4, TNF-α, iNOS, and COX-2. The results revealed that the alterations in protein expression induced by Dox were restored in rats that were co-treated with diosmin (Figs. 3A–D and 4A–D). These findings indicated that diosmin has anti-inflammatory properties.

3.4. Diosmin modulates Dox-induced cardiac hypertrophy

We explored the impact of Dox on cardiac muscle by measuring the body weight (BW), heart weight (HW), and ratio of heart weight to body weight (HW:BW), which is considered as an indicator of cardiac hypertrophy. Our data revealed significant changes in the BW, HW, and HW:BW ratio in the Dox group compared with the control group (Fig. 5A–C). Notably, animals co-treated with HDios showed minimal changes compared with the control group (Fig. 5A–C). Furthermore, to validate our hypothesis, we measured the hypertrophic gene markers that are reported to increase in response to cardiac damage, such as beta myosin heavy chain (β-MHC) and brain natriuretic peptide (BNP). Interestingly, we found a significant increase in the expression of these genes in the Dox-treated group (Fig. 5D and E). However, co-administration of diosmin mitigated these gene alterations. Overall, these findings indicated that diosmin treatment protected the heart from Dox-induced hypertrophy.

3.5. Diosmin mitigates Dox-induced alterations in histology

Histopathological analysis of cardiac tissue revealed a normal myocardium and architecture in the control group (Fig. 6A). In contrast, the group treated with Dox had interstitial edema and chronic inflammation with focal distortion of the myocardial fibers (Fig. 6B). The group treated with Dox and LDios had residual interstitial edema, but only minimal chronic inflammatory cell infiltration (Fig. 6C). The group treated with Dox and HDios exhibited no residual inflammation, necrosis, or edema (Fig. 6D).

4. Discussion

Chemotherapeutic agents, such as Dox, are often used to treat a variety of cancers, including those of the stomach, ovary, breast, and thyroid and pediatric cancers. However, long-term Dox treatment may have detrimental effects on healthy tissues, such as the kidney, liver, and heart, and this severely limits its use in clinical settings (Carvalho et al., 2009; Wenningmann et al., 2019; Damodar et al., 2014; Ayla et al., 2011). According to the results of the present study, a single injection of Dox led to the onset of cardiac hypertrophy, that was demosntrated by histological examinations, HW:BW ratio, and the induction of hypertrophic gene markers (i.e., β-MHC and BNP). Furthermore, we found that rats that were treated with Dox exhibited cardiac injury, which led to a significant increase in the release of cardiac enzymes, including CK-MB and cTnI, into the bloodstream due to damaged cardiomyocytes; this is considered to be an important indicator of cardiac damage. Our findings were in line with those of previously published studies (Guo et al., 2020). Consequently, our study showed the attenuation of serum levels of CK-MB and cTnI by diosmin pretreatment in a dose-dependent manner (Senthamizhselvan et al., 2014; Sabarimuthu et al., 2017).

The existing literature has suggested the involvement of reactive oxygen species (ROS) and lipid peroxidation in cardiotoxicity caused by Dox (Xu et al., 2001; Simůnek et al., 2009; Rawat et al., 2021). In the current study, we analyzed lipid peroxidation markers through MDA analysis. Several previously published reports have highlighted elevated MDA levels in rats treated with Dox (Zhao et al., 2018; Erdogmus Ozgen et al., 2022). We also observed a significantly higher MDA content in the group treated with Dox compared with the control group; however, the

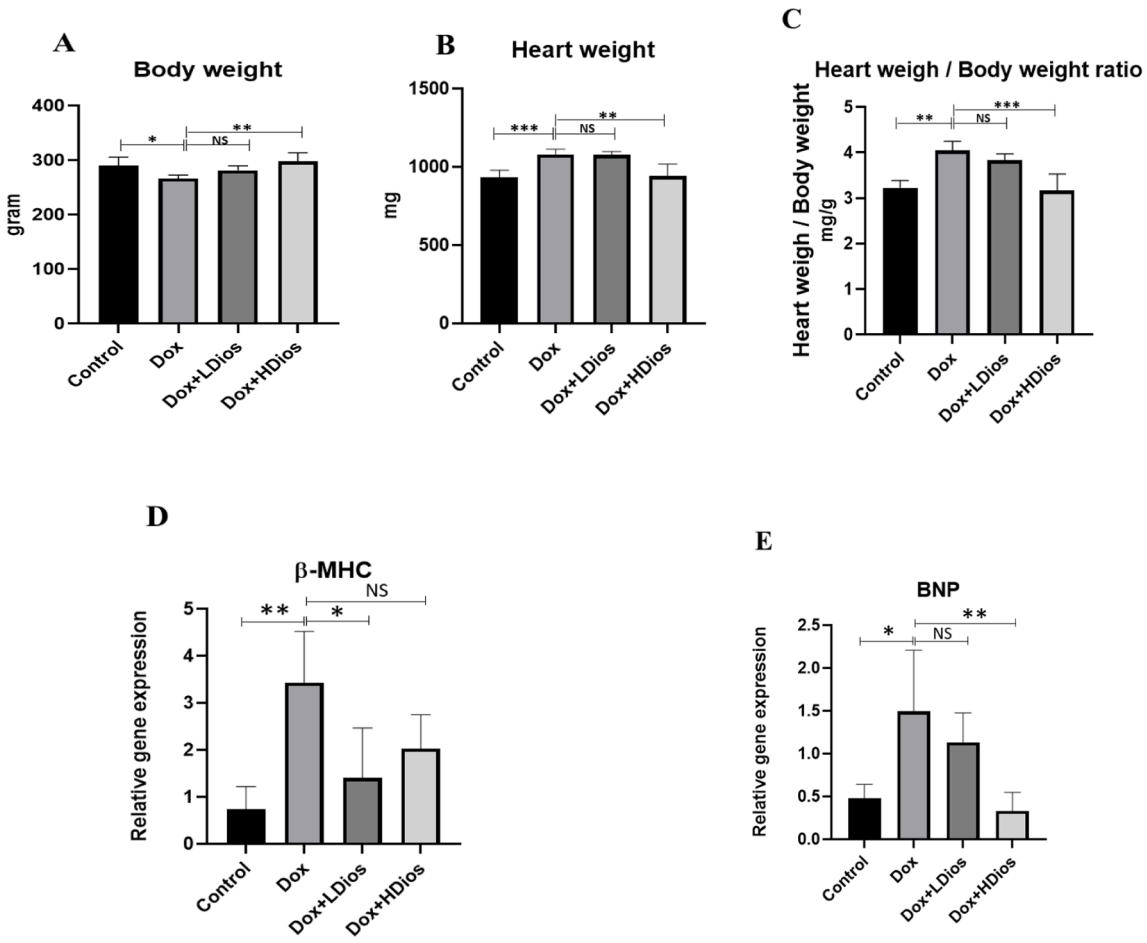

Fig. 5. Diosmin modulated Dox-induced cardiac hypertrophy. (A) represents the effects of drug treatments on body weight. (B) represents the effects of drug treatments on heart weight (C) represents the effects of drug treatments on heart weight and body weight ratio. (D) represents the effects on β-MHC. (E) represents the effects on BNP. Where NS: Nonsignificant ($p > 0.05$), *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$ ($n = 5$).

group that was pretreated with diosmin had a significantly lowered MDA content. Glutathione (GSH) is an endogenous substance crucial for detoxifying reactive oxygen species (ROS) generated by internal or external stimuli. This process is essential for maintaining the homeostasis necessary for normal cellular function. Catalase (CAT) is an enzyme renowned for its antioxidant activity, as it decomposes hydrogen peroxide (H_2O_2) into oxygen (O_2) and water (H_2O). This enzymatic reaction serves as a vital preventive mechanism against ROS production (Ali et al., 2021; Mohan et al., 2010; Rašković et al., 2011). Our current study showed significantly lower GSH content and lower CAT activity in rats exposed to Dox. Our findings were in agreement with those of Olorundare and colleagues (Olorundare et al., 2020). These changes were mitigated by diosmin pretreatment, suggesting that diosmin protects cardiac tissue from oxidative damage through its antioxidative properties.

Published reports suggest that oxidative stress has the capability of triggering inflammatory responses by activating signaling pathways such as NF-kB (Farag et al., 2021; CDC, 2023). Moreover, inflammation has been reported in Dox-induced cardiotoxicity (Wang et al., 2021). NF-kB is a group of transcription factors that are activated to control the expression of a wide range of genes that play important roles in many aspects of immunological and inflammatory responses (Imam et al., 2018). Several studies have reported the role of NF-kB activation in Dox-induced cardiotoxicity (Munir et al., 2023; El-Agamy et al., 2019). The current study reported an elevated level of NF-kB following a single Dox injection and the mitigation of this elevation by diosmin co-administration. Our findings were in support of published reports that suggested the ability of naturally occurring compounds to inhibit the activity of NF-kB and reduce cardiotoxicity (Qi et al., 2020; Munir et al., 2023). Furthermore, NF-kB has been reported to be a regulator of inflammation-related signaling cascades, such as COX-2, TNF-α, IL-6, and iNOS (Alanazi et al., 2020). Published data have shown the upregulation of COX-2, IL-6, iNOS, and TNF-α in Dox-treated animals (Alanazi et al., 2020; Ekinci Akdemir et al., 2021). In accordance with the published data, the current study demonstrated that a single injection of Dox led to an increase in the protein expression of TLR-4, IL-6, COX-2, iNOS, and TNF-α. These alterations in protein expression were significantly blunted by the prophylactic administration of diosmin. Therefore, our results suggested that this protection might be due to the anti-inflammatory properties of diosmin.

These results were supported by the histopathological analysis of cardiac tissue. Previously published reports have stated that Dox treatment induced alterations in the cardiac architecture (Ekinci Akdemir et al., 2021). In the current study, we observed the presence of parallel and normal myocardial fibers with detectable cross-striations in the control group, but in the Dox-treated group, the presence of interstitial edema and inflammation with focal distortion of myocardial fibers were seen. Prophylactic treatment with diosmin caused a decrease in interstitial edema with minimal inflammatory cell infiltration.

5. Conclusion

Our research findings indicate that diosmin has a significantly protective effect against cardiac damage that is caused by Dox

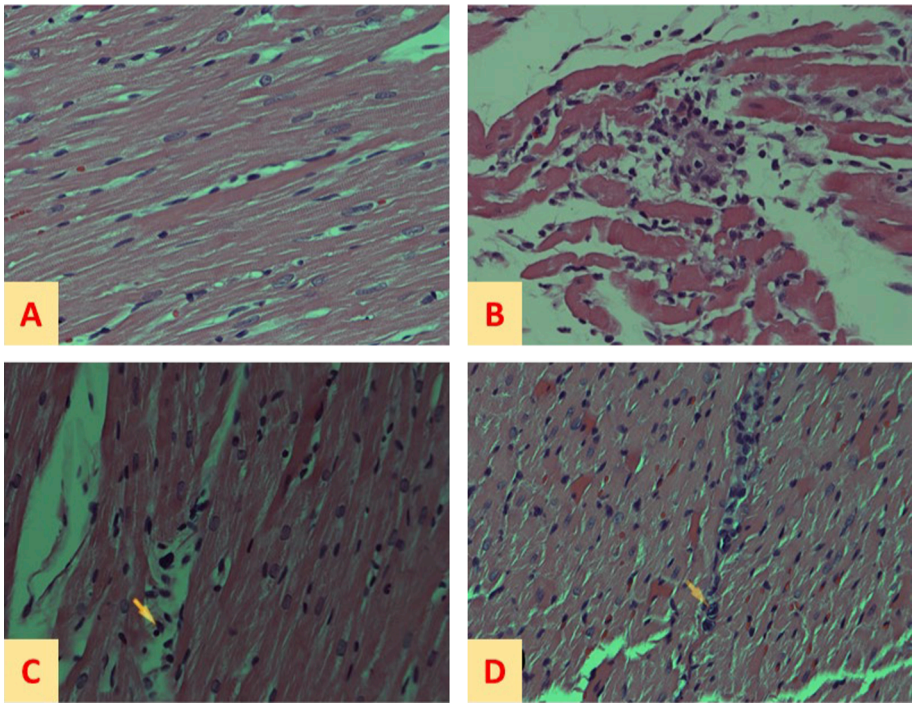

Fig. 6. Diosmin modulated Dox-induced alteration in histology. (A) Section of myocardium obtained from the control group shows the presence of normal architecture.. (B) Dox treated group shows the presence of interstitial edema and chronic inflammation with focal distortion of myocardial fibres. (C) Dox and LDios group shows residual interstitial oedema with minimal chronic inflammatory cell infiltration. (D) Dox and HDios group shows No residual inflammation, necrosis, or oedema. 400× magnification was used to capture the images.

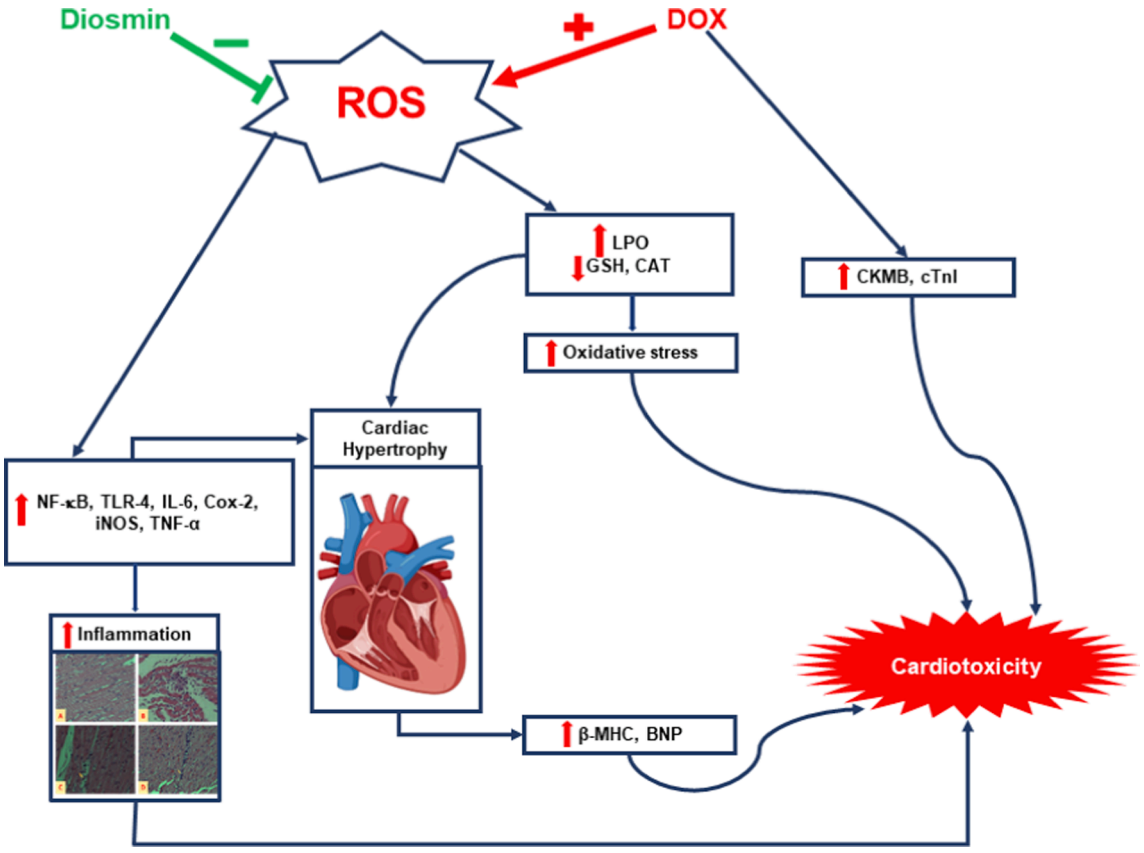

Fig. 7. Schematic representation of cardiotoxicity mechanism of Dox and the protective effects of diosmin.

administration. This was evidenced by improved cardiac function, reduced oxidative stress, decreased levels of inflammatory proteins, decreased expression of hypertrophic genes, and histopathological improvement (Fig. 7). The potentially protective mechanism may be partially related to the suppression of oxidative stress and the inflammatory process.

CRediT authorship contribution statement

Abdullah F. AlAsmari: Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mohammed M. Al-Shehri:** Writing – review & editing, Methodology, Formal analysis. **Nasser Algarini:** Methodology, Data curation. **Nada A. Alasmari:** Writing – review & editing, Formal analysis, Data curation. **Alabid Alhazmi:** Writing – review & editing, Visualization, Software, Methodology. **Mohammed AlSwayyed:** Software, Methodology, Data curation. **Metab Alharbi:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Fawaz Alasmari:** Writing – review & editing, Validation, Supervision, Methodology, Data curation. **Nemat Ali:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors acknowledge and extend their appreciation to the Researchers Supporting Project Number (RSPD2024R940), King Saud University, Riyadh, Saudi Arabia for funding this study.

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