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Nutraceuticals in cardiovascular diseases and their associated risk conditions

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Nutraceuticals in cardiovascular diseases and their associated risk conditions

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Editorial: Nutraceuticals in cardiovascular diseases and their associated risk conditions

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KEYWORDS

cardiovascular diseases, inflammation, nutraceuticals, oxidative stress, comorbidity, cardioprotection

Editorial on the Research Topic
Nutraceuticals in cardiovascular diseases and their associated risk conditions

Cardiovascular diseases and their associated risk conditions including dyslipidemia, hypertension, coronary heart disease and diabetes are one of the main health issues worldwide. The mechanisms underlying cardiovascular disorders are complex and multifactorial including oxidative stress, inflammation and mitochondrial dysfunction as well as modulating the activities of several kinases and phosphatases. Importantly, pharmacotherapies available for the management of these diseases are considered insufficient and show several limitations and side effects especially in high-risk patients.

Nutraceuticals are bioactive food components or phytochemicals that provide benefits including the prevention or treatment of several diseases. Nutraceuticals such as flavonoids, vitamins and other natural substances have shown pleiotropic antioxidant and anti-inflammatory properties. These natural components enriched in dietary substances are believed to target multiple pathways in slow and effective ways without causing significant side effects. Current evidence potentiates the application of nutraceuticals to enhance the efficacy of pharmacological therapies and to reduce their adverse effects. A number of studies have shown that cardiovascular diseases are strongly correlated with dietary habits of patients. However, limited studies are available about the use nutraceuticals in cardiovascular diseases, their long-term safety and effectiveness especially in clinical trials and thus more research is required in this direction. This issue was designed to shed more light on the use of nutraceuticals for the management of cardiovascular diseases and their associated risk conditions with emphasis on their protective potentials in both experimental and clinical studies and the underlying mechanisms.

Hyperactivation of blood platelets leads to various thrombotic events, including embolies, thrombotic stroke, etc., and could be influenced by various dietary components from vegetables, fruits, teas, wines, cocoa and its products. The cocoa products including chocolate and coffee are very widespread in the world and their cardioprotective function and interaction with other drugs regarding hypercoagulation and bleeding are of special interest. Olas examined the impact cocoa and its products, including chocolate, on the number and function of thrombocytes, and the anti-platelet activity of their phenolic compounds. In conclusion, it was shown that cocoa

consumption, especially in the form of dark chocolate, mostly due to the high flavanol content, had anti-platelet activity and might play a significant role in cardioprotection. Thus, the high cocoa products consumption may be an excellent strategy in primary prevention of cardiovascular events through decreasing cardiovascular risk, including hyperactivation of thrombocytes.

Coronary and systemic atherosclerosis is a significant cause of cardiovascular and cerebrovascular diseases, with a greater impact on men than women. Wang and Wang investigated the association between dietary antioxidants intake and the ankle brachial pressure index (ABPI), the marker of arterial compliance and endothelial dysfunction which could be also used for assessing the progression of atherosclerosis. In this cross-sectional designed study with more than thousand participants from the NHANES survey (USA), the six antioxidants (selenium, carotenoids, zinc, and vitamins A, C, E) and a composite dietary antioxidant index (CDAI) were investigated as risk factors of ABPI. The multiple regression analysis revealed that among men but not women, the dietary intake of selenium, zinc, and vitamin A was positively associated with a higher ABPI and between CDAI and ABPI, a U-shaped association was presented. The important issue was also that CDAI, high vitamin A, C, and E intake had a direct effect on all-cause mortality. In this regard, there was a significant interaction between high intake of vitamin A and gender, with a superior effect for women. As a result, the intake of dietary nutrients with antioxidant properties may prevent vascular atherosclerosis and improve vascular compliance in a sex-dependent manner.

Carnosine is an antioxidant and an endogenous dipeptide that has been widely assessed as a protective agent in various studies. A study by Berdaweel et al. revealed the antifibrotic potential of L-carnosine and its ability to mitigate the adverse cardiac remodeling in an experimental mouse model that mimics pathophysiological basis of type II diabetes and metabolic diseases in patients. Either wild type or GPx4+/−mice were exposed to high sucrose high fat diet (HSHF) for 16 weeks. Carnosine treatment moderately improved glycemic control with non-significant effect on insulin resistance. Interestingly, carnosine significantly upregulated cardiac GPx4 expression and reduced levels of iron and protein carbonyls in all genotypes exposed to HSHF diet. Furthermore, *in vitro* experiments demonstrated the carnosine's ability to inhibit crosslinking of collagen. In summary, this research provides a valuable information regarding the antifibrotic potential of carnosine and the possible underlying mechanisms.

Cafestol is a natural diterpene that presents in coffee. It shows distinctive anti-inflammatory and antioxidant in addition to antimutagenic properties in various investigations. A study by Al-Kenany and Al-Shawi provided data about the obvious protective effect of cafestol in cardiotoxicity induced by doxorubicin in rats. Cafestol significantly improved cardiac injury induced by doxorubicin and improved histopathological changes through inhibition of cardiac oxidative stress and enhancement of Nrf2 expression and the associated downstream antioxidant genes. Moreover, cafestol significantly inhibited inflammatory markers (IL-1β and TNF-α) and cardiac apoptosis (TUNEL-positive cardiomyocytes, Bax, and Casp 3). These outcomes reveal the potential of cafestol use as an adjuvant to chemotherapy to alleviate doxorubicin-induced toxicities.

Therefore, the current special issue revealed the safety and efficacy of nutraceuticals such as cocoa products, dietary composite antioxidants, carnosine, cafestol, etc. in various cardiovascular diseases. We aim that this research will motivate more experimental and clinical studies, providing new directions for the early prevention and management of various cardiovascular conditions.

Author contributions

LA: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. NZ: Supervision, Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Protective effect of cafestol against doxorubicin-induced cardiotoxicity in rats by activating the Nrf2 pathway

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Doxorubicin (DOX) is an efficient antineoplastic agent with a broad antitumor spectrum; however, doxorubicin-associated cardiotoxic adverse effect through oxidative damage and apoptosis limits its clinical application. Cafestol (Caf) is a naturally occurring diterpene in unfiltered coffee with unique antioxidant, antimutagenic, and anti-inflammatory activities by activating the Nrf2 pathway. The present study aimed to investigate the potential chemoprotective effect of cafestol on DOX-induced cardiotoxicity in rats. Wistar albino rats of both sexes were administered cafestol (5 mg/kg/day) for 14 consecutive days by oral gavage alone or with doxorubicin which was injected as a single dose (15 mg/kg intraperitoneally at day 14) to induce toxicity. The result showed that Caf significantly improved cardiac injury induced by doxorubicin, decreased serum levels of CK-MB, LDH, ALP, and ALT, and improved histopathological changes. In addition, cafestol significantly inhibited DOX-induced cardiac oxidative stress as seen in the reduced level of MDA and increased GSH, SOD, CAT, and Gpx-1 cardiac tissue levels; cafestol significantly enhanced Nrf2 gene and protein expression and promoted the expression of downstream antioxidant genes HO-1 and NQO-1 and downregulated Keap1 and NF-κB genes' expression; in addition, Caf significantly reduced inflammatory mediators, TNF-α, and IL-1β levels and inhibited cardiac apoptosis by modulating Bax and Casp 3 tissue levels and reduced TUNEL-positive cardiomyocytes. In conclusion, the present study confirmed that cafestol improved the cardiotoxic effects induced by doxorubicin through the regulation of apoptosis and oxidative stress response through the Nrf2 pathway; this study suggests that cafestol may serve as a potential adjuvant in chemotherapy to alleviate DOX-induced toxicities.

KEYWORDS

doxorubicin, cafestol, oxidative stress, Nrf2 pathway, cardiotoxicity

1 Introduction

Doxorubicin (DOX), known as "adriamycin," is an important member of the anthracycline antibiotics that has a broad antitumor spectrum, where it is used worldwide in the treatment of many human malignant cancers as a component of various chemotherapeutic regimens (Sritharan and Sivalingam, 2021). Antitumor activity of doxorubicin, through direct DNA damage, by interfering with the function of many enzymes necessary for DNA replication (Chen, Tzu, Wu, Yan, Chung, Yu, Hwu, Yeukuang, Cheng, Hsun, Mou, Yuan, 2012) (Nebigil and Laurent, 2018) in addition to oxidative stress and overproduction of free radicals, which deplete antioxidants and increase lipid

peroxidation, impacts not only cancerous cells but also normal cells leading to toxicity (Quiles et al., 2002) (Swift et al., 2006) (Sirwi et al., 2022). As such, doxorubicin causes severe cardiotoxicity, which is dose-dependent; often results in systolic cardiac dysfunction, dilated cardiomyopathy, and, in extreme cases, heart failure, which has a substantial negative impact on the patient's health; and poses a significant hurdle in doxorubicin clinical application (Hitchcock-Bryan et al., 1986) (Karim et al., 2001).

The mechanism that mediates doxorubicin-induced cardiotoxicity is unclear, but it is thought to be associated with oxidative stress, inflammatory cascade disorder, autophagy, and apoptotic cell death, in which increased lipid peroxidation, disrupted Ca^{2+} homeostasis, and mitochondrial damage, might account for the leading cause of myocardial injury (Hajra et al., 2018) (Rocca et al., 2020) (Rawat et al., 2021). On the other hand, the nuclear factor erythroid 2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) antioxidant pathway activation displayed a protective effect against cardiovascular diseases, including hypertension, myocardial ischemia, and reperfusion myocardiopathy injury (Barančík et al., 2016). Therefore, studies have concentrated on investigating the possibility of decreasing doxorubicin-mediated cardiotoxicity through attenuating oxidative stress and inhibiting apoptosis via the endogenous antioxidant pathway activation (Dundar et al., 2016) (Gu et al., 2021). Although dexrazoxane is currently the sole chemoprotective agent to prevent and manage cardiotoxicity induced by doxorubicin, its use carries a substantial risk of inducing hematological abnormalities and myelosuppression (Reichardt et al., 2018) (Dallons et al., 2020).

Cafestol is a natural diterpene extracted from the lipid fraction of the coffee bean, which predominantly exists in unfiltered coffee as a fatty ester (Cruchten et al., 2010); it has been shown to counteract oxidative stress (Lee et al., 2007), has protective activity against genetic abnormalities as it prevents the early mutagenic events (Tao et al., 2008), and has an anti-inflammatory effect (Kim, Jung, and Jeong, 2004). Cafestol's antioxidant effects are related to its ability to activate the Nrf2/antioxidant response element (ARE) pathway, leading to the induction of the expression of antioxidant proteins and enzymes and inhibiting the expression of the inflammatory mediator (Hao et al., 2018); cafestol showed to inhibit high-glucose-mediated cardiac fibroblast proliferation and collagen synthesis by inhibiting proteasomal degradation of Nrf2 and promote its nuclear translocation, causing HO-1 expression upregulation, reducing ROS production, and inhibiting oxidative stress (LiuChen et al., 2020). Furthermore, cafestol exhibited to possess a protective effect against I/R (ischemia-reperfusion) injury by ameliorating inflammation, apoptosis, and autophagy (Ji et al., 2020). In addition, cafestol reduced inflammatory mediators' production induced by the cyclic strain in human umbilical vein endothelial cells (HUVECs) through activation of the Nrf2/HO-1 pathway and Sirt1 upregulation (Hao et al., 2018). Cafestol treatment might be a new approach to managing oxidative stress-mediated pathophysiological injury.

Therefore, the present study was designed to investigate cafestol's possible chemoprotective effect on doxorubicin-induced cardiotoxicity through the modulation of the Nrf2 pathway, inflammatory mediator production, and apoptosis inhibition in Wistar albino rats.

2 Materials and methods

2.1 Chemicals and materials

Cafestol (purity >98%, CAS no. 81760-48-7) was acquired from Sigma-Aldrich (St. Louis, MO 63103, United States). Doxorubicin (doxorubicin HCl 50 mg powder for injection, batch no. 30495, Khandelwal Labs, India) was purchased from local pharmacies. Polysorbate 20 (Tween-20) was purchased from Sinopharm Chemical Reagent Co., Ltd., China. All solvents and chemicals used were of analytical grade.

2.2 Dose selection, preparation, and mode of administration

Doxorubicin was dissolved in 0.9% normal saline, and a single dose of 15 mg/kg body weight was administered intraperitoneally based on its success in inducing cardiotoxicity in Wister rats (Sirwi et al., 2022).

Cafestol was prepared as a suspension using 5% Tween-20 in distilled water (D.W.). It was freshly prepared each day just before treatment and was administered orally by oral gavage as a single dose of 5 mg/kg body weight which was optimized based on the previously shown protective effect (Lee et al., 2007) and our preliminary experiment.

2.3 Animal care and experimental design

A total of 32 Wistar albino experimental rats of both sexes aged 6 weeks with an average weight of 150 g were used in this study; animals were acquired and maintained in the College of Pharmacy Experimental Animal House, University of Baghdad, Iraq. The experimental animals were kept under controlled conditions of a light/dark cycle (12 h) at a temperature of 23°C ± 2°C and humidity of 50% ± 5%. They had free access to standard commercial diet purchased from the local market and tap water *ad libitum*. The rats were acclimatized prior to the start of the experiment for 1 week. The study protocol was approved by the Graduate Studies and Ethics Committees of the College of Pharmacy, University of Baghdad.

Experimental animals were randomly assigned into four groups (n = 8) as follows:

Group I: each rat was given vehicle only (5% Tween in DDW) orally via oral gavage for 14 consecutive days. Then, a single dose of NaCl (0.9%), 10 mL/kg (Turner et al., 2011), was injected intraperitoneally 1 h after the last vehicle administration on day 14. This group served as the normal (negative control) group.

Group II: each rat was orally given cafestol (5 mg/kg/day) for 14 consecutive days.

Group III: each rat was given vehicle only (5% Tween in DDW) via oral gavage for 14 consecutive days. Then, a single dose of doxorubicin (15 mg/kg) was injected intraperitoneally 1 h after the last vehicle administration on day 14 to serve as the positive control group.

Group IV: each rat received cafestol (5 mg/kg/day) orally for 14 consecutive days, and then, a single dose of doxorubicin (15 mg/kg) was injected intraperitoneally 1 h after the last cafestol treatment on day 14.

2.4 Serum collection and tissue preparation

Twenty-four hours after doxorubicin dose administration (i.e., day 15), the animals were anaesthetized, and blood samples were collected from the jugular vein in non-heparinized tubes and were left to clot at room temperature. Then, the samples were centrifuged for 20 min at 4,000 rpm to obtain serum and stored at −20°C for biochemical analysis. The animals were euthanized by cervical dislocation, and the heart of each rat was excised and rinsed with cold PBS; then, a small section of the heart was homogenized with an electric homogenizer in cold saline (1: 10, w/v) on ice. Homogenates were then centrifuged at 4°C for 10 min at 12,000 rpm, and the supernatants were preserved at −20°C for further analysis. Another slice of the cardiac tissue was fixed in 10% buffered formalin for histopathological examination.

2.5 Estimation of cardiac injury markers

Creatine kinase myocardial band (CK-MB) and lactate dehydrogenase (LDH) serum levels and other toxicity markers, including ALT, AST, and ALP activities, were estimated spectrophotometrically by HumaReader HS (Human, Germany) using available commercial kits (Linear Chemicals, Barcelona, Spain).

2.6 Assessment of oxidative stress and inflammatory markers

The supernatant obtained from homogenized heart tissue was used to assess the oxidative/antioxidant status by measuring malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) levels; in addition, inflammatory biomarkers including interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) levels were determined using commercially available ELISA kits obtained from MyBioSource (San Diego, United States).

2.7 Assessment of cardiac apoptosis markers

The activity of caspase 3 and Bax was assessed using ELISA kits obtained from MyBioSource (San Diego, United States), in accordance with the manufacturer's instructions.

2.8 Real-time quantitative reverse transcription polymerase chain reaction analysis

Following the manufacturer's protocol, total RNA was extracted from heart tissues using the TransZol Up Plus RNA Kit (TransGen Biotech, China). The purity of RNA was determined by 260/280 ratio using NanoDrop 2000c (Thermo Fisher Scientific, United States). cDNA was synthesized using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen, China), and quantification of mRNA using qPCR was carried out utilizing TransStart® Top Green qPCR SuperMix (TransGen Biotech, China) with forward and reverse primers on rotor gene Q, Qiagen. The relative changes in mRNA expression normalized to the GAPDH level (housekeeping gene) for every gene were calculated using the ΔΔCt method ($2^{-\Delta\Delta CT}$), also known as the "Livak method". Primer sequences were designed using "Primer Quest" (IDT, United States); the primer sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase (NQO-1), Kelch-like ECH-associated protein 1 (Keap1), and nuclear factor kappa B (NF-κB) are shown in Supplementary Table S1. Primers were purchased from Alpha DNA, Canada.

2.9 Western blot analysis

The heart tissues were washed with cold PBS and lysed using a strong RIPA lysis buffer in the presence of protease phosphatase inhibitor cocktail (cat. no. E-BC-R327; Elabscience Technologies, Inc.). The protein concentration was determined using the BCA Protein Assay Kit (cat. no. E-BC-K318; Elabscience Technologies, Inc.). Aliquots of 30 μg of protein from each sample were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Bio-Rad, United States). After blocking with 5% skim milk for 90 min, membranes were incubated with specific antibodies, namely, anti-Nrf2 polyclonal antibody (MyBioSource: MBS8001991, 1:1,000), anti-HO-1 (MyBioSource: MBS2538072, 1: 1,000), and B-actin (Elabscience: E-AB-40517, 1:1,000) overnight at 4°C, followed by incubation with the secondary antibody, namely, HRP-conjugated anti-rabbit IgG at 1:1,000 dilution (Elabscience: E-AB-1003) for 1 h at room temperature. Antibody binding with the protein of interest was detected with chemiluminescence (ECL, Bio-Rad, United States). Quantification was performed by densitometry. The results were normalized to beta-actin.

2.10 Histopathological cardiac damage assessment

Fixed 10% formalin heart tissues were dehydrated with ethanol and embedded in paraffin; the tissues were cut into ~5 μm thick sections, then stained with hematoxylin and eosin (H&E) stain and examined under a light microscopic. The images were scored semiquantitatively depending on the extent of necrosis of the cardiac tissue and inflammatory cell infiltration following the scoring scale: score 0, normal; score 1, damage up to 25%; score 2, between 25% and 50%; score 3, between 50% and 75%; and score 4, above 75% (Kanda et al., 2004); the examination was carried out by a senior pathologist, blinded to the study.

2.11 *In situ* cell death (apoptosis)/TdT-mediated dUTP-biotin nick end labeling assay

Cellular apoptosis in cardiac tissue was determined at the nuclear level using the One-Step TUNEL *In Situ* Apoptosis Kit

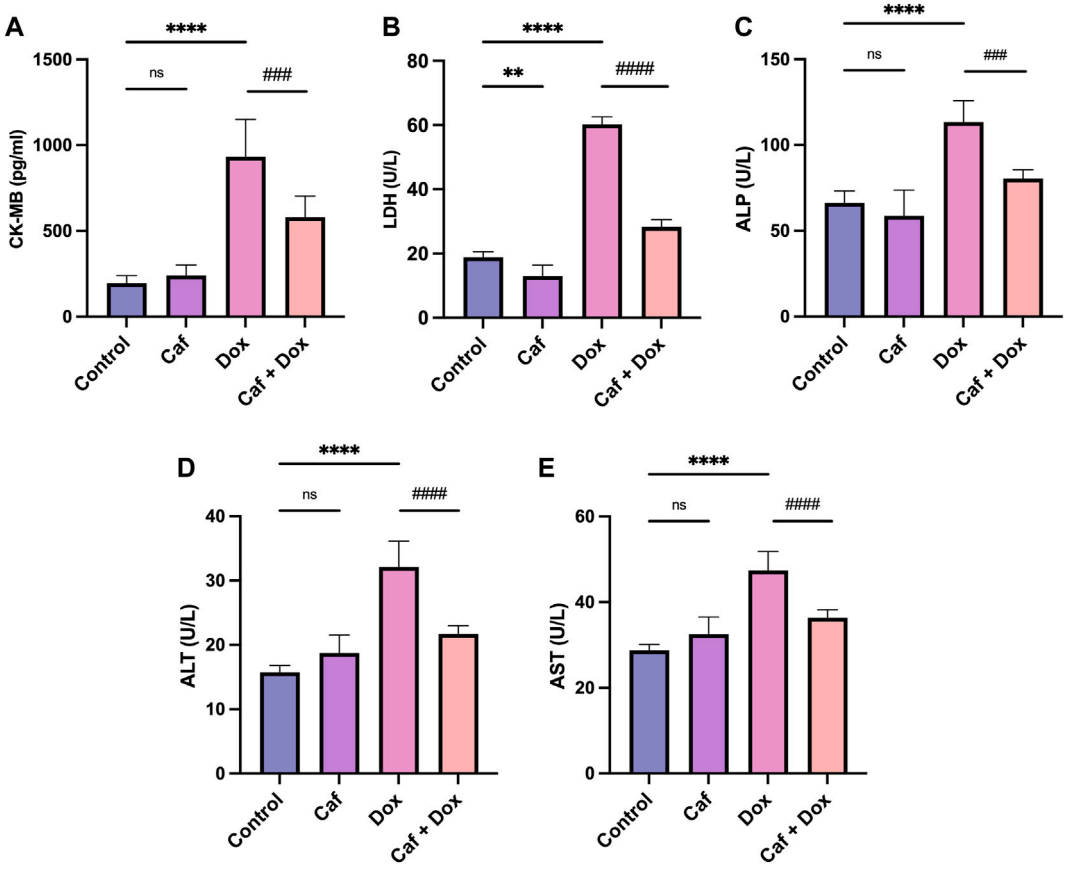

FIGURE 1
Effect of cafestol on cardiac tissue injury markers in Wister rats. **(A)** Effect of cafestol on CK-MB serum levels. **(B)** Effect of cafestol on LDH serum levels. **(C)** Effect of cafestol on ALP serum levels. **(D)** Effect of cafestol on ALT serum levels. **(E)** Effect of cafestol on AST serum levels. Data are expressed as mean ± SD, $n = 8$. **, $p < 0.01$; ****, $p < 0.0001$ vs. control group; ###, $p < 0.001$; ####, $p < 0.0001$ vs. DOX group; ns ($p > 0.05$), no significant difference.

(green, FITC) (Cat No: E-CK-A320, Elabscience, United States), in accordance with the manufacturer's protocol. TUNEL-positive cells were detected under a fluorescence microscope, and photomicrographs were taken at ×1000 magnification. For TUNEL quantification, seven selected fields in each slide were examined, and the mean and the percentage were calculated; the examination was carried out by a senior pathologist who was blinded to the study. The apoptosis indicator (apoptotic index) was estimated as the fraction (%) of the labeled nuclei to the total number of nuclei counted.

2.12 Statistical analysis

The data of this study were demonstrated as mean ± standard deviation (SD), RT-qPCR data were expressed as mean ± standard error of mean (SEM), and the statistical significance among groups was determined using the one-way analysis of variance (ANOVA) test followed by Tukey's *post hoc* test for multiple comparisons between groups using GraphPad Prism software (version 9.5.0). *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; ###, $p < 0.001$; and ####, $p < 0.0001$ values across all figures were statistically significant.

3 Results

3.1 Effect of cafestol on cardiac injury marker levels and histopathological changes

Figure 1 shows that IP-administered DOX alone (group III) caused cardiac injury, as seen in the significant ($p < 0.0001$) elevated serum levels of CK-MB, LDH, AST, ALT, and ALP compared to the control group (group I). However, pre-treatment with cafestol at 5 mg/kg b.w. prior to DOX exposure (group IV) showed a significant reduction in the measured parameters' serum levels compared to the DOX-only group, which indicated that cafestol has a protective role against DOX-induced cardiac injury.

Consistently, in Figures 2A, B, the rats' heart tissues exposed to DOX alone (group III) showed a significantly higher ($p < 0.05$) cardiac pathological score as characterized by the myocardial tissue architectural disturbance (fragmentation and degenerated wavy myofibers with absent striations), necrosis, and inflammatory cell infiltration than the control group (group I), which showed normal myocardial structure (branching and anastomosing cardiac myofibers) with no necrosis or infiltration with inflammatory cells. However, pre-treatment with cafestol (group IV) maintained cardiac myofiber organization with less

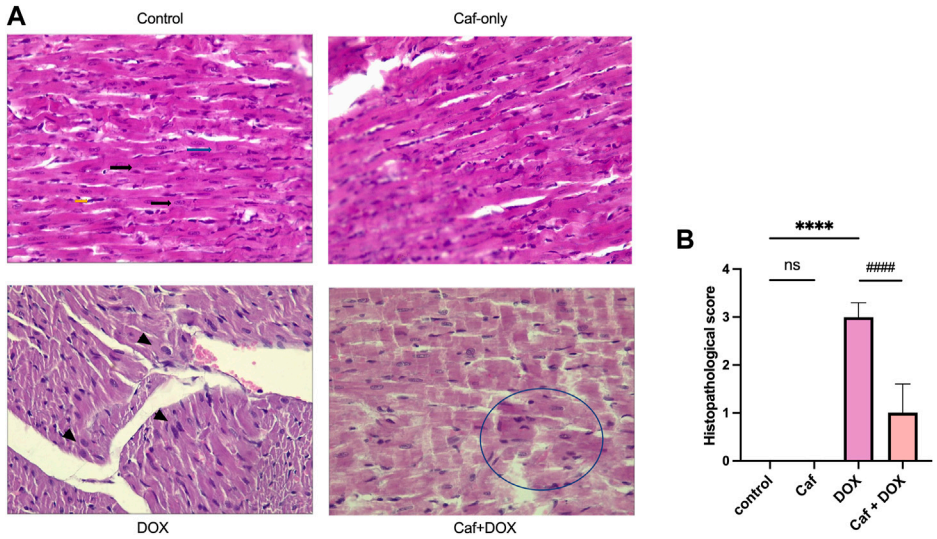

FIGURE 2
Effect of cafestol on cardiac tissue histopathological change in Wister rats. **(A)** Photomicrographs showing the pathological morphology of myocardial tissue (H&E ×400); the control group has normal histology of longitudinally oriented cardio-myofibers from the left ventricle of the rat, with large oval nuclei containing granular chromatin (black arrows) and with faint intercalated discs that are also well demonstrated (blue arrows). The typical branched appearance of the cardiac muscle is also well preserved (yellow arrows). The DOX group showed dilated cardiomyopathy and myocardial necrosis within the longitudinal section in a rat's heart, manifested as areas of pallor in the myocardium with loss of cellular details, and myocardium coalesces, with large nuclei starting to appear (arrowhead). In the caf group, cardiac tissue myofibers are well preserved and appeared with normal architecture. In the caf + DOX group, cardiac tissue general architecture of the left ventricle showed reduced signs of myocardial damage with some losses of cellular architecture in the form of "merged" myofibers (encircled area). **(B)** Semiquantitative analysis of the histological score in the rat myocardium following the scale: score 0, normal; score 1, lesion up to 25%; score 2, between 25% and 50%; score 3, between 50% and 75%; and score 4, above 75%; seven selected fields in each slide were examined for necrosis and inflammatory cells infiltration. Data are expressed as mean ± SD, $n = 8$. ****, $p < 0.0001$ vs. control group; ####, $p < 0.0001$ vs. DOX group; ns ($p > 0.05$), no significant difference.

myofibrillar loss (Figure 2A); additionally, cafestol pre-treatment at 5 mg/kg b.w. showed significantly ($p < 0.05$) lower cardiac semiquantitative injury scores than group III as demonstrated in Figure 2B. Notably, cafestol alone (group II) showed comparable changes in the levels of cardiac injury markers, as exhibited by the control group (group I), with no significant difference (Figure 1). In addition, cafestol alone did not cause histopathological changes in the cardiac tissue with an appearance comparable to that of the control group (Figure 2A), with a cardiac pathological score similar to that exhibited by the control group (Figure 2B), indicating that cafestol at 5 mg/kg b.w. is safe and has no toxic effect.

3.2 Effect of cafestol on cardiac oxidative stress

The cardiac antioxidant activity of cafestol was assessed in rats following acute DOX exposure, as oxidative stress is important in DOX-mediated myocardial damage. Administration of DOX alone (group III) caused a highly significant increase ($p < 0.0001$) in the MDA level compared to the control group (group I); however, pre-treatment with cafestol (group IV) significantly ameliorated the increase in the MDA level (Figure 3A). On the other hand, DOX alone (group III) caused a significant GSH depletion and SOD, CAT, and GPx-1 exhaustion compared to the control group (group I), while pre-treatment with cafestol (group IV) significantly increased the levels of GSH and restored SOD, CAT, and GPx-1 activity in comparison to the DOX group (group III) (Figures 3B–E). Furthermore, cafestol alone (group II) caused no significant difference in the MDA level ($p > 0.05$) Figure 3A and significantly increased the SOD tissue level when compared to the control group (group I) Figure 3C.

3.3 Effect of cafestol on cardiac inflammatory marker levels and NF-κB gene expression

Inflammation is one of the leading triggers of cardiomyocyte death and the subsequent cardiac dysfunction; for this, the proinflammatory cytokines' (IL-1β and TNF-α) cardiac tissue levels and NF-κB gene expression were analyzed. Figures 4A, B show that there was a significant increase ($p < 0.0001$) in the cardiac tissue level of TNF-α and IL-1β in the DOX group compared to the normal control group. Furthermore, the expression of the NF-κB gene in the cardiac tissue significantly increased ($p < 0.001$) in the model group (group III), as shown in Figure 4C. However, cafestol pre-treatment in group IV significantly reduced cardiac tissue levels of TNF-α and IL-1β and inhibited the expression of the NF-κB gene compared to the model group (group III). Cafestol alone (group II) showed comparable cardiac levels of TNF-α, IL-1β, and NF-κB gene expression to those of the normal control group (group I).

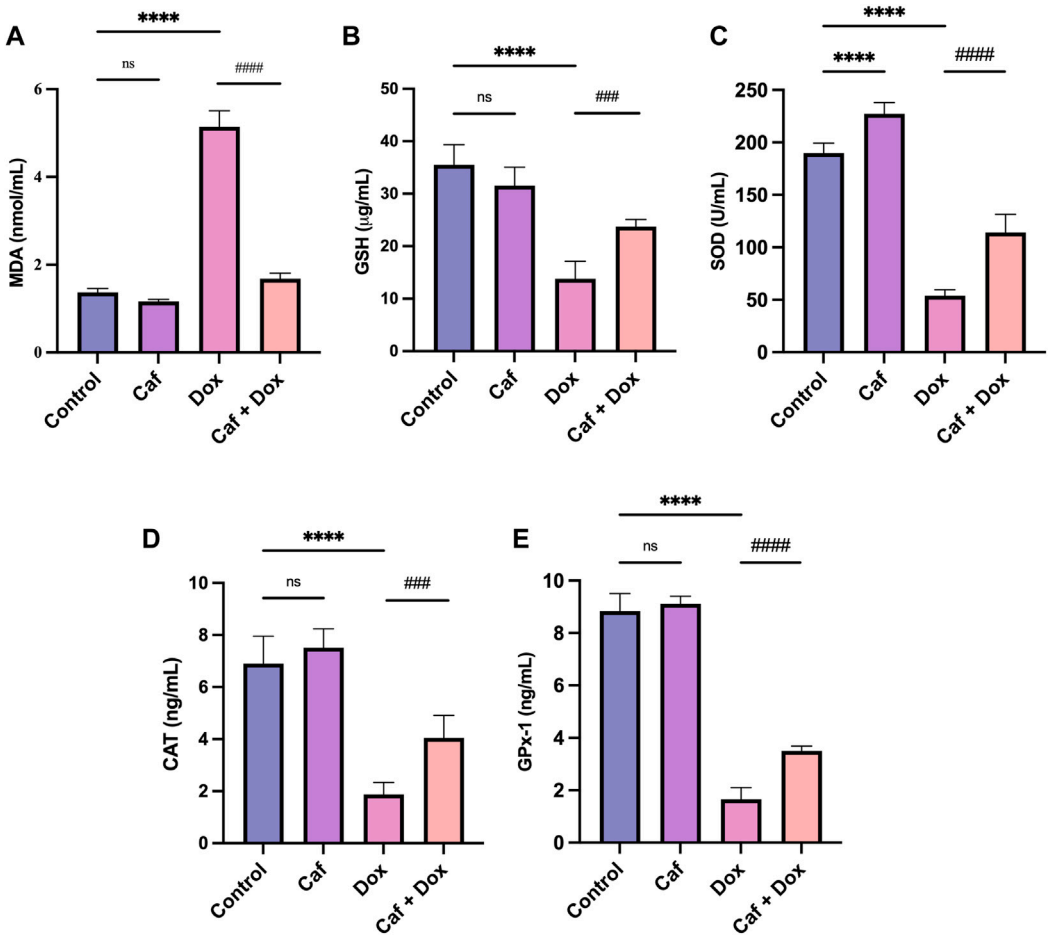

FIGURE 3
Effect of cafestol on cardiac tissue oxidative stress markers in Wister rats. **(A)** Effect of cafestol on MDA. **(B)** Effect of cafestol on GSH. **(C)** Effect of cafestol on SOD. **(D)** Effect of cafestol on CAT. **(E)** Effect of cafestol on GPx-1. Data are expressed as mean ± SD, $n = 8$. ****, $p < 0.0001$ vs. control group; ###, $p < 0.001$; ####, $p < 0.0001$ vs. DOX group; ns ($p > 0.05$), no significant difference.

3.4 Effect of cafestol on cardiomyocyte apoptosis

Apoptotic cell death of the cardiomyocytes is a causative factor of heart failure following the use of doxorubicin; thus, we examined the cardiac tissue levels of both caspase 3 and Bax. As shown in Figures 5A, B, there was a significant increase ($p < 0.0001$) in the cardiac tissue levels of both caspase 3 and Bax in the model group (group III) compared to the control group (group I); however, pre-treatment with cafestol prior to acute exposure to DOX (group IV) induced a significant inhibition in the apoptotic markers compared to the model group, while cafestol alone (group II) has no significant effect on Casp 3 and Bax cardiac tissue levels compared to the control group.

On the other hand, the evaluation of apoptotic cells at the nuclear level using the TUNEL assay (TUNEL-positive cardiomyocytes) (Figure 5C) showed that acute exposure to DOX (group III) induced an increase in the appearance of TUNEL-positive cardiomyocytes in the form of hyperchromatic patches of bright green (indicating DNA fragmentation along adjacent rows of cells) with a significantly higher ($p < 0.0001$) apoptosis rate (Figure 5D) than the control group. However, pre-treatment with cafestol reduced the number of TUNEL-positive cardiomyocytes (seen as scattered apoptotic cells) and significantly reduced the apoptosis rate compared to the model group. However, cafestol alone (group II) showed a TUNEL-positive cardiomyocyte appearance comparable to that of the control group with no significant difference ($p > 0.05$) in the apoptotic index in comparison to the control group.

3.5 Effect of cafestol on the Nrf2 pathway and its downstream antioxidant genes

To explain the mechanisms underlying the cardioprotective effects of cafestol pre-treatment, we assessed the effects of cafestol on Nrf2, Keap1, HO-1, and NQO-1 gene expression in myocardial tissue. Figure 6 shows that the Nrf2 and HO-1 genes' expression were significantly decreased ($p < 0.0001$) and ($p < 0.01$), respectively, and there was a significant increase ($p < 0.01$) in the expression of the Keap1 gene in the model group (group III) compared to group I. In addition, doxorubicin alone caused a

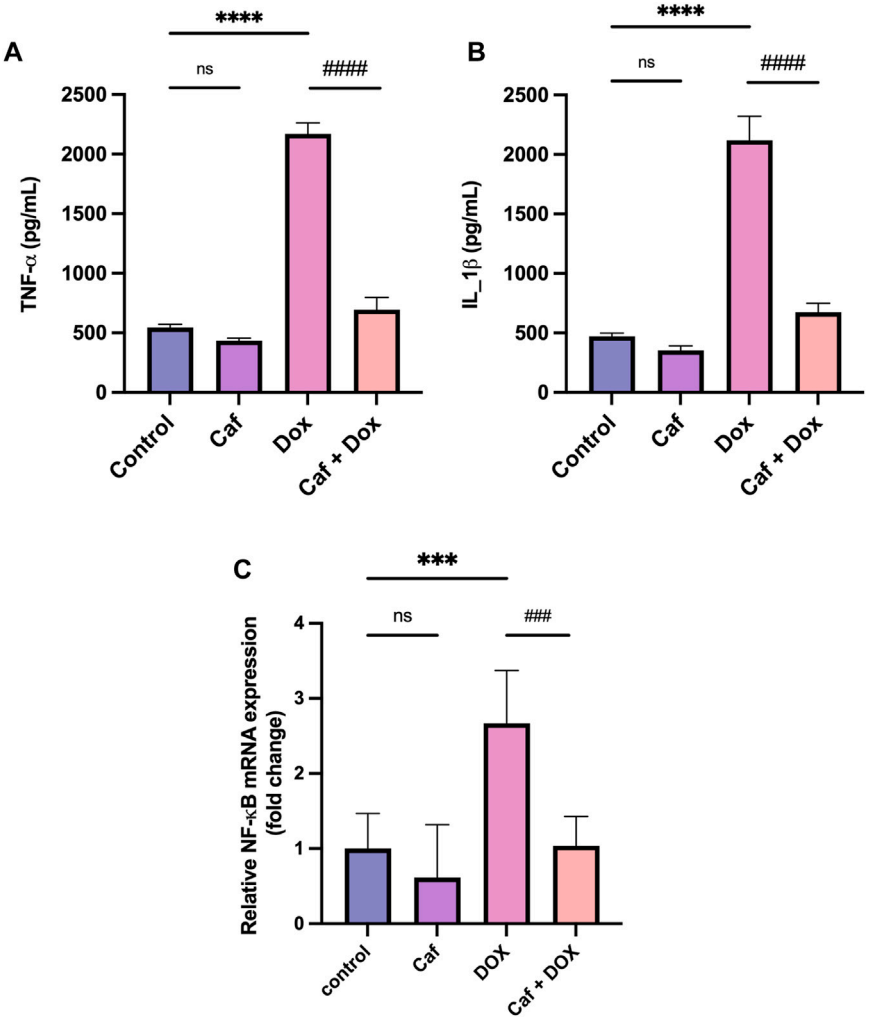

FIGURE 4
Effect of cafestol on cardiac tissue inflammatory markers in Wister rats. **(A)** Effect of cafestol on TNF-α. **(B)** Effect of cafestol on IL-1β. Data are expressed as mean ± SD, $n = 8$. **(C)** Effect of cafestol on NF-κB. Data are expressed as mean ± SEM, $n = 8$. ***, $p < 0.001$; ****, $p < 0.0001$ vs. control group; ###, $p < 0.001$; ####, $p < 0.0001$ vs. DOX group; ns ($p > 0.05$), no significant difference.

non-significant change in the NQO-1 gene expression in comparison to group I (control group).

However, the mRNA expression levels of Nrf2, HO-1, and NQO-1 in group IV (cafestol pre-treatment + DOX) were significantly higher ($p < 0.0001$) than those in group III (doxorubicin only); in addition, cafestol pre-treatment significantly reduced ($p < 0.0001$) the cardiac tissue gene expression of Keap1 compared to the model group. On the other hand, cafestol alone (group II) significantly increased cardiac tissue mRNA levels of Nrf2, HO-1, and NQO-1 with no significant effect on Keap1 levels compared to the control group.

In addition, the effect of cafestol on Nrf2 protein expression was examined in the rats' heart tissue; Figure 7A reveals that group III (acute exposure to doxorubicin) exhibited a significantly reduced ($p < 0.05$) Nrf2 protein expression; in contrast, cafestol pre-treatment prior to DOX exposure (group IV) significantly increased ($p < 0.01$) the Nrf2 protein expression in the heart tissue. Moreover, the administration of cafestol alone (group II) increased the cardiac tissue Nrf2 protein expression significantly ($p < 0.05$).

For cardiac tissue HO-1 protein levels, as demonstrated in Figure 7B, the exposure to doxorubicin (group III) caused a significant reduction ($p < 0.05$) in HO-1 protein expression; while in group IV, the HO-1 protein expression in the heart tissue showed a significant increase. Furthermore, in group II, there was a significant increase in HO-1 protein expression.

4 Discussion

Although DOX is used for treating a broad range of solid tumors, its use is associated with severe cardiotoxicity, including arrhythmia, reduced ejection fraction, cardiomyopathies, and heart failure (Skovgaard, Hasbak, and Kjaer, 2014). The mechanism that mediates doxorubicin-induced cardiotoxicity is unclear; however, it might be related to oxidative stress, inflammatory cascade,

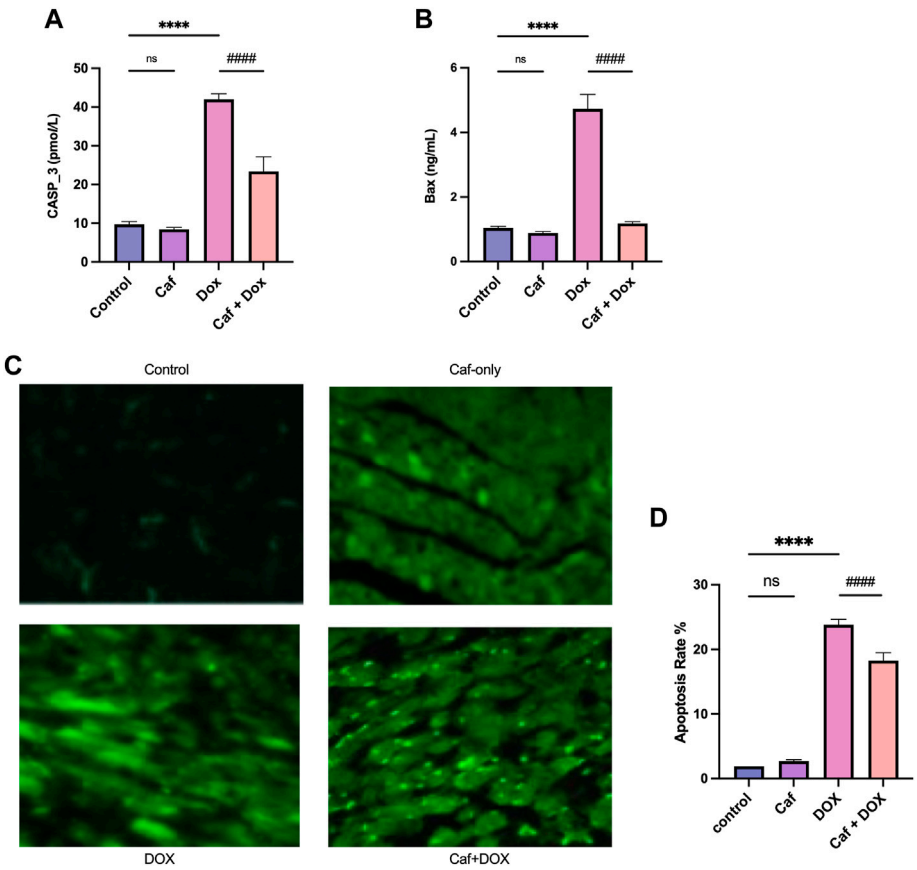

FIGURE 5
Effect of cafestol on cardiac tissue apoptosis in Wister rats. **(A)** Effect of cafestol on Casp3 cardiac tissue levels. **(B)** Effect of cafestol on Bax cardiac tissue levels. **(C)** Photomicrographs showing TUNEL-positive cardiomyocytes (hyperchromatic-bright green), ×1,000 magnification. **(D)** Analysis of TUNEL-positive cells quantitatively in the rat myocardium. Data are expressed as mean ± SD, $n = 8$. ****, $p < 0.0001$ vs. control group; ####, $p < 0.0001$ vs. DOX group; ns ($p > 0.05$), no significant difference.

apoptosis, and DNA damage; among them, oxidative stress plays an important role in DOX-induced toxicities through the generation of free radicals that cause depletion of antioxidants, increasing lipid, protein, and nucleic acid peroxidation and disturbing mitochondrial function (Rocca et al., 2020) (Ridha and Nada, 2019).

As Nrf2 plays a critical role in regulating oxidative stress within cells, once oxidative stress occurs, Nrf2 translocates into the nucleus after dissociating from Keap1 due to the oxidation of the active site of Keap1, triggering the expression of different antioxidant genes; in addition, Nrf2 signaling pathway activation can efficiently preserve cellular redox homeostasis and modulate apoptotic protein levels with an efficient anti-inflammatory function, which helps alleviate myocardial infarction and other cardiovascular disorders (Wu et al., 2022) (Saha et al., 2020).

In the current study, the data showed that acute exposure to DOX produced cardiac injury, represented by the change in the histological appearance of cardiac tissue and the significant increase in serum levels of CK and LDH as indicators of clinical myocardial injury; in addition, DOX caused oxidative stress as seen in the elevated cardiac tissue level of MDA, depleted GSH and antioxidant enzymes, and induced the apoptosis and inflammatory cascade by increasing the Bax and caspase 3 and TNF-α and IL-1β cardiac tissue levels. Furthermore, DOX alone suppressed the Nrf2 pathway by enhancing Keap1 gene expression and reducing Nrf2 and HO-1 mRNA tissue levels compared to group I (control group). These results are agreeable with previous studies affirming that DOX caused cardiac damage and oxidative stress and induced apoptosis and inflammation (Dundar et al., 2016) (Sirwi et al., 2022) (Zhou et al., 2022) (Ridha and Nada, 2019); thus, DOX-induced cardiotoxicity was successfully established.

Our result revealed that cafestol (5 mg/kg/day) alone caused a non-significant difference in CK and LDH serum levels with a comparable cardiac tissue histological appearance to that of the control group; this indicates that cafestol alone has no cardiotoxic effect on rats' heart *in vivo*. However, cafestol pretreatment efficiently protected against doxorubicin-induced cardiac injury as it significantly reduced the serum levels of CK and LDH and other cellular injury biomarkers; in addition, cafestol ameliorated the histopathological changes and significantly reduced the apoptosis and inflammatory markers. Furthermore, cafestol pre-treatment significantly increased the cardiac tissue levels of GSH, SOD, CAT, and GPx-1, reduced MDA tissue levels, and promoted Nrf2 and HO-1 proteins and

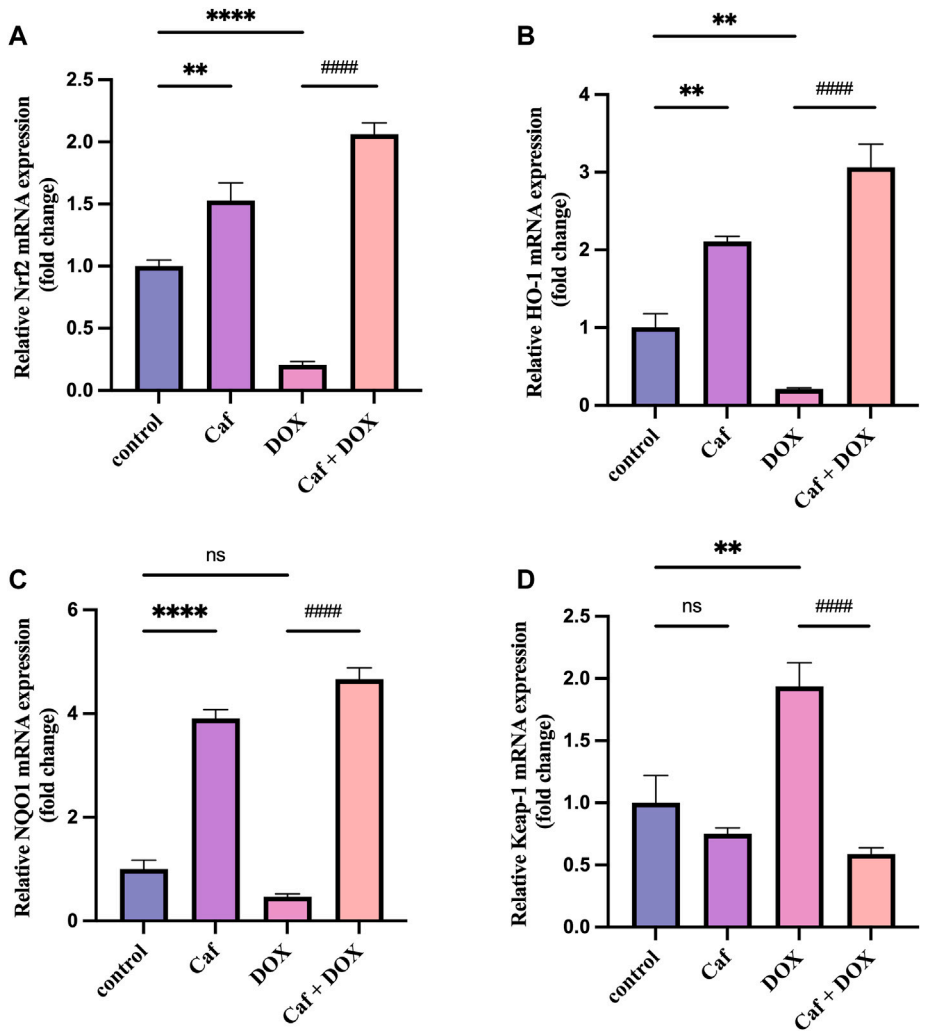

FIGURE 6
Effect of cafestol on the Nrf2/Keap1 pathway. **(A)** Effect of cafestol on Nrf2 gene expression. **(B)** Effect of cafestol on HO-1 gene expression. **(C)** Effect of cafestol on NQO-1 gene expression. **(D)** Effect of cafestol on Keap1 gene expression. Data are expressed as mean ± SEM, $n = 8$. **, $p < 0.01$; ****, $p < 0.0001$ vs. control group; ####, $p < 0.0001$ vs. DOX group; ns ($p > 0.05$), no significant difference.

gene expression and NQO-1 gene expression; in addition, cafestol significantly decreased the expression of the Keap1 gene, thus inhibiting the oxidative stress-mediated damage in cardiomyocytes when compared to animals treated with doxorubicin alone; therefore, cafestol might have a beneficial role in alleviating doxorubicin-mediated acute cardiac injury.

The mechanism that may explain the reduction of doxorubicin-induced cardiotoxicity might be related to the cafestol protective effect as a result of its antioxidant activity through the activation of the Nrf2 pathway, as it may have reduced doxorubicin-mediated free radicals' generation since doxorubicin is well recognized to induce cellular oxidative stress. Studies showed that cafestol, a coffee-specific diterpene, has antioxidant and anticarcinogenic effects in animal models as a result of modulating the Nrf2 axis (Lima et al., 2017) (Cavin et al., 2002) (Miller et al., 1991), which is related to cafestol's ability to enhance the expression of glutathione-S-transferases (GSTs) (Lam, Sparnins, and Wattenberg, 1982). Cafestol in a mixture with kahweol was previously reported to prevent aflatoxin B1-induced genotoxicity through a dual mechanism that involved the modulation of xenobiotic-activating enzymes that have the ability to activate potential carcinogens and increase the expression of GST that, in turn, provides a chemoprotective effect (Cavin et al., 1998). Furthermore, cafestol activates the Nrf2/HO-1 pathway, thus inhibiting the redox signaling (Hao et al., 2019). Furthermore, cafestol has been reported to interfere with NF-κB-dependent transcriptional activity, thus reducing inflammatory-mediated cardiac damage induced by doxorubicin (Kim, Jung, and Jeong, 2004) (Kathem, Abdulsahib, and Zalzala, 2022). Similarly, cafestol has been found to attenuate apoptosis and protect cells against oxidative stress and DNA damage induced by hydrogen peroxide (Ji et al., 2020) (Lee and Jeong, 2007). The results of the present study indicated that the protective effect of cafestol against DOX-induced cardiotoxicity depended on the activation of the Nrf2 signaling pathway.

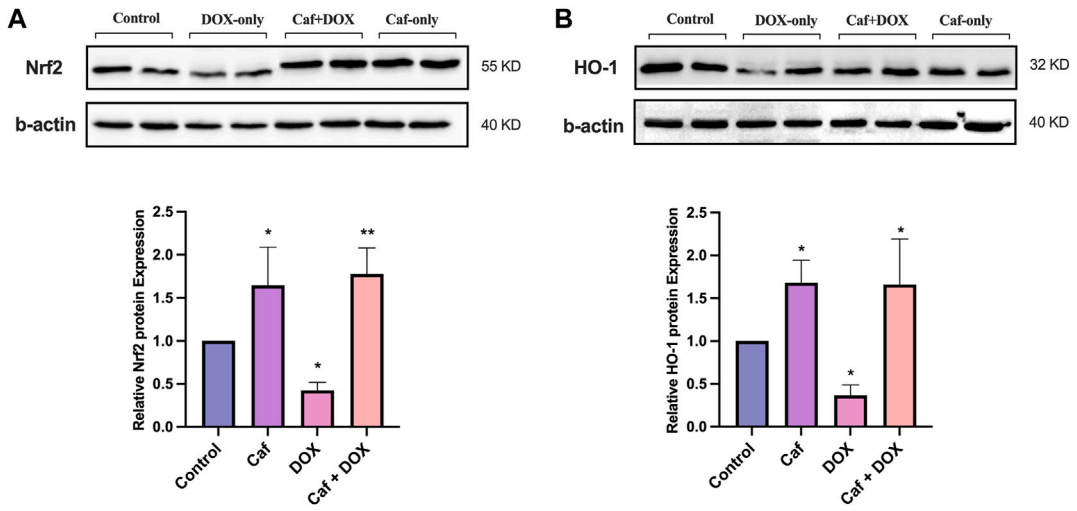

FIGURE 7
Effect of cafestol on cardiac tissue Nrf2 and HO-1 protein expression in Wister rats. **(A)** Representative western blot bands and the relative quantification of Nrf2 protein expression. **(B)** Representative western blot bands and the relative quantification of the level of HO-1 protein level. Data are expressed as mean of fold change relative to the control ±SD; $n = 4$. *, $p < 0.05$; **, $p < 0.01$ vs. control group.

5 Conclusion

According to results obtained from this study, it can be concluded that oral administration of cafestol (5 mg/kg/day) alone has no cardiotoxic effect; cafestol pre-treatment mitigated the DOX-induced cardiotoxicity and myocardial injury, inhibited apoptosis, and ameliorated oxidative stress and inflammatory response through the activation of the Nrf2 signaling pathway. Cafestol might be a potential chemoprotective agent against doxorubicin-induced adverse effect in cancer chemotherapy. However, additional *in vivo* and *in vitro* experiments on the antioxidant and antiapoptotic roles of cafestol on the cardiotoxicity model induced by doxorubicin, further investigation of the mechanisms involved, and the evaluation of other routes of administration and different doses, in addition to comparing the efficacy of effect of cafestol to other cardioprotective agents, are required.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Graduate Studies and Ethics Committees of the College of Pharmacy, University of Baghdad.

Author contributions

All authors contributed substantially to the study' conception and design; SA-K contributed to the experimental design, conducted the experiments, analyzed the data, and wrote the original manuscript. NA-S contributed to the experimental design and supervised and confirmed the authenticity of all raw data and participated in revising the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1206782/full#supplementary-material

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Association of gamma-tocopherol serum concentrations and blood pressure among adults in the United States: a cross-sectional study

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Background: hypertension is one of the major preventable risk factors for numerous diseases. The role of vitamin E in blood pressure (BP) has been controversial. We aimed to investigate the relationship between gamma-tocopherol serum concentration (GTSC) and BP

Methods: Data from 15,687 US adults from the National Health and Nutrition Examination Survey (NHANES) were analyzed. The correlations of GTSC with systolic BP (SBP), diastolic BP (DBP), and prevalence of hypertension were investigated by multivariate logistic regression models, generalized summation models, and fitted smoothing curves. Subgroup analyses were performed to investigate possible effect modifiers between them.

Results: With each natural log increase in GTSC, SBP, and DBP increased by 1.28 mmHg (β 1.28, 95% CI 0.71–1.84) and 1.15 mmHg (β 1.15, 95% CI 0.72–1.57), respectively, both P for trend < 0.001; the prevalence of hypertension increased by 12% (OR 1.12, 95% CI 1.03–1.22), P for trend 0.008. In subgroup analysis, in drinkers, with each natural log increase in GTSC, SBP, and DBP increased by 1.77 mmHg (β 1.77,95% CI 1.13–2.41) and 1.37 mmHg (β 1.37,95% CI 0.9–1.85), respectively, whereas they were not correlated in non-drinkers.

Conclusion: GTSC was linearly and positively associated with SBP, DBP, and the prevalence of hypertension, and alcohol consumption may modify the relationship of GTSC with SBP and DBP.

KEYWORDS

blood pressure, hypertension, gamma-tocopherol, vitamin E, alcohol consumption

1. Introduction

In recent decades, hypertension has been the major single factor in all causes of death and disability worldwide and is one of the major preventable risk factors for numerous diseases such as cardiovascular disease (CVD), renal insufficiency, and Alzheimer's disease (1). In the United States, hypertension is the largest contributor to CVD deaths compared to other modifiable risk factors and is second only to smoking in terms of all-cause mortality (2). The progression of disease due to blood pressure (BP) is known to be graded and continuous, and a BP of 115/75 mmHg or less is considered to be within the perfectly normal range (3). The Global Burden of Disease Study shows that 9.4 million deaths and 212 million lost healthy life yearly due to exceeding optimal BP levels. It is worrying that over 3.5 billion people worldwide have systolic BP (SBP) outside the ideal range (i.e., >115/mmHg) and 874 million people have SBP pressure above 140 mmHg, and this data will gradually increase with economic and social development (4).

Therefore, controlling BP is an important step in reducing the burden of disease and increasing the life expectancy of the world's population.

Many factors have been shown to be associated with BP increase, such as socio-demographic factors like gender, age, ethnicity, and environmental and behavioral factors. There are also many changeable exposure elements such as a high sodium diet, low potassium diet, obesity, and a sedentary lifestyle that could increase BP (5). In addition, studies have shown that oxidative stress plays an important role in the development of hypertension (6–8). Oxidative stress increases the generation of endothelium-derived contractile factors and decreases the biological availability of nitric oxide, resulting in impairment of vascular relaxation and endothelial dysfunction in hypertensive patients (9). Vitamin E is an antioxidant, however, its effect on BP has been controversial. Some interventional trials and animal experiments indicated that vitamin E could produce beneficial effects on BP by inhibiting oxidative stress (10–12). Some studies also argue that vitamin E has no effect on BP and may even be harmful (13–16). In addition, it has recently been shown that the effect of vitamin E on BP has a multisegmented effect, with an inverted J-shaped relationship (17). Differences in study design, sample size, ethnic distribution, and control of confounding factors may explain the controversial results among these studies. More importantly, most of these studies did not separately investigate the single active form of vitamin E.

Vitamin E is made up of tocopherols and tocotrienols (α, β, γ, and δ) and has two main dietary forms in the body, α-tocopherol (ATC) and γ-tocopherol (GTC), of which the most studied is ATC, because ATC is most bioactive and abundant in the blood, and vitamin E deficiency can be corrected by taking this supplement (18). More recently, there has been a growing body of research discussing the role of GTC in public health. GTC is the most common form of vitamin E in the diet, and its concentration in tissues is much greater than its concentration in the blood (19, 20). Differences in morphology, biological activity, and tissue distribution lead to different biological effects of ATC and GTC. Because vitamin E may have an impact on the development of hypertension and there are few studies on the role of GTC, we propose to conduct cross-sectional studies in U.S. adults to explore the correlation between GTC serum concentration (GTSC) and BP, including SBP, diastolic BP (DBP), and the prevalence of hypertension.

In this study, we extracted data from a representative National Health and Nutrition Examination Survey (NHANES) database of 4 cycles with GTSC data to investigate the correlation between GTSC and BP, and possible modifiers of this relationship.

2. Method

2.1. Study design and population

The data used in this study were all from four cycles in the NHANES database. The NHANES is a continuous representative survey of the U.S. national population that provides a wealth of data on the nutrition and health of adults and children in the U.S. using a complex, multi-stage, probability sampling design. The study was approved by the Ethics Review Committee of the National Center for Health Statistics and with the written consent of each participant. More information can be found at https://www.cdc.gov/nchs/nhanes/index.htm.

We performed this cross-sectional study using information from participants aged ≥18 years ($n = 23032$) in the NHANES (2001–2002, 2003–2004, 2005–2006, 2017–2018) study. Exclusion criteria are as follows: participants with cancer ($n = 2000$), patients with missing GTSC data ($n = 2494$), and participants with missing BP data or hypertension history ($n = 2851$). Finally, 15,687 participants were included in the statistical analysis (**Figure 1**).

2.2. The exposure and outcome variables

No fasting or special diet is required before the blood was collected by a phlebotomist. The exposure variable was the GTSC, which was measured by high-performance liquid chromatography with multiwavelength photodiode-array absorbance detection. Tocopherols have absorption maxima

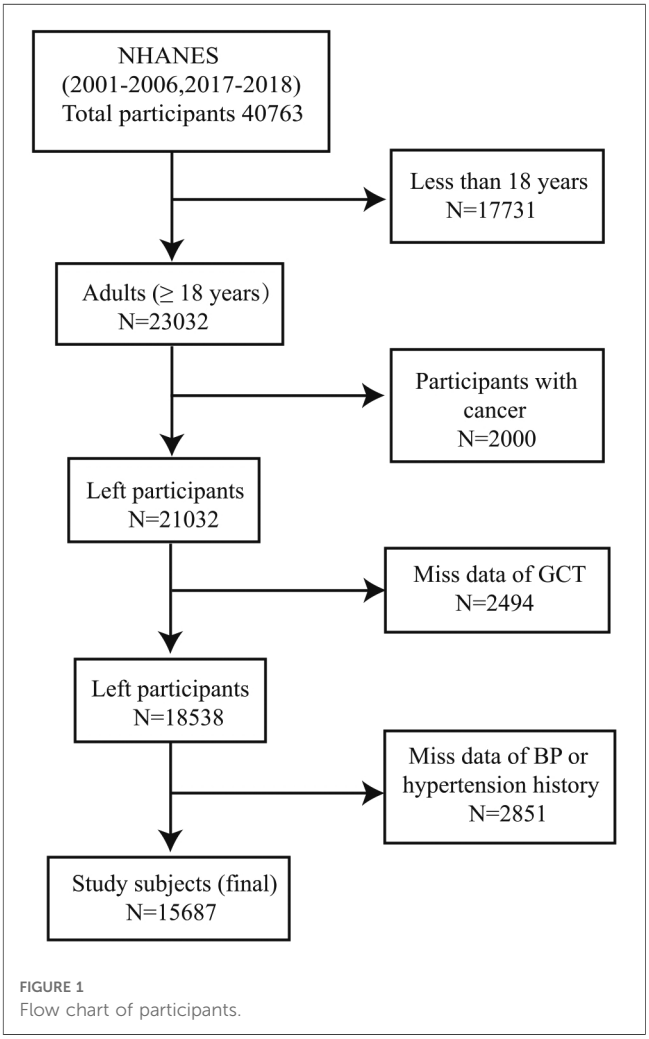

FIGURE 1
Flow chart of participants.

between 292 and 300 nm and chromatograms are recorded using a computer data system. Spectrophotometric methods are used for quantitative analysis. GTSC was a skewed distribution in our study, and we convert it to a natural logarithm with base e (Ln GTSC) in the data analysis. The outcome variables were SBP, DBP, and the prevalence of hypertension. The SBP and DBP were defined respectively as the average of the SBP and DBP measured at three different times. Hypertension was defined as a self-reported diagnosis of hypertension, or SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg, or the use of antihypertensive medication.

2.3. Potential covariates

In our study, covariates included demographic data [sex, age, race, education levels, and poverty income ratio (PIR)], examination data (weight (kg), height (cm), body mass index (BMI, kg/m^2), and waist (cm)), laboratory data (Albumin (g/L), blood urea nitrogen (BUN, mmol/L), uric acid (UA, umol/L), serum creatinine (Scr, mol/L), estimated glomerular filtration rate (eGFR, we calculated by the modification of diet in renal disease equation, ml/min/1.73 m^2), fasting blood glucose (FBG, mmol/L), glycated haemoglobin A1c (HbA1c, %), total cholesterol (TC, mmol/L), triglycerides (TG, mmol/L), high-density lipoprotein cholesterol (HDL-C, mmol/L)), and questionnaire data (History of diabetes, heart failure (HF), coronary artery disease (CAD), smoking, and drinking). Diabetes was defined as a self-reported diagnosis of diabetes or having an HbA1c level ≥ 6.5%, or FPG ≥ 7 mmol/L. HF and CAD were defined as the self-reported diagnosis of HF and CAD, respectively.

2.4. Statistical analysis

Described continuous data by mean ± standard deviations (SDs) or median (interquartile ranges, Q1–Q3) and categorical data by number (%), respectively. Differences between the tertiles of Ln GTSC groups were compared using one-way ANOVA for continuous variables and the chi-square test for categorical variables. In multivariate logistic regression models, covariates were proven traditional or suspected risk factors for hypertension, or the Ln GTSC estimates for SBP, DBP, and hypertension changed by more than 10% (21). We investigated the relationship between GTSC (continuous and categorical) and BP values and the prevalence of hypertension using multivariate logistic regression analysis. Model 1 represents unadjusted data. Model 2 adjusts for demographic information: age, sex, race, education level, and PIR. Model 3 additionally adjusts for smoking status, drinking status, diabetes, CAD, HF, BMI, albumin, BUN, UA, Scr, eGFR, FBG, HbA1c, TC, TG, and HDL-C. The shape of the relationship between GTSC and BP and the prevalence of hypertension, respectively, was described by using the generalized additive model (GAM) and smoothed curve fit (penalized spline method). Subgroup analysis using stratified multiple regression analysis for the following variables: age (<60 vs. ≥60 years), sex (male vs. female), race (Mexican American vs. Other Hispanic vs. Non-Hispanic White vs. Non-Hispanic Black vs. other races), BMI ((<24 vs. ≥ 24 kg/m^2), CAD (yes vs. no), HF (yes vs. no), diabetes (yes vs. no), smoking status (yes vs. no), drinking status (yes vs. no), and eGFR (<60 vs. ≥60 ml/min/1.73 m^2).

All analyses were conducted with package R (http://www.R-project.org) and EmpowerStats (http://www.empowerstats.com), with a P value <0.05 considered statistically significant.

3. Results

3.1. Baseline participant characteristics

Table 1 presents the baseline characteristics of all participants and participants grouped by the tertiles of GTSC. Overall, 15,687 participants with a mean age of 44 ± 18.97 years were included in our study, and 7,629 (48.63%) were males. Among all participants, 5,711 (36.41%) had hypertension and the mean Lg GTSC was 1.50 ± 0.57 umol/L. In the three GTSC groups, differences were statistically significant for all variables except sex. The participants in the GTSC T3 group were likely to be younger, non-Hispanic black; have hypertension, diabetes, HF, CAD; be smokers and non-drinkers; have the higher level for SBP, DBP, weight, BMI, waist circumference, BUN, UA, Scr, FBG, HbA1c, TC and TG; and have lower levels of poverty income ratio, eGFR, height, and HDL-C, than those in the lower GTSC group.

3.2. Association of GTSC with BP and prevalence of hypertension

As shown in Table 2, in logistic regression, GTSC was significantly and positively associated with BP increase and the prevalence of hypertension in all participants, regardless of whether they were adjusted for confounders. After adjusting for age, sex, race, education level, PIR, smoking status, drinking status, diabetes, CAD, HF, BMI, albumin, BUN, UA, Scr, eGFR, FBG, HbA1c, TC, TG, and HDL-C, each natural log increase in GTSC was associated with a 1.28 mmHg increase in SBP (β 1.28, 95% CI 0.71–1.84) and a 1.15 mmHg increase in DBP (β 1.15, 95% CI 0.72–1.57), and a 12% increase in the prevalence of hypertension (OR 1.12, 95% CI 1.03–1.22). We grouped GTSC in tertile groups and used the T1 group as the reference group to further evaluate the relationship between GTSC and BP values and the prevalence of hypertension. Compared with the T1 reference group, SBP increased by 0.71 mmHg (β 0.71,95%CI −0.01–1.43) and 1.39 mmHg (β 1.39,95%CI 0.62–2.15) in the T2 and T3 groups, respectively, P for trend <0.001; DBP increased by 0.53 mmHg (β 0.53, 95%CI −0.02–1.07) and 1.39 mmHg (β 1.39,95%CI 0.82–1.97), respectively, P for trend <0.001; and the relative risk of hypertension incidence increased by 8% (OR 1.08,95%CI 0.96–1.21) and 17% (OR 1.17,95%CI 1.04–1.32), P for trend 0.008. In summary, SBP, DBP, and the prevalence of

TABLE 1 Baseline characteristics of study participants.

Characteristics[a]	GTSC[b] (umol/L) tertiles				
	Total	Tertiles 1	Tertiles 2	Tertiles 3	*P*-value
GTSC[b] rang	−1.78–4.32	−1.78–1.30	1.31–1.76	1.76–4.32	
N	15687	5198	5245	5244	
Sex					0.341
female	8058 (51.37%)	2689 (51.73%)	2651 (50.54%)	2718 (51.83%)	
male	7629 (48.63%)	2509 (48.27%)	2594 (49.46%)	2526 (48.17%)	
Age (years)	45.21 ± 18.97	47.54 ± 20.04	42.21 ± 18.58	45.87 ± 17.85	<0.001
Race					<0.001
Mexican American	3179 (20.27%)	1024 (19.70%)	1078 (20.55%)	1077 (20.54%)	
Other Hispanic	823 (5.25%)	313 (6.02%)	319 (6.08%)	191 (3.64%)	
Non-Hispanic White	6940 (44.24%)	2444 (47.02%)	2158 (41.14%)	2338 (44.58%)	
Non-Hispanic Black	3402 (21.69%)	824 (15.85%)	1228 (23.41%)	1350 (25.74%)	
Other Race	1343 (8.56%)	593 (11.41%)	462 (8.81%)	288 (5.49%)	
Education level					<0.001
<9th grade	1672 (10.66%)	553 (11.58%)	553 (11.93%)	566 (11.63%)	
9–11th grade	1975 (12.59%)	533 (11.16%)	596 (12.86%)	846 (17.39%)	
High school	3434 (21.89%)	993 (20.80%)	1145 (24.70%)	1296 (26.63%)	
AA degree	4162 (26.53%)	1343 (28.13%)	1392 (30.03%)	1427 (29.33%)	
College or above	3032 (19.33%)	1352 (28.32%)	949 (20.47%)	731 (15.02%)	
PIR	2.60 ± 1.62	2.76 ± 1.65	2.56 ± 1.63	2.47 ± 1.58	<0.001
Hypertension					<0.001
No	9976 (63.59%)	3384 (65.10%)	3566 (67.99%)	3026 (57.70%)	
yes	5711 (36.41%)	1814 (34.90%)	1679 (32.01%)	2218 (42.30%)	
Diabetes					<0.001
no	13592 (86.64%)	4597 (88.44%)	4646 (88.58%)	4349 (82.93%)	
yes	2095 (13.36%)	601 (11.56%)	599 (11.42%)	895 (17.07%)	
Heart failure[c]					0.036
No	13904 (88.63%)	4652 (97.42%)	4533 (97.82%)	4719 (96.98%)	
yes	371 (2.37%)	123 (2.58%)	101 (2.18%)	147 (3.02%)	
Coronary artery disease[c]					<0.001
No	13308 (84.83%)	4425 (92.67%)	4384 (94.61%)	4499 (92.46%)	
yes	967 (6.16%)	350 (7.33%)	250 (5.39%)	367 (7.54%)	
Smoking status[c]					<0.001
none	7872 (50.18%)	2826 (57.77%)	2582 (54.85%)	2464 (50.38%)	
current	6618 (42.19%)	2066 (42.23%)	2125 (45.15%)	2427 (49.62%)	
Drinking status[c]					<0.001
none	3312 (21.11%)	1004 (21.69%)	1054 (23.73%)	1254 (27.04%)	
current	10396 (66.27%)	3625 (78.31%)	3388 (76.27%)	3383 (72.96%)	
SBP (mmHg)	123.43 ± 19.28	122.52 ± 19.55	121.83 ± 18.36	125.93 ± 19.67	<0.001
DBP (mmHg)	70.37 ± 12.88	69.13 ± 12.39	69.88 ± 12.74	72.08 ± 13.30	<0.001
Weight (kg)	80.26 ± 20.42	74.84 ± 18.01	79.84 ± 19.93	86.07 ± 21.60	<0.001
Height (cm)	167.58 ± 10.11	167.03 ± 10.20	167.94 ± 10.12	167.79 ± 10.01	<0.001
BMI (kg/m2)	28.51 ± 6.52	26.75 ± 5.57	28.25 ± 6.40	30.53 ± 6.95	<0.001
Waist(cm)	97.20 ± 16.04	93.05 ± 14.91	96.29 ± 15.70	102.25 ± 16.11	<0.001
Albumin (g/L)	41.67 ± 3.81	41.94 ± 3.75	41.74 ± 3.87	41.36 ± 3.79	<0.001
BUN (mmol/L)	4.71 ± 2.05	4.93 ± 2.11	4.55 ± 1.92	4.65 ± 2.10	<0.001
UA (umol/L)	317.93 ± 85.97	306.53 ± 82.88	315.98 ± 84.14	331.14 ± 88.97	<0.001
Scr (mol/L)	79.28 ± 37.90	78.87 ± 35.22	78.60 ± 41.29	80.36 ± 36.90	0.039
eGFR (ml/min/1.73 m^2)	95.52 ± 28.51	95.06 ± 29.01	97.59 ± 27.65	93.90 ± 28.75	<0.001
FBG (mmol/L)	5.43 ± 1.86	5.28 ± 1.45	5.32 ± 1.66	5.70 ± 2.33	<0.001
HBA1C (%)	5.60 ± 1.00	5.51 ± 0.79	5.51 ± 0.87	5.76 ± 1.25	<0.001
TC (mmol/L)	5.05 ± 1.11	4.75 ± 1.04	4.92 ± 1.01	5.46 ± 1.15	<0.001
Triglycerides (mmol/L)	1.61 ± 1.47	1.30 ± 0.74	1.43 ± 0.92	2.10 ± 2.16	<0.001
HDL-C (mmol/L)	1.38 ± 0.41	1.43 ± 0.40	1.39 ± 0.40	1.33 ± 0.42	<0.001

GTSC, gamma-tocopherol serum concentration; PIR, poverty-income ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; BUN, blood urea nitrogen; UA, uric acid; SCR, Serum creatinine; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c, TC total cholesterol; HDL-C, high-density lipoprotein cholesterol.

[a]Data are presented as mean ± standard deviation or median (Q1–Q3) and numbers (%) as appropriate.

[b]GTSC value was log e-transformed (e = 2.718).

[c]Numbers that do not add up to 100% are attributable to missing data.

TABLE 2 Association of GTSC with SBP, DBP, and the prevalence of hypertension among American adults.

Exposure	SBP (mmHg, β, 95%CI)			DBP (mmHg, β, 95% CI)			Hypertension (OR, 95% CI)		
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
Ln GTSC	1.95 (1.42, 2.48)	2.85 (2.36,3.35)	1.28 (0.71, 1.84)	2.29 (1.93, 2.64)	2.50 (2.12, 2.87)	1.15 (0.72, 1.57)	1.19 (1.12, 1.26)	1.45 (1.35, 1.56)	1.12 (1.03, 1.22)
Ln GTSC									
T1	reference	Reference	reference	reference	reference	reference	reference	reference	reference
T2	−0.69 (−1.43, 0.05)	1.60 (0.90, 2.30)	0.71 (−0.01, 1.43)	0.74 (0.25, 1.24)	1.22 (0.68, 1.75)	0.53 (−0.02, 1.07)	0.88 (0.81, 0.95)	1.22 (1.10, 1.35)	1.08 (0.96, 1.21)
T3	3.40 (2.67, 4.14)	3.66 (2.97, 4.36)	1.39 (0.62, 2.15)	2.95 (2.46, 3.44)	3.18 (2.65, 3.71)	1.39 (0.82, 1.97)	1.37 (1.26, 1.48)	1.64 (1.48, 1.81)	1.17 (1.04, 1.32)
P for trend	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.008

GTSC, gamma-tocopherol serum concentration; Ln GTSC was GTSC log e-transformed (e = 2.718); SBP, systolic blood pressure; DBP, diastolic blood pressure. Model 1 adjusts for none; Model 2 adjusts for age, sex, race, education level, and poverty income ratio; Model 3 adjusts for age, sex, race, education level, and poverty income ratio; smoking status, drinking status, diabetes, CAD, HF, BMI, albumin, BUN, UA, Scr, eGFR, FBG, HbA1c, TC, TG, and HDL-C.

hypertension were all linearly and positively correlated with GTSC. The above findings are consistent with the results of the smoothed curve fitting (Figure 2).

3.3. Subgroup analyses

To further verify the reliability of the results in the presence of confounding factors and whether there are factors that may modify the relationship between GTSC and BP and the incidence of hypertension, we conducted subgroup analyses by stratifying the main covariates. Except for drinking status, there were no other covariates that significantly modified the relationship between GTSC and BP values and the incidence of hypertension, including sex, age, race, BMI, eGFR, HF, CAD, DM, and smoking status (all P-interaction > 0.05) (Figure 3). In individuals who consumed alcohol, each natural log increase in GTSC was associated with an increase in SBP of 1.77 mmHg (β 1.77,95% CI 1.13–2.41) and an increase in diastolic BP of 1.37 mmHg (β 1.37,95% CI 0.9–1.85). There was no statistically significant relationship between GTSC and BP (either diastolic or systolic) in individuals who did not

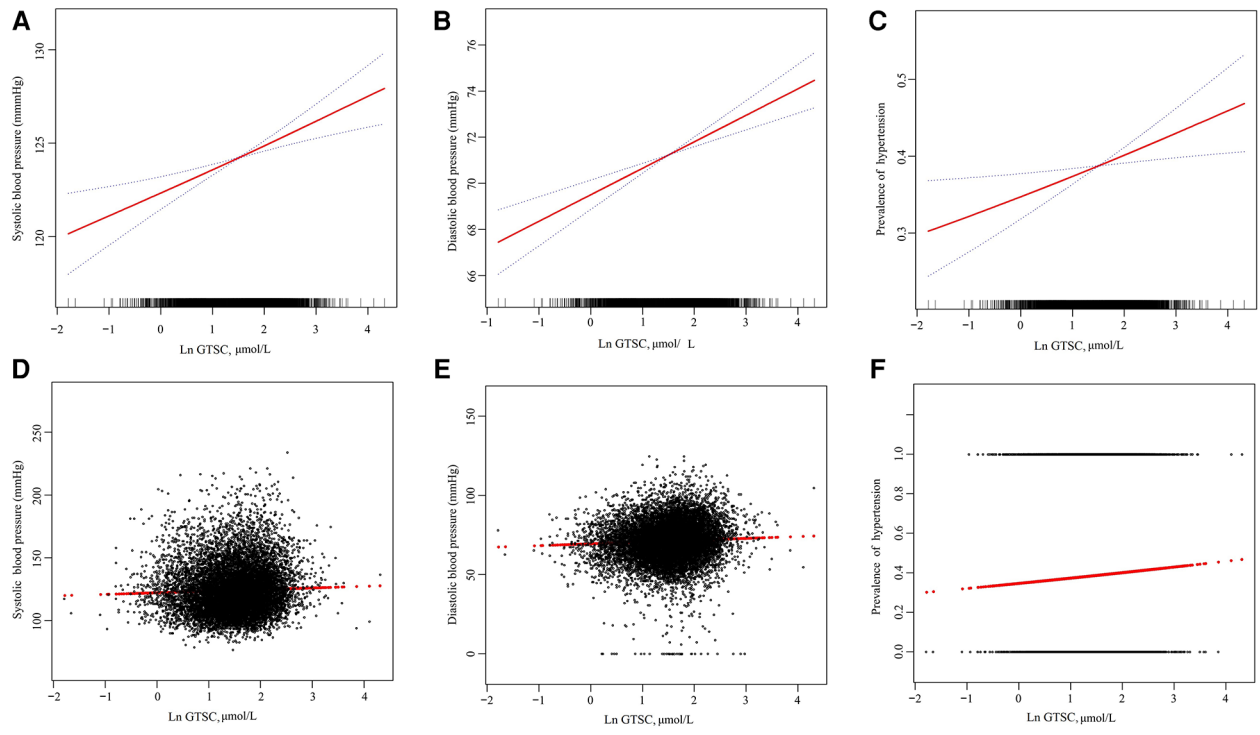

FIGURE 2
The association of GTSC with SBP (A,D), DBP (B,E), and prevalence of hypertension (C,F). (A–C): The solid line and dashed line represent the estimated values and their corresponding 95% confidence interval, respectively. (D–F): Each black point represents a sample. Age, sex, race, education level, and poverty income ratio; smoking status, drinking status, diabetes, CAD, HF, BMI, albumin, BUN, UA, Scr, eGFR, FBG, HbA1c, TC, TG, and HDL-C were adjusted.

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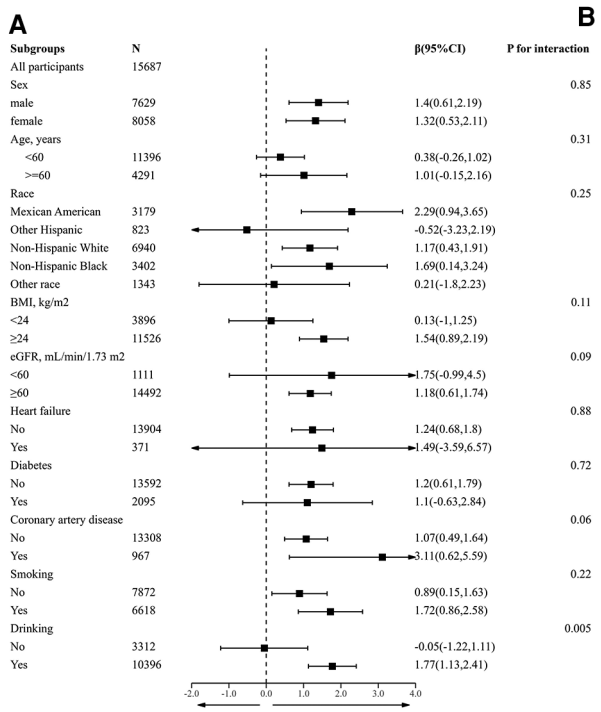

FIGURE 3
Stratified analyses by potential modifiers of the association between GTSC and SBP (**A**), DBP (**B**), and prevalence of hypertension (**C**). *Each subgroup analysis adjusted for Age, sex, race, education level, and poverty income ratio; smoking status, drinking status, diabetes, CAD, HF, BMI, albumin, BUN, UA, Scr, eGFR, FBG, HbA1c, TC, TG, and HDL-C were adjusted. except for the stratifying variable. *Numbers that do not add up to 100% are attributable to missing data

consume alcohol. There may be an interaction between GTSC and alcohol consumption in terms of BP (SBP: P for interaction = 0.005, DBP: P for interaction = 0.02), but not in terms of hypertension incidence (P for interaction = 0.28) (**Figure 3**).

4. Discussion

In this large sample cross-sectional study, we found that GTSC was positively associated with SBP, DBP, and the prevalence of hypertension, and this relationship remained significant after

adjustment for confounders. Furthermore, we noted that alcohol consumption modifies the relationship of GTSC with SBP and DBP, but not the relationship with the incidence of hypertension.

To date, there is still controversy regarding the role of vitamin E in hypertension. Boshtam et al. conducted a randomized triple-blind placebo-controlled trial including 78 patients with mild hypertension in Isfahan and indicated that compared to the placebo group, the oral vitamin E group was able to significantly reduce SBP (−24% in the vitamin E group versus −1.6% in the placebo group) and DBP (−12.5% in the vitamin E versus −6.2% in the placebo group) ($p < 0.05$) (11). Although this study followed a strict clinical randomized study design, it had significant limitations, such as its small sample size, which added significant bias, and weight (kg), which is known to be an important risk factor for hypertension, was different between the two groups (88.91 ± 20.03 vs 75.92 ± 10.67). In addition, there are some cross-sectional studies, meta-analyses, and animal studies that also suggest that taking vitamin E can lower BP (15, 22, 23). However, other studies hold a different view. Ward and his colleagues also performed a randomized, double-blind, placebo-controlled trial including 58 patients with type 2 diabetes to investigate the effects of vitamin E on hypertension (24). Compared with the placebo group, the α-tocopherol group (500 mg/day for 6 weeks) increased SBP by 7.0 mmHg (β 7, 95% CI 5.2–8.8) and DBP by 5.3 mmHg (β 5.3, 95% CI 4.0–6.5); the mixed tocopherol group (60% γ-, 25% δ- and 15% α-tocopherol, 500 mg /day for 6 weeks) increased SBP by 6.8 mmHg (β 6.8, 95% CI 4.9–8.6) and DBP by 3.6 mmHg (β 3.6, 95% CI 2.3–4.9). Moreover, the prospective cohort study by Lai et al. including 684 pregnant women, and the cross-sectional study by Francis et al. both concluded that vitamin E (in either form) was not significantly associated with BP (13, 25). A recent large sample prospective cohort study showed an inverse J-shaped relationship between dietary vitamin intake and the incidence of new-onset hypertension, with participants in the second to fourth quintiles (Q2-Q4) having the lowest incidence of new-onset hypertension. Compared with Q2-Q4, the incidence of new-onset hypertension increased by 40% in Q1 participants (OR 1.4, 95% CI 1.29–1.52), and by 18% in Q5 (OR 1.18, 95% CI 1.09–1.29) (17). The controversial results in these studies may be explained by differences in study design, sample size, study population, and confounding factors. However, what is more, important is that several different forms of vitamin E are known both *in vivo* and in dietary supplementation, and most of these studies have not been studied separately.

Based on previous studies, the present study is the first to individually investigate the association of GTSC with BP using cross-sectional data from a large sample, adding more evidence to the field. Our study has several important findings. First, our study found that GTSC was linearly and positively correlated with SBP, DBP, and the prevalence of hypertension, with a trend toward higher BP and hypertension prevalence with higher GTC concentrations. It is not clear the specific mechanism by which GTSC increases SBP, DBP, and the prevalence of hypertension, but the following mechanisms might explain it. Firstly, GTC has a dual role in oxidative stress. GTC is known to have antioxidant and anti-inflammatory effects, but some studies indicated that excess GTC could promote the production of nitric oxide, an inflammatory mediator through its oxidation products, leading to an enhancement of cellular immune response and an increase of lipid peroxidation (26). Studies have proven that endothelial damage, vascular dysfunction, cardiovascular remodeling, renal dysfunction, immune cell activation, and systemic inflammation during oxidative stress underlie the pathophysiology of hypertension (27–30). Another possible mechanism is that GTC could increase the excitability of the sympathetic nervous system. This hypothesis was supported by a randomized clinical trial in which heart rate was significantly increased by either ATC or mixed tocopherol treatment, indicating a central effect of tocopherol (24). However, it contradicts another study, which concluded that the cardiac autonomic nervous system improved after treatment with vitamin E (31).

Second, we noted that alcohol consumption could modify the relationship of GTSC with both SBP and DBP. In participants who consumed alcohol, a potentiation of the GTC-driven increase in SBP and DBP was observed, but not in non-drinking participants. Numerous studies have demonstrated that alcohol consumption is positively associated with BP increase, even in small amounts (32–34). The potential mechanisms responsible for this outcome are complex and varied, including effects on the central nervous system, inhibition of the vagal, excitation of the sympathetic nerve, activation of the renin-angiotensin-aldosterone system, increased cortisol secretion, insulin resistance and impaired glucose tolerance, oxidative stress, and impaired endothelial function (35, 36). Overall, our findings encouraged people with high levels of GTSC to lower their GTSC appropriately to control BP, especially those who consume alcohol. However, it is worth noting that the above results are only hypothesis generation and further investigations need to be conducted to support the findings of our study.

Although our study is based on nationally representative U.S. general population data and has a large sample size that minimizes bias and has good extrapolation, we must also acknowledge several important limitations of this study. First, this study was a retrospective cross-sectional design, and thus no causal inferences could be made about the association of GTSC with SBP, DBP, and the prevalence of hypertension, which is an inherent drawback of all cross-sectional designs. Second, although we adjusted for numerous confounding factors, we still cannot exclude the interference of unknown confounders (e.g., daily lifestyle, medication use) on the study results. In this study, only a single blood test was performed to assess the status of GTC, and repeat sampling may be required to overcome the daily variability of individuals, but this process would become very complex and expensive. Fourth, our study was limited to adults in the United States, so generalization to other countries or age groups requires caution.

5. Conclusion

In summary, this study investigated the relationship between GTSC and SBP, DBP, and the prevalence of hypertension in the

general U.S. population and found that GTSC was linearly and positively associated with SBP, DBP, and the prevalence of hypertension, and found that this correlation of GTSC with SBP and DBP was more significant in those who consumed alcohol.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Review Committee of the National Center for Health Statistics. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZW and YW conceived and designed the study; ZW drafted the manuscript and participated in the literature search, data analysis, and interpretation. KL, LH, and JF collected the data and contributed to the statistical analysis. All authors contributed to the review/editing of key intellectual content of the manuscript. YW provided key revisions. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Iron scavenging and suppression of collagen cross-linking underlie antifibrotic effects of carnosine in the heart with obesity

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Oral consumption of histidyl dipeptides such as l-carnosine has been suggested to promote cardiometabolic health, although therapeutic mechanisms remain incompletely understood. We recently reported that oral consumption of a carnosine analog suppressed markers of fibrosis in liver of obese mice, but whether antifibrotic effects of carnosine extend to the heart is not known, nor are the mechanisms by which carnosine is acting. Here, we investigated whether oral carnosine was able to mitigate the adverse cardiac remodeling associated with diet induced obesity in a mouse model of enhanced lipid peroxidation (i.e., glutathione peroxidase 4 deficient mice, GPx4$^{+/-}$), a model which mimics many of the pathophysiological aspects of metabolic syndrome and T2 diabetes in humans. Wild-type (WT) and GPx4$^{+/-}$male mice were randomly fed a standard (CNTL) or high fat high sucrose diet (HFHS) for 16 weeks. Seven weeks after starting the diet, a subset of the HFHS mice received carnosine (80 mM) in their drinking water for duration of the study. Carnosine treatment led to a moderate improvement in glycemic control in WT and GPx4$^{+/-}$mice on HFHS diet, although insulin sensitivity was not significantly affected. Interestingly, while our transcriptomic analysis revealed that carnosine therapy had only modest impact on global gene expression in the heart, carnosine substantially upregulated cardiac GPx4 expression in both WT and GPx4$^{+/-}$mice on HFHS diet. Carnosine also significantly reduced protein carbonyls and iron levels in myocardial tissue from both genotypes on HFHS diet. Importantly, we observed a robust antifibrotic effect of carnosine therapy in hearts from mice on HFHS diet, which further *in vitro* experiments suggest is due to carnosine's ability to suppress collagen-cross-linking. Collectively, this study reveals antifibrotic potential of carnosine in the heart with obesity and illustrates key mechanisms by which it may be acting.

KEYWORDS

obesity, cardiac fibrosis, lipid peroxidation, carnosine, iron chelation, carbonyl stress, collagen, extracellular matrix

1 Introduction

Obesity and overweight are chronic metabolic disorders characterized by excessive body fat accumulation with a BMI >30 and 25 kg/m^2, respectively. Both conditions have approached epidemic proportions globally and are established risk factors for cardiometabolic disorders such as type 2 diabetes mellitus and nonalcoholic fatty liver disease (Piché et al., 2020). "Obesity cardiomyopathy" is a unique clinical condition characterized by cardiac structural remodeling and functional abnormalities independent of any cardiovascular risk factor (e.g., hypertension, coronary artery disease) (Chockalingam, 2022) that is associated with increased risk of premature death (Adams et al., 2006).

Western dietary patterns rich in saturated fat and sucrose are strongly obesogenic and known to cause glucose intolerance, insulin resistance and associated cardiometabolic abnormalities in rodent models (Kopp, 2019). Moreover, this type of diet induces significant lipid peroxidation and protein glycation in cardiovascular tissues (Hauck et al., 2019; Świątkiewicz et al., 2023). Lipid peroxidation is an oxidative reaction between a polyunsaturated fatty acid (PUFA) and a transition metal, usually iron, which is required for its initiation and propagation. If not neutralized by antioxidants, lipid peroxides ultimately degrade into a wide variety of biogenic aldehydes (e.g., 4-hydroxynonenal, HNE, malondialdehyde, MDA) and due to their high reactivity and toxicity these reactive carbonyl species (RCS) have been implicated in the pathology of many obesity related disorders (Davì et al., 2005; Ramana et al., 2013; de Souza Bastos et al., 2016). Pathogenicity of RCS is mainly related to their irreversible modification of proteins and other macromolecules which ultimately lead to changes in cellular metabolism and signaling pathways (Marisa et al., 2012). Lipid peroxidation and its associated RCS are known to be involved in profibrotic signaling through stimulation of collagen production and activation of key mediators such as transforming growth factor β (TGF-β) and other inflammatory chemo-/cytokines (Poli and Schaur, 2000; Macdonald et al., 2001; Albano et al., 2005; Tsubouchi et al., 2019).

As the only member of the glutathione peroxidase superfamily capable of neutralizing lipid peroxides, glutathione peroxidase-4 (GPx4) is a master regulator of ferroptosis and experimental studies have shown that this selenoenzyme has important pathophysiological roles in many cardiometabolic, neurodegenerative, autoimmune diseases and malignancies (Park et al., 2019; Ursini et al., 2022; Wang et al., 2022; Xu et al., 2022). A large number of genetic epidemiological studies have linked *gpx4* variants and GPx4 content/activity with obesity (Rupérez et al., 2014; Costa-Urrutia et al., 2020) cardiovascular and inflammatory diseases (Polonikov et al., 2012; Berdaweel et al., 2022), and cancer (Méplan et al., 2010).

Histidyl dipeptides such as l-carnosine are endogenous molecules endowed with potent carbonyl-scavenging capacity and exist in abundant quantities in excitatory tissues such as skeletal muscle, brain and heart. Carnosine is also capable of buffering protons and chelating divalent cations such as Ca^{2+}, properties that have been implicated as mechanisms of cardioprotection in models of cardiac injury (Zhao et al., 2020; Gonçalves et al., 2021). Oral carnosine has shown therapeutic potential in several clinical and experimental models of cardiometabolic diseases in which RCS are known to have a pathogenic role, including insulin resistance and glucose tolerance (Matthews et al., 2021; Rohit et al., 2023), prevention of diabetes related complications (Menini et al., 2020) and cardiovascular disorders (de Courten et al., 2015; Lombardi and Metra, 2016). Most of the beneficial effects of carnosine have been ascribed to its carbonyl-scavenging properties, although carnosine has limited ability to neutralize reactive oxygen species (ROS) directly. In a recent comprehensive study (Anderson et al., 2018), we reported that a carnosine analog which is resistant to degradation by carnosinase can mitigate insulin resistance and liver steatosis/fibrosis in diet induced obesity, even in mice with enhanced lipid peroxidation due to GPx4 haploinsufficiency (i.e., GPx4$^{+/-}$ mice). What remained unclear is whether the beneficial effects of oral carnosine therapy in obesity also extended to the heart, and the potential mechanisms by which it might be acting. In this study, we investigated the effectiveness of oral carnosine supplementation in mitigating the structural and functional changes in the heart that are known to accompany obesity in both wild-type (WT) and GPx4$^{+/-}$ mice. Our findings reveal carnosine to have potent antifibrotic effects in the heart with obesity and identify mechanisms involved in these effects.

2 Results

2.1 Impact of HFHS diet and oral carnosine supplementation on cardiometabolic parameters

In a previous study we found significantly lower GPx4 enzyme in myocardial tissue from type 2 diabetes patients, corresponding to greater levels of lipid peroxidation and RCS (i.e., HNE adducts) in the tissue from these patients compared with age-matched normoglycemic patients (Katunga et al., 2015). Here, both WT and GPx4$^{+/-}$ mice were randomly assigned to either standard chow diet (CNTL) or HFHS diet for 16 weeks, with half of the HFHS diet group also receiving l-carnosine supplementation in their drinking water (80 mM). The overall study flow and design is summarized in Supplementary Figure S1. After the HFHS diet, the overall body weight, body fat and lean body mass composition were similar among the obese mice with and without carnosine, independent of genotype as shown in Table 1. However, HFHS diet dramatically increased gonadal body fat content (as a percent of body weight) in GPx4$^{+/-}$ mice compared with WT, yet this increase was fully suppressed with carnosine treatment in both genotypes. Notably, WT obese mice developed higher brown fat percent compared to lean WT mice. On the other hand, among the obese GPx4$^{+/-}$ mice, only the carnosine treated group developed significantly higher brown fat content. Carnosine treatment demonstrated a mild enhancement in glycemic control but not insulin sensitivity (data not shown), in both WT mice. Obese mice maintained a normal cardiac output, ejection fraction and stroke volume regardless of carnosine treatment in both genotypes.

2.2 Effect of HFHS diet and carnosine on GPx4 expression and RCS in myocardial tissue

We observed substantial upregulation of myocardial GPx4 mRNA and protein content with HFHS diet and carnosine

TABLE 1 Body composition and cardiometabolic parameters.

Parameter	WT CNTL	WT HFHS	WT HFHS + Carnosine	GPx4$^{+/-}$CNTL	GPx4$^{+/-}$HFHS	GPx4$^{+/-}$HFHS + Carnosine	*p-value**
Final body weight (g)	31.02 ± 0.67	50.86 ± 0.95^{a}	50.01 ± 0.9^{a}	31.56 ± 0.7	49.93 ± 0.4^{b}	49.45 ± 0.6^{b}	<0.0001
Body fat %	15.79 ± 3.1	43.02 ± 0.6^{a}	43.89 ± 0.9^{a}	15.82 ± 2.3	46.96 ± 1.1^{b}	43.78 ± 0.7^{b}	<0.0001
Lean mass%	64.98 ± 2.3	44.87 ± .09^{a}	45.17 ± 0.7^{a}	65.03 ± 2.0	43.59 ± 0.8^{b}	45.82 ± 0.4^{b}	<0.0001
Gonadal fat %	2.18 ± 0.1	2.98 ± 0.1^{a}	2.59 ± 0.1^{c1}	2.08 ± 0.2	4.17 ± 0.1^{b,c2}	2.55 ± 0.1^{d}	a = 0.0012
							c1 <0.05
							c2, b, d < 0.0001
Brown fat %	1.04 ± 0.1	1.35 ± 0.1^{a}	1.38 ± 0.1^{a}	1.00 ± 0.1	1.26 ± 0.2	1.49 ± 0.1^{b,d}	a, d < 0.05
							b = 0.0003
Glucose AUC (GTT)	4,891 ± 1,046	24,578 ± 1635^{a}	18,210 ± 2015^{a,c}	11,331 ± 2445	23,145 ± 2589^{b}	17,626 ± 4,498 ^{d}	a< 0.0001
							b < 0.0006
							d, c < 0.05
Stroke volume (μL	30.88 ± 1.2	37.47 ± 2.3	41.05 ± 3.0	33.60 ± 2.6	44.27 ± 3^{b}	41.32 ± 1.184	b < 0.05
Ejection fraction (%)	0.8301 ± 0.003	0.7757 ± 0.02	0.8251 ± 0.01	0.8343 ± 0.01	0.8339 ± 0.02	0.8418 ± 0.01	-
Cardiac output (μL/min)	22,262 ± 1,074	24,037 ± 1,487	27,531 ± 1716	23,068 ± 973.6	28,668 ± 1,679	29,371 ± 1042^{b}	b < 0.05
Left ventricle thickness (mm)	0.7011 ± 0.02	0.6897 ± 0.02	0.7591 ± 0.02	0.7694 ± 0.2	0.7638 ± 0.02	0.7376 ± 0.02	-

Values are mean ± SEM., n = 6–12 per group.* Statistical differences between groups were compared using one-way ANOVA, with *post hoc* Tukey's tests for multiple comparisons between treatments within each genotype.^{a} $p < 0.05$ vs WT-CNTL.^{b} $p < 0.05$ vs GPx4$^{+/-}$CNTL.^{c} $p < 0.05$ vs WT-HFHS.^{d} $p < 0.05$ vs GPx4$^{+/-}$HFHS., Abbreviations: WT; wild type, CNTL; control diet, HFHS; high fat high sucrose diet, GTT; intraperitoneal glucose tolerance test.

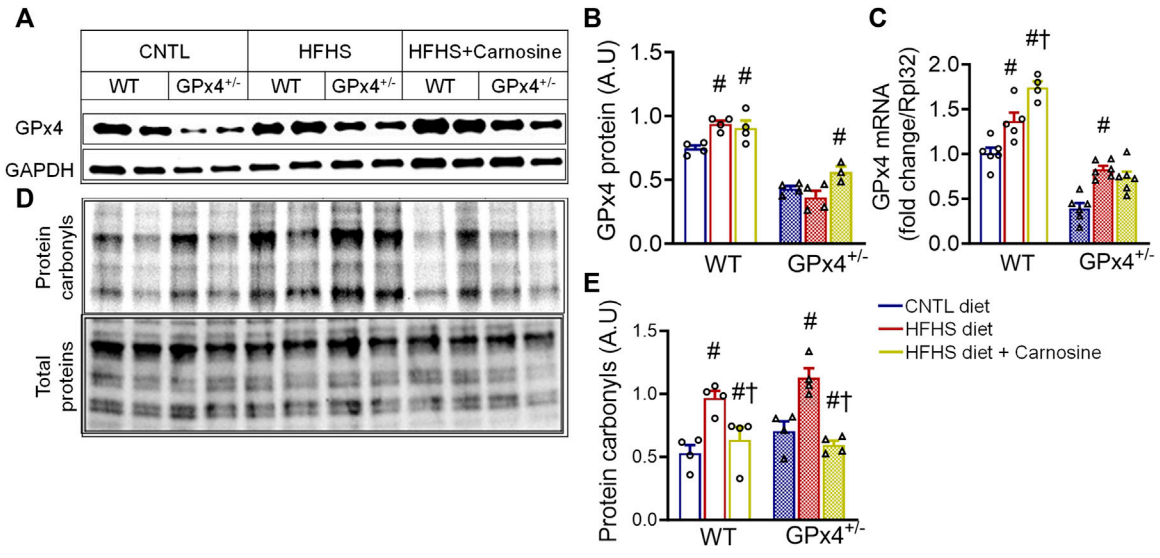

FIGURE 1
Myocardial GPx4 expression and protein carbonylation. Shown in **(A)** are representative immunoblots of GPx4 protein in left venticular samples with the corresponding densitometric analysis in **(B)**. GPx4 mRNA gene expression is shown in **(C)**. Representative image of Cy5.5 labeled protein carbonyls in whole heart homogenates, n = 2 mice per treatment group is shown in **(D)** with corresponding densitometric analysis shown in **(E)**. One = way ANOVA with *post hoc* Tukey's tests for multiple comparisons within the same genotype: #$p < 0.05$ vs. CNTL, †$p < 0.05$ vs HFHS. n = 4–6 mice per treatment group. WT, wild type; CNTL, control diet; HFHS, high fat high sucrose diet.

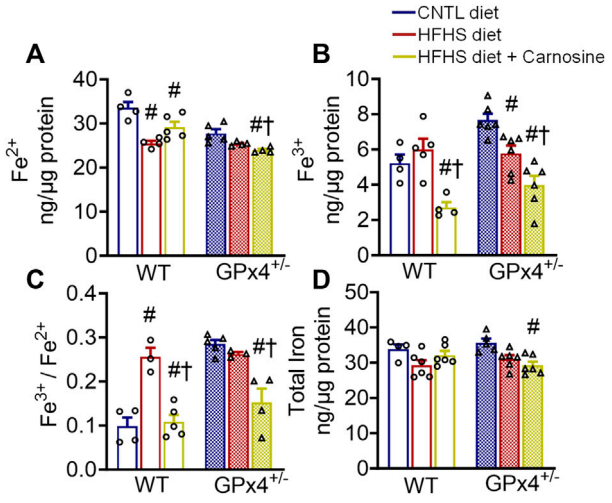

FIGURE 2
Myocardial iron content. Concentration of ferrous **(A)**, ferric **(B)**, and ferric/ferrous iron ratio **(C)**, in left venticular tissue samples are shown, along with total iron **(D)**. Values represent mean ± SEM. One-way ANOVA with *post hoc* Turkey's tests for multiple comparisons within the same genotype: #$p < 0.05$ vs. CNTL, †$p < 0.05$ vs. HFHS. n = 4–6 mice per treatment group. WT, wild type; CNTL, control diet; HFHS, high fat high sucrose diet.

(Figures 1A–C). To test if this increase in GPx4 was accompanied by other redox enzymes as part of a more global antioxidant response we analysed expression of several other key antioxidant genes and found rather unremarkable changes, with only a small upregulation in glutathione S-transferase (GST) and catalase mRNA with HFHS diet or carnosine (Supplementary Figure S2).

Given that HFHS diet is known to induce lipid peroxidation and RCS formation in the heart, particularly in GPx4$^{+/-}$ mice, (Méndez et al., 2014; Kobi et al., 2023), we next sought to the evaluate the effect of carnosine on protein carbonylation in myocardium from these mice. HFHS diet did indeed increase myocardial protein carbonyls, particularly in GPx4$^{+/-}$ mice, while carnosine treatment effectively blunted protein carbonylation in both genotypes (Figures 1D,E). Since carbonyl stress has been linked with inflammation and fibrosis in many studies (Tanase et al., 2016; Maruyama and Imanaka-Yoshida, 2022), we examined expression of several pro-inflammatory and pro-fibrotic genes and observed a small but significant increase in TGF-β and RAGE expression in the obese GPx4$^{+/-}$ mice as we have previously shown (Katunga et al., 2015), and this upregulation was fully abolished with carnosine treatment. We also observed a significant reduction in iNOS gene expression in the carnosine treated obese mice (Supplementary Figure S3). No significant changes in expression of IL-6, IL-1β and TNF-α were found (data not shown).

We next investigated whether HFHS diet and/or carnosine influenced protein carbonyl levels in the circulation. To do this, we measured serum 4-HNE-protein adducts after food restriction and ~30 min following glucose challenge. No significant differences in serum 4-HNE protein adducts were found between groups in the fasted (i.e., food restricted) state, but adducts were substantially increased after glucose challenge with all groups, although the greatest increase appeared with HFHS diet. Carnosine treatment blunted the increase in 4-HNE adducts following glucose challenge (Supplementary Figure S4). No differences in overall 4-HNE adducts were found between WT and GPx4$^{+/-}$ mice in any of the treatment groups (data not shown).

2.3 Myocardial iron levels following HFHS diet and carnosine

Carnosine treatment has been shown to have modest antioxidant effects in several *in vitro* and *in vivo* models, and this effect has been linked partly to its metal chelation ability (Reddy et al., 2005; Hider et al., 2021). Considering the role of iron in the initiation and the propagation of lipid peroxidation (Chen et al., 2021), we next examined myocardial iron levels in the mice from each treatment group. Interestingly, carnosine treatment moderately decreased Fe^{2+} and substantially decreased Fe^{3+} levels in both WT and GPx4$^{+/-}$ mice (Figures 2A–C). Although no significant differences in total myocardial iron levels were found between WT and GPx4$^{+/-}$ mice, carnosine significantly decreased Fe^{3+}/Fe^{2+} in HFHS diet in both genotypes (Figures 2C,D). To determine whether this effect on the Fe^{3+}/Fe^{2+} ratio is mediated by changes in iron transporters or other regulators, we analyzed expression of several iron homeostasis proteins and found that transferrin receptor 1 (TfR1) expression is increased by carnosine treatment in GPx4$^{+/-}$ mice but not WT. No significant differences in ferritin heavy chain 1 and ferritin light chain were observed, however (Supplementary Figure S5).

2.4 Effect of carnosine on cardiac fibrosis and collagen cross-linking with HFHS diet

Many previous studies have shown that obesity is accompanied by extracellular matrix protein expansion as a result of collagen deposition in the heart (Tikellis et al., 2008; Natarajan et al., 2019). Histological staining of fixed myocardial tissue with Masson's trichrome confirmed that fibrosis was mitigated by carnosine in both WT and GPx4$^{+/-}$ mice with HFHS diet (Figure 3A). Quantitative analysis of myocardial hydroxyproline, a marker for collagen, was significantly higher in both WT and GPx4$^{+/-}$ mice with HFHS diet when compared to lean mice, and carnosine treatment completely normalized these levels (Figure 3B). Furthermore, concentrations of the insoluble form of hydroxyproline, which indicates degree of collagen cross-linking in the tissue, was also normalized by carnosine treatment (Figure 3C).

To gain mechanistic insights on the antifibrotic mechanisms of carnosine therapy, we took advantage of the rheological properties of collagen suspensions, namely, its elastic moduli, and how elasticity changes occur when collagen becomes 'stiff' in response to cross-linking. Here, using a modified protocol previously established (Aslanides et al., 2016) we performed an *in vitro* study with soluble collagen to determine the effect of a mixture of reactive biogenic aldehydes and glucose on collagen cross-linking using rheometry. The aldehyde mixture included 4-HNE (lipid-derived aldehyde), 3,4-dihydroxyphenylacetaldehyde (DOPAL) and 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL). The latter are dopamine and norepinephrine metabolites, respectively, which we

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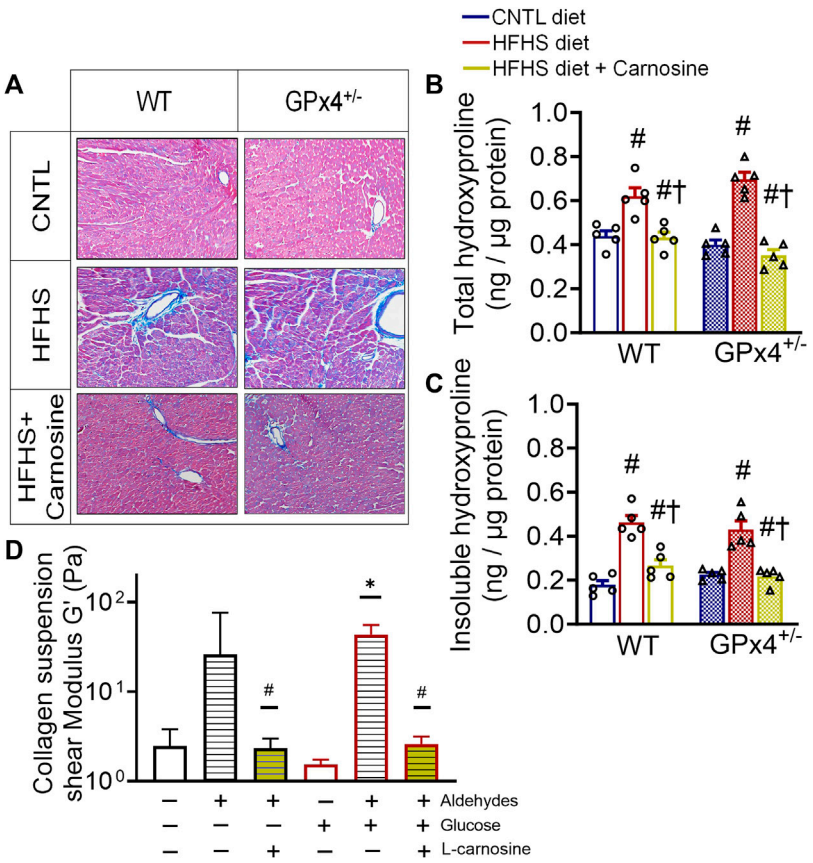

FIGURE 3
Myocardial collagen, fibrosis and *in vitro* collagen cross-linking. Representative images of Masson's trichrome staining in fixed myocardial tissue slices shown in **(A)**, n = 2 mice per treatment group, images are representative of 20 image fields captured per mouse under ×20 magnification. Total hydroxyproline concentration **(B)** and insoluble hydroxyproline concentration **(C)** in left ventricular tissue samples, n = 5, values are mean ± SEM, oneway ANOVA with *post hoc* Tukey's tests for multiple comparisons within the same genotype: #$p < 0.05$ vs. CNTL, †$p < 0.05$ vs HFHS. n = 5 mice per treatment group. Shown in **(D)** is effect of carnosine on collagen cross-linking stimulated by aldehydes and glucose *in vitro* as outlined in the Methods section. Suspensions of collagen were treated with a mixture of biogenic aldehydes (HNE 50 M, DOPAL 10 M, DOPEGAL 10 M) alone or with L-carnosine (10 mM). Elastic moduli of collagen were measured by rheometer at w = 2.122 Hz *$p < 0.05$ *versus* untreated control, #$p < 0.05$ *versus* aldehyde-treated group for each respective ± glucose. WT, wild type; CNTL, control diet; HFHS, high fat high sucrose diet; GTT, oral glucose tolerance test.

have previously shown are biogenic aldehydes formed in heart and are increased in myocardial tissues from patients with T2 diabetes (Monroe and Anderson, 2021; Nelson et al., 2021). We found that collagen cross-linking was stimulated by these aldehydes in the absence ($p = 0.0545$) and presence of glucose, as indicated by higher shear modulus of the suspension (Figure 3D). However, addition of carnosine to the mixture completely suppressed the aldehyde induced cross-linking.

2.5 Effect of carnosine on global cardiac gene expression with HFHS diet

To further interrogate potential mechanisms of carnosine's antifibrotic effects, we performed unbiased mRNA sequencing analysis on myocardial tissue from mice in each experimental group. As expected, major changes in gene expression were found in hearts between WT and GPx4$^{+/-}$ mice (not shown) and between CNTL and HFHS diet (not shown). Interestingly, carnosine only influenced a modest amount of overall gene expression in the hearts when compared with HFHS diet alone, with <50 transcripts passing the threshold of significance in our analysis for either up- or downregulation (Figures 4A,B). Of note, KEGG pathway analysis of differentially expressed gene pathways did list AGE-RAGE and TGF-β as significantly changing with carnosine (Figure 4C and Supplementary Table S3), which support our qRT-PCR results.

3 Discussion

Fibrosis is a significant pathogenic contributor to most cardiac disorders including valve disease, cardiomyopathies, arrhythmias and heart failure. Unfortunately, at present there are essentially no pharmacotherapies used in the clinic that specifically target fibrosis. This is partly due to the complex role of fibrosis in wound healing combined with the fact that the mechanisms controlling extracellular matrix expansion and collagen turnover in heart remain incompletely understood. Moreover, obesity induced cardiac fibrosis is a complex heterogenous condition involving a wide variety of molecular pathways. Efforts to understand fibrosis

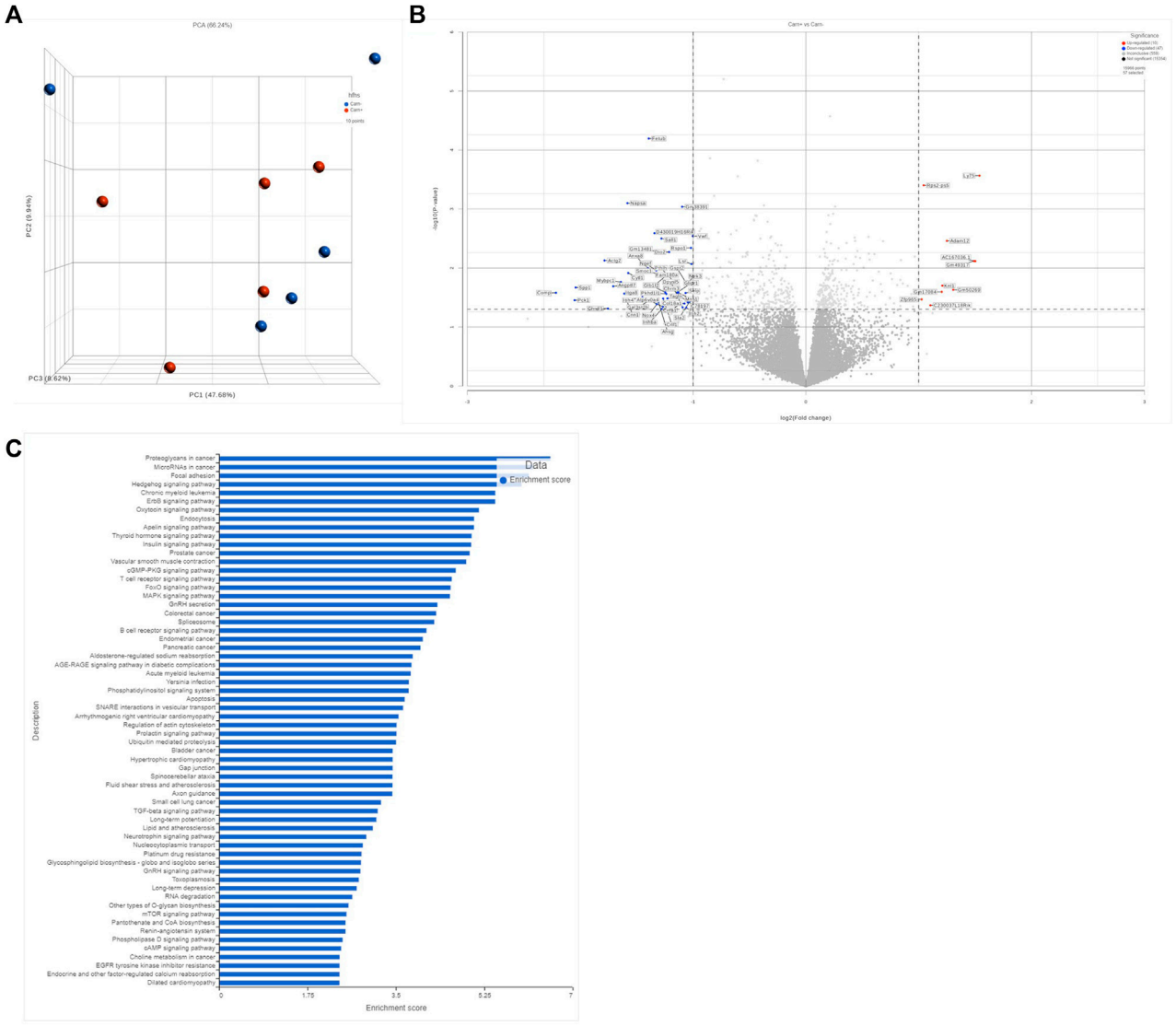

FIGURE 4
Transcriptomic anlysis of bulk myocardial tissue. Comparison of unbiased transcript expression in myocardial tissues from WT mice on HFHS diet with and without carnosine treatment. N = 5 per treatment group. Shown in **(A)** is a PCA plot accessing sample variance among group. Shown in **(B)** is a volcano plot depicting the top 250 differentially expressed sp transcripts, with the significant upregulated and downregulated genes annotated. *Y*-axis represents significance as –Log10 (*p*-value). *X*-axis represents the Beta value (natural log of the fold change between groups). Shown in **(C)** is a gene ontology pathway analysis based on the filtered list of genes with minimum *p*-value of 0.1 and minimum read count of 50 in at least 50 samples.

and its mechanisms could be of great benefit for developing new or existing therapies for cardiac fibrosis that could help lower incidence of cardiovascular diseases and their global public health burden (Wong et al., 2012; Travers et al., 2016; Hinderer and Schenke-Layland, 2019; Travers et al., 2022). To this end, our study demonstrates that oral administration of the histidyl dipeptide L-carnosine mitigates carbonyl stress in the heart with diet induced obesity and this consequently leads to less cross-linked collagen and ultimately, less cardiac fibrosis. Furthermore, the cardioprotective effects of oral carnosine are likely multifactorial and indirect, given that cardiac transcriptomic analysis of carnosine treated mice showed only modest differences compared with untreated animals. Much of the indirect effects of carnosine involve its iron chelating ability and its role as an indirect antioxidant, enhancing GPx4 expression in the heart following HFHS diet even in mice with GPx4 haploinsufficiency.

Carnosine is an endogenous dipeptide that has been extensively investigated as a cardio/neuroprotectant in numerous studies including atherosclerosis (Barski et al., 2013), diabetes and its associated micro and macrovascular complications (Lee et al., 2005; Caruso et al., 2023), aging (Hipkiss et al., 2016) and various neurodegenerative disorders (Caruso et al., 2019; Solana-Manrique et al., 2022). It is widely accepted that carnosine does have some direct antioxidant effect, including ability to neutralize ROS, RCS and reactive nitrogen species (Hipkiss, 2009; Boldyrev et al., 2013; Caruso et al., 2017; Aldini et al., 2021). However, the direct antioxidant effect of carnosine is unlikely to be a primary therapeutic mechanism *in vivo* given the high concentrations of carnosine

required for these effects, and accumulating evidence suggests that alternative therapeutic mechanisms are involved. In this study, we found that oral carnosine supplementation induces significant GPx4 expression and content in the heart. Such an effect of carnosine has been shown previously by other groups, where carnosine treatment normalized lipid peroxidation levels and glutathione ratio, increased superoxide dismutase (SOD) and catalase (CAT) activities in rat brain treated with salsolinol (a toxic pesticide) (Zhao et al., 2017). Similarly, carnosine supplementation was found to increase the expression and activities of SOD, CAT, and glutathione peroxidase (GPX) in the liver, muscles, and plasma of pigs (Caruso et al., 2017).

Studies have also shown carnosine's effectiveness as a chelator of divalent cations, including iron. Iron is a transition metal that exists in either ferric (Fe^{3+}) or ferrous (Fe^{2+}) forms in biological systems. In our study, carnosine treatment modulated the redox equilibrium between Fe^{3+}/Fe^{2+} ions in the myocardium of obese mice (Figure 2), leading to dramatic decrease in the Fe^{3+}/Fe^{2+} ratio. Fe^{2+} ions react with hydrogen peroxide to produce Fe^{3+} ions as well as hydroxyl free radicals ($OH^{\bullet}$) that initiate lipid peroxidation through a Fenton-based mechanism (Minotti and Aust, 1987a; Latunde-Dada, 2017). The classical dogma regarding this reaction has always maintained that Fe^{2+} is the form required to initiate lipoperoxidation. Importantly, it has been shown that the presence of Fe^{2+} alone is not sufficient to induce lipid peroxidation, while the addition of Fe^{3+}/Fe^{2+} in 1:1 ratio potently induces lipid peroxidation (Minotti and Aust, 1987b; Braughler et al., 1987). This finding was confirmed by a recent study which showed that addition of Fe^{3+} to the reaction mixture stimulates lipoperoxidation (Ohyashiki et al., 2002). Moreover, the importance of the Fe^{3+}/Fe^{2+} ratio under different peroxidation conditions was demonstrated in classic redox equilibria studies which concluded that this ratio directly regulates the rate and extent of lipid peroxidation (Braughler et al., 1986; Minotti and Aust, 1987b; Ohyashiki et al., 2002). Although the absolute ratio of Fe^{3+}/Fe^{2+} was variable among these studies depending on the reaction components (e.g., oxidants, lipids), what is undeniably clear is that higher Fe^{3+}/Fe^{2+} favors a more rapid and extensive lipid peroxidation (Tadolini and Hakim, 1996; Tadolini et al., 1997). This might be partly explained by the possibility that Fe^{3+} ions could complex with polyunsaturated fatty acids at their Δ^9 or Δ^{11} carbon constituent, thus facilitating their peroxidation, as shown recently (Morrill et al., 2004). Given the known link between lipid peroxidation and ferroptosis, it is notable that a higher Fe^{3+}/Fe^{2+} ratio was found to be associated with ferroptosis in serum of Eales disease patients and in the amyloid plaque regains of mouse brains in an Alzheimers' model. Both conditions are associated with lipid peroxidation and advanced glycation end product (AGE) accumulation (Swamy-Mruthinti et al., 2002; Selvi et al., 2007; Wu et al., 2023). However, the prooxidant effect of iron in these studies is typically examined in the context of total iron levels rather than reporting the Fe^{3+}/Fe^{2+} ratio, which makes the *in vivo* and translational significance of this observation somewhat limited.

In addition to its ability to chelate metals, it is well established that carnosine is a potent RCS scavenger, thereby inhibiting protein carbonyl formation and its consequent effects in cells and tissues. Consistent with this, our findings show that carnosine treatment reduces the overall level of oxidative protein damage in the myocardium, as indicated by reduced protein carbonyls in the tissue (Figure 1). Protein carbonyls are considered a form of irreversible post-translational protein modification and have been found to cause derangements in cellular metabolism and signaling and implicated in the pathogenesis of obesity (Macdonald et al., 2001; Grimsrud et al., 2008; Méndez et al., 2014; Katunga et al., 2015; de Souza Bastos et al., 2016). Products of lipid peroxidation such as the α,β-unsaturated carbonyls (e.g., 4-HNE) react with the nucleophilic residues in proteins (e.g., Cys, Lys, His and Arg) resulting in the formation of inter and intramolecular cross-links (Spickett and Pitt, 2019). Protein carbonylation is also caused by AGEs (i.e., sugar-amines) which are forms of oxidized glucose-derived aldehydes generated under chronic hyperglycemic conditions (Gaar et al., 2020). Structural changes in proteins caused by carbonylation leads to protein unfolding and polymerization, ultimately leading to formation of insoluble cross-linked protein aggregates. These protein aggregates evade proteasomal degradation, which prolongs their turnover rate and enhances their accumulation (Negre-Salvayre et al., 2008; Dilek, 2022).

It is in this context where the major antifibrotic effect of carnosine appears to exist, based on our findings. The extracellular matrix is primarily a collagen-based network (96%) that is highly susceptible to crosslinking on Lys and Arg residues within the network (Maruyama and Imanaka-Yoshida, 2022). Measuring the extent of collagen crosslinking by assessing the concentration of insoluble compared with soluble collagen is routinely used as an index of collagen cross-linking in tissues, and enhanced crosslinking in the heart has been reported in cardiometabolic disorders such as hypertension (Woodiwiss et al., 2001; Badenhorst et al., 2003) and diabetes (Avendano et al., 1999; Liu et al., 2003). Importantly, collagen crosslinking has been recognized as a key modulator of left ventricular diastolic stiffness in patients with heart failure (Asif et al., 2000; Klotz et al., 2005). The anti-carbonyl and anti-crosslinking effect of carnosine was initially reported by Hipkiss et al. (Hipkiss and Brownson, 2000) and others (Carini et al., 2003; Baba et al., 2013; Albrecht et al., 2017), the mechanism of which has mainly been attributed to carnosine's ability to react with RCS through Michael addition resulting in carnosine-adducts that are detoxified by dehydrogenases (Bispo et al., 2016). *In vitro* studies have shown that carnosine's anti-crosslinking effect was able to preserve activities of several enzymes such as SOD (Hipkiss et al., 1995), esterase (Yan and Harding, 2005) and aspartate aminotransferase (Swearengin et al., 1999). In the present study, carnosine was able to block *in vitro* collagen crosslinking stimulated by a mixture of biogenic aldehydes and sugars (Figure 3), and this was reflected in the obese mice where carnosine substantially decreased the concentration of insoluble collagen in the heart.

To conclude, our study has revealed that oral carnosine therapy has potent antifibrotic and carbonyl detoxifying effects in the heart with diet induced obesity. Translational significance of these findings remains to be determined and there are challenges with oral carnosine therapy in humans due to serum carnosinase activity (Menini et al., 2012; Vistoli et al., 2012; Boldyrev et al., 2013). However, carnosinase-resistant analogs of carnosine have shown similar beneficial effects in rodent models of obesity and cardiovascular disease (Menini et al., 2012; Anderson et al.,

2018). It is noteworthy that multiple small clinical trials of oral carnosine therapy have shown modest but significant cardiometabolic benefits in obese, prediabetic individuals (Côté et al., 2012; de Courten et al., 2016; Baye et al., 2017; Baye et al., 2018). Future work is obviously needed to optimize and exploit therapeutic efficacy of carnosine in humans.

4 Materials and methods

4.1 Mouse model, diet and oral carnosine intervention

Animal care and experimental procedures were reviewed and approved by the University of Iowa Institutional Animal Care and Use Committee (IACUC) prior to beginning the study. $GPx4^{+/-}$ mice were generated as previously described and maintained by backcross with C57BL6/J (Jackson Laboratory) mice. At 8–10 weeks, male $GPx4^{+/-}$ and WT littermates were randomly assigned to either normal chow diet (CNTL, D20122207, Research Diets, Inc) or high fat high sucrose diet (HFHS), D09071704, Research Diets, Inc) for 16 weeks. Seven weeks after starting the diet, a subgroup of the mice on HFHS diet in both genotypes was started on 80 mM carnosine supplemented in their drinking water and the diet intervention was continued. The mice randomization was continued until there were 10 mice per treatment group Diets were matched for protein and macronutrients except that the HFHS diet is comprised of lard-based fat (35.5% daily kcal) and cholesterol (1.5% daily kcal), plus sucrose (38% daily kcal), compared with the CNTL diet with low fat (6% daily kcal), and starch-based carbohydrates (~75% daily kcal). HFHS diet and water were refreshed every 3–4 days. Carnosine was confirmed to be completely stable for this length of time in drinking water using HPLC (Supplementary Table S1). At the end of the diet −/+ carnosine intervention, the mice were euthanized after 8 h fast followed by either a 1 g/kg bolus (i.p.) of 50% dextrose in saline, or normal saline.

4.2 Metabolic and cardiovascular parameters

Body weight was recorded at baseline and then once every week during the diet intervention. An intraperitoneal glucose tolerance test (GTT) was performed 14 weeks after starting the diet using standard technique. Briefly, a dose of 1 g/kg of 50% dextrose in saline was administered by i. p. injection after 6 h of fasting, followed by blood glucose measurements using glucometer (OneTouch, Verio Flex). Body composition was determined in each animal within 24 h of euthaniasia by the *Time Domain NMR Analyzer* (LF50, Beckman Coulter). Echocardiography in mice was performed 1 week prior to euthanasia in conscious mice using a 30 MHz transducer (Vevo 2100, VisualSonics, Toronto, ON), by staff in the cardiovascular phenotyping core facility at University of Iowa.

4.3 Masson's trichrome stain

Mice were anesthetized with ketamine xylazine and perfused via cardiac puncture with 4% paraformaldehyde (PFA) for 20 min. Hearts were then sectioned and stained at the University of Iowa Pathology Core, according to standard procedures. Briefly, paraffin-embedded hearts were sectioned at 5-µm thickness, then deparaffinized and rehydrated with serial dilutions of concentrated alcohol (100% alcohol, 95% alcohol 70% alcohol). Sections were then re-fixed in Bouin's solution for 15 min at 56°C and rinsed with water for 5–10 min to improve staining quality. Sections were then incubated in Weigert's iron hematoxylin solution for 10 min then washed in running warm water buffer for 5 min. Then sections were stained with Biebrich scarlet acid fuchsin solution for 10–15 min and washed in distilled water. Sections were then subjected to differentiation in phosphomolybdic-phosphotungstic acid solution for 10–15 min and transferred immediately to aniline blue solution and stained for additional 5–10 min. This last stain was followed by a brief rinse in distilled water and differentiation in 1% acetic acid solution for ~5 min. Lastly, sections were dehydrated using 95% ethyl alcohol, absolute ethyl alcohol and xylene. Stained sections were visualized by a EVOS FL AUTO2 microscope (ThermoFisher, Inc.) and images captured by camera with ×10 objective.

4.4 Immunoblot analysis

Samples of left ventricular tissue (10–15 mg) were homogenized in TEE buffer (10 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100 pH 7.4) with a protease/phosphatase inhibitor cocktail (Roche) using a glass grinder (Kimble Chase, Vineland, NJ, United States). Lysates were then mixed with 10% β-mercaptoethanol and Laemmli buffer and loaded onto 4%–20% gradient polyacrylamide gel (Bio-Rad, Hercules, CA) and subjected to electrophoresis. Proteins were then transferred from gel to a PVDF membrane via semi-dry apparatus, blocked in 3% bovine serum albumin, and incubated with primary antibodies for GPx4 and GAPDH (Abcam, Cambridge, UK). Membranes were then incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody and scanned by the iBright Imaging System (iBright FL1000, ThermoFisher Scientific, Waltham, MA, United States). Densitometric analysis was performed using ImageJ (NIH).

4.5 Quantitative analysis of serum 4-HNE adducts

For quantitative analysis of 4-HNE adducts in serum, an enzyme-linked immunosorbent assay (ELISA) established by our group was used (Monroe and Anderson, 2019). In brief, standards of 4-HNE-protein adducts were first made by incubating serial dilutions of 4-HNE (Cayman chemicals, United States) in 1 mg/mL BSA/50 mM sodium phosphate buffer (pH = 7.4) at 37 C° for 24 h. Serum and standards were loaded in duplicate on immunolon-coated 96-well plates (Nunc MaxiSorp, Invitrogen, United States) and incubated overnight at 4C° with a continuous rocking. The plate was then blocked with 10% BSA for 2 h followed by overnight incubation with 4-HNE polyclonal primary antibody (Sigma-Aldrich). Next the plate was incubated with a HRP-conjugated mouse anti-goat secondary antibody followed by incubation with

55 μM Amplex red (fluorogenic reagent) for 30 min at room temperature. Fluorescence intensity of each well was then measured using the BioTek Synergy microplate reader at 530/595 nm (ex/em), concentrations of 4-HNE protein adducts in the serum samples were determined against the standard curve.

4.6 Hydrazide labeling of carbonyl-modified proteins in myocardial tissue

For unbiased labeling of protein carbonyls in the myocardial tissues we used a protocol previously established by our group (Katunga et al., 2015). Homogenates of left ventricular tissue (~10 mg) were prepared using TissueRuptorII (Qiagen) under anaerobic conditions in a nitrogen-saturated glove box (Coy Laboratory Products, Grass Lake, MI) using degassed TEE buffer. Hydrazide Cy5.5 dye (Lumiprobe, Maryland, United States), was incubated with the protein lysates at final concertation of 25uM for 2 hours on an orbital shaker at room temperature, then incubated overnight at 4 C°. Lysates were then loaded into 4%–20% gradient acrylamide gels (Bio-Rad, Hercules, CA) and subjected to electrophoresis. The Cy5.5 label on carbonyls was then captured by imaging the gel with the iBright Imaging System, and total protein was captured using No-Stain protein labeling reagent (ThermoFisher Scientific, Waltham, MA, United States). Images were analyzed by densitometry using ImageJ (NIH).

4.7 Myocardial iron content

Iron content of myocardial tissue samples was calculated using a colorimetric iron assay kit (Abcam) according to the manufacturer's instructions. Briefly, around 15 mg of the heart tissue were lysed using the assay buffer provided with the kit. Standards and samples were loaded into a 96-well plate and incubated with iron reducer at 37 C for 30 min. Next, iron probes were added to the wells and incubated at 37 C for 30 min. Sample absorption was then measured in duplicate at 593 nm with a plate-reader (Epoch, Bio-Tek).

4.8 Quantitative analysis of myocardial collagen concentrations

To measure the amount of the soluble and cross-linked collagen separately in the heart samples, we used a protocol for measuring hydroxyproline as a surrogate for collagen using a method established previously by our group (Anderson et al., 2018), which involves first separating the soluble from insoluble (i.e., cross-linked) collagen fractions as described below.

4.8.1 Soluble and insoluble collagen fractionation

Pepsin (Sigma-Aldrich) was dissolved in phosphate-buffered saline (pH 3.0) and then incubated with frozen left ventricular tissue sample (40 μg pepsin/mg tissue) to digest the heart samples at 37C° for 30 min with gentle shaking. The digestion was then stopped by adding 2% SDS, 0.6M β-mercaptoethanol solution to each tissue suspension. This was followed by a 30-min sonication step to enhance the release of the soluble portion into the solution. Soluble protein fraction was then separated from the insoluble fraction by centrifuging at 10000xg for 90 min at 4C°. The soluble fraction (the supernatant) was then separated from the insoluble protein fraction (pellet) into prelabeled glass tubes. The pellets were then resuspended in DDI water, and both sets of samples were completely dried by heating at 100C° overnight. Hydroxyproline concentration in each sample was then quantified by a colorimetric assay as described below.

4.8.2 Hydroxyproline assay

Dried protein within the glass tubes were hydrolyzed using HCl (6 M) at 100C° for 24 h. The HCl was then evaporated by heating at 100C° overnight and samples were resuspended in 50% isopropanol. Next, commercially obtained hydroxyproline (Sigma-Aldrich) was used to prepare standards in DDI water, and then standards and unknown samples were oxidized with chloramine-T solution (1.4%) for 5–7 min at room temperature. The oxidized standards and samples were then mixed with a solution of 4-dimethylaminobenzaldehyde (DMAB) in 60% perchloric acid and incubated for 17 h at room temperature in the dark. After that, absorption of the samples was measured in duplicates at 568 nm using a plate reader (Epoch, Bio-Tek). Concentration of hydroxyproline in the samples was then calculated using the hydroxyproline standard curve within each plate and normalized to the total protein concentration in each sample.

4.9 Rheometric collagen cross-linking assay

Collagen suspensions were prepared in phosphate buffered saline (PBS) and were mixed with either 10 μM DPEGAL or 25 mM glucose. Also, the same mixtures were prepared in addition to 10 mM carnosine treatment and incubated for 48 h at 37 C°. The elastic moduli of these collagen suspensions were measured at ω = 1.193 Hz using HAAKE™ RheoStress™ 1 Rheometer (Thermo Scientific) that utilizes HAAKE™ RheoWin3 software. The samples were poured between the bottom and the top plates and then a dynamic strain sweep to determine the linear viscoelastic range and the small amplitude oscillatory shear (SOAS) measurements were performed at a frequency ranging between 0.0895 Hz and 15.92 Hz.

4.10 RNA extraction and gene expression analysis using quantitative RT-PCR

Total RNA was extracted from left ventricular tissues (~10 mg), using the RNeasy Fibrous Tissue Kit (QIAGEN, Germantown, Maryland). Reverse transcription was performed with Superscript IV (ThermoFisher Scientific, Waltham, MA, United States) kit according to manufacturer's instructions. Quantitative PCR (qPCR) was performed according to the manufacturer's protocol on the ViiA 7 real-time PCR system (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, United States) using the PowerTrack™ SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA, United States). Primers used for each target are listed in Supplementary Table S2. Amplification curves were established and expression of each target mRNA was calculated using the ΔΔ Ct

(threshold cycle) method. Fold-change differences in target mRNAs were expressed relative to WT-CNTL as the baseline.

4.11 Transcriptomic profiling of myocardial tissue

mRNA sequencing of total bulk RNA myocardial tissue was performed by the University of Iowa Genomics Division, using commercial protocols provided by vendors. Briefly, 500 ng of total RNA (DNAse-1 treated) was used to prepare sequencing libraries using the Illumina TruSeq stranded mRNA library preparation kit (Cat. #RS-122–2101, Illumina, Inc., San Diego, CA). Final concentrations of the resulting indexed libraries were determined using the Fragment Analyzer (Agilent Technologies, Santa Clara, CA) and pooled equally for sequencing. Library pool concentration was determined using the Illumina Library Quantification Kit (KAPA Biosystems, Wilmington, MA) and sequenced on a SP flowcell of the Illumina NovaSeq 6,000 genome sequencer using 150 bp paired end SBS chemistry, at a read depth of ~35M reads per sample. RNA sequencing data was aligned using STAR version 020,201 to the GRCm38.98 release. The average mapping rate across samples was ~90% and read depth was ~25M aligned reads per sample. BAM files were uploaded to Partek Flow and feature counts performed on ensemble gene annotations. Gene level differential expression was performed using DESeq2 after median ratio normalization. Data visualizations (Volcano, PCA, cluster plots) were created using Partek flow software.

4.12 Statistical analysis

All the data in Table 1 are presented as mean ± SEM. Statistical analysis on mouse model variables were performed with GraphPad Prism (GraphPad Prism, La Jolla, Ca.). One-way ANOVA was performed on continuous variables followed by Tukey's post-tests for multiple comparisons between treatment groups within the same genotype. Statistical significance between groups was defined as $p < 0.05$. For RNA-seq data, the level of significance has been set at $p < 0.05$ and fold change >1 up or down. See Supplementary Table S3.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE250315.

Ethics statement

The animal study was approved by University of Iowa Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

IB: Conceptualization, Investigation, Methodology, Formal Analysis, Data curation, Writing–original draft. TM: Investigation, Methodology, Writing–review and editing, Formal Analysis. AA: Investigation, Methodology, Writing–review and editing. JM: Investigation, Methodology, Writing–review and editing. IL: Investigation, Methodology, Writing–review and editing. KB: Investigation, Methodology, Writing–review and editing. DG: Investigation, Methodology, Writing–review and editing. JM: Investigation, Methodology, Formal Analysis, Writing–review and editing. EA: Investigation, Methodology, Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1275388/full#supplementary-material

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Dietary antioxidant intake increases ankle brachial pressure index in men but not in women: a cross-sectional study

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Objective: Atherosclerosis is a significant cause of cardiovascular and cerebrovascular diseases, with a greater impact on men than women. Dietary antioxidant intake is inversely related to the risk of atherosclerosis development. We aimed to investigate the association between dietary composite antioxidant intake and the ankle brachial pressure index (ABPI). The ABPI is not only used for assessing the progression of arterial lesions but also for stratifying the risk of atherosclerotic disease.
Methods: We conducted a cross-sectional analysis involving 1,049 participants from the National Health and Nutrition Examination Survey (NHANES). We examined six antioxidants (zinc, selenium, carotenoids, and vitamins A, C, and E) and a composite dietary antioxidant index (CDAI) derived from these antioxidants as exposure variables. The primary outcomes encompassed cardio-metabolic parameters, including body mass index (BMI), body fat mass (BFM), systolic and diastolic blood pressure, triglycerides, HDL and LDL cholesterol, C-reactive protein, and the Ankle-Brachial Pressure Index (ABPI). Associations and interactions between variables were assessed using linear regression analyses. Moreover, mediation and moderation analysis is employed.
Results: Hierarchical multiple regression analysis revealed that among men, dietary intake of zinc, selenium, and vitamin A remained positively associated with a higher ABPI even after adjusting for covariates. Conversely, in the stratified regression analysis based on CDAI quartiles, a U-shaped association between CDAI and ABPI was suggested. Notably, no significant association between dietary antioxidant intake and ABPI was observed among women. CDAI, intake of Vitamin A, Vitamin C, and Vitamin E do not influence all-cause death through mediation by abpi, but rather have a direct effect on all-cause death. Moreover, there is a significant interaction between the intake of Vitamin A and gender, where a daily intake of Vitamin A more than 776 ug is especially beneficial for women.
Conclusion: The combined intake of nutrients with antioxidant properties may prevent the initiation and progression of atherosclerosis and influence the outcome in a sex-specific manner.

KEYWORDS

ABPI, CDAI, antioxidant, NHANES, mortality

1 Introduction

Ankle-Brachial Pressure Index (ABPI), a non-invasive diagnostic tool, measures the ratio of systolic blood pressure at the ankle to that at the brachial artery in the arm, serving as an indicator of peripheral artery disease (PAD) and vascular health (1). Antioxidants, encompassing an array of compounds such as vitamins, minerals, and phytochemicals, play a crucial role in counteracting oxidative stress, a process implicated in various chronic diseases (2). The ABPI and the intake of antioxidants have both emerged as influential factors in cardiovascular health, with recent research highlighting the importance of considering sex-specific differences in their association.

Sex-specific differences in cardiovascular health have gained prominence, underscoring the need to explore the interplay between ABPI and antioxidant intake in a nuanced manner. Cardiovascular diseases often manifest differently between males and females, with variations in risk factors, disease prevalence, and outcomes (3, 4). Factors like hormonal differences, genetics, and lifestyle choices contribute to these disparities, motivating the investigation of sex-specific associations between ABPI and antioxidant intake. The ABPI serves as a valuable marker of arterial stiffness and peripheral vascular function. Sex-specific variations in vascular health are well-documented, with differences in arterial compliance and endothelial function between men and women (5). These disparities could potentially influence the relationship between ABPI and antioxidant intake. Moreover, oxidative stress, a driving force behind cardiovascular diseases, may be influenced by sex-related factors, leading to divergent responses to antioxidants. Antioxidants have been extensively studied for their ability to combat oxidative stress and mitigate the risk of chronic diseases. Their impact on cardiovascular health, however, might differ between sexes due to varying physiological and hormonal profiles. This raises intriguing questions about the potential modulatory effects of antioxidant intake on ABPI in a sex-specific context.

This study seeks to provide an in-depth exploration of the association between the ABPI and antioxidant intake, emphasizing the importance of considering sex-specific differences. We aim to elucidate how sex-related factors might influence the relationship between ABPI and antioxidants, shedding light on potential sex-specific interventions and contributing to a more tailored approach to cardiovascular health management.

2 Methods

2.1 Study population

The National Health and Nutrition Examination Survey (NHANES) is a cornerstone of public health research and epidemiological studies conducted by the Centers for Disease Control and Prevention (CDC) in the United States. The NHANES data are released every 2 years. Data from the 1999–2004 NHANES cycle were used in this analysis, because these cycle specifically provided data for ABPI. All participants aged 40 years and over were eligible, as participants aged 40 years and older were asked to undergo the ABPI measurement. Participants were excluded if (1) Their dietary intakes of vitamins, carotenoids, zinc, or selenium are missing; (2) Their ABPI data are missing or ABPI > 1.5, which is considered an outlier (6, 7); (3) Their data for anthropometric measurements, including height, weight, and body fat mass (BFM), are missing; (4) Their data from physical examinations and biochemical analyses, including blood pressure (BP), total cholesterol, low-density lipoprotein cholesterol (LDL-cholesterol), high-density lipoprotein cholesterol (HDL-cholesterol), triglycerides, glycated hemoglobin (HbA1c), glucose, creatinine, urea nitrogen, and C-reactive protein (CRP), are missing; (5) The available information does not encompass their socio-demographic characteristics (such as age, gender, education level, and income), lifestyle factors (such as smoking habits, alcohol consumption, and physical activity), or medical records related to diabetes, hypertension, and hyperlipidemia. Figure 1 illustrated the inclusion and exclusion criteria employed in the selection of the final study population (Figure 1). The protocol was approved by the National Center for Health Statistics Institutional Review Board, and all participants provided written informed consent.

2.2 Dietary assessment

Dietary intake reports of six antioxidants (zinc, selenium, carotenoids and vitamins A, C and E), and total energy intake were included as exposures. All participants in the NHANES study are eligible for two 24-hour dietary recall interviews (8). The initial dietary recall interview is conducted face-to-face within the Mobile Examination Center, while the subsequent interview is conducted via telephone within a span of 3–10 days. In this analysis, dietary intakes of six antioxidants and total energy intake were assessed using 1-day values for individuals with single recalls and 2-day means for others. To comprehensively assess the synergistic impact of dietary antioxidants on ABPI, we employed a modified rendition of the Composite Dietary Antioxidant Index (CDAI), originally devised by Wright et al. (9).

2.3 ABPI measurement

Participants assume a supine position on the examination table for the assessment. Systolic pressure measurements are taken on the right arm (brachial artery) as well as on both ankles (posterior tibial arteries). If a participant has conditions such as a rash, an open wound on the right arm, a dialysis shunt, or a history of right-sided radical mastectomy that could compromise accurate measurement or lead to discomfort, the brachial pressure measurement is conducted using the left arm. Systolic blood pressure is recorded twice at each site for participants aged 40–59 years, while those aged 60 years and older undergo one measurement at each site. The computer system automatically

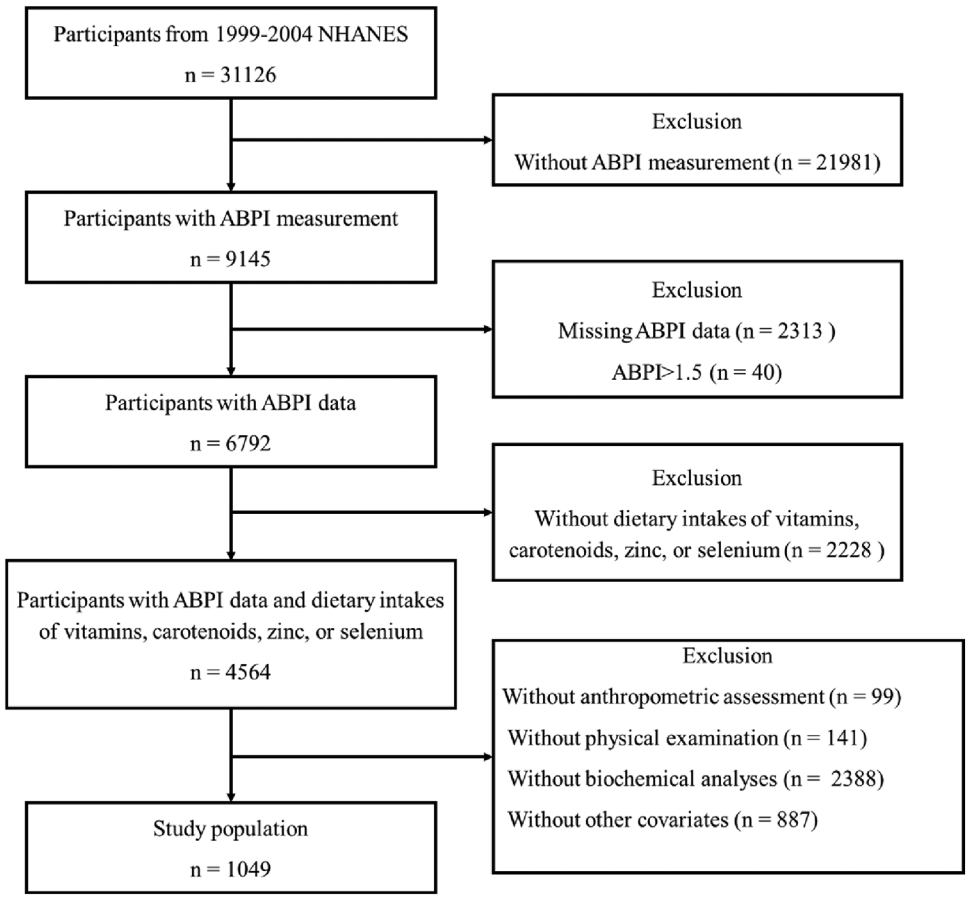

FIGURE 1
Illustration of inclusion and exclusion criteria as utilized to select the final study population.

calculates the Ankle-Brachial Pressure Index (ABPI). Specifically, the right ABPI is determined by dividing the mean systolic blood pressure in the right ankle by the average blood pressure in the arm. Similarly, the left ABPI is calculated by dividing the mean systolic blood pressure in the left ankle by the arm's mean blood pressure. The mean blood pressure values for both the arm and ankles are derived from the first and second readings taken at each respective site. For individuals aged 60 years and older, since the second reading is absent, the mean values represent the initial recorded blood pressure reading at the given site. This also applies to individuals aged 40–59 years who have a missing value for either the first or second blood pressure reading. In this analysis, the lower ABPI value from both the left and right sides is regarded as the definitive ABPI. If the calculated ABPI $\geq$ 1.3, the higher of the two values is chosen.

2.4 Anthropometric assessment

BMI was calculated as weight in kilograms divided by height in meters squared. Body fat mass was computed employing prediction equations derived from the NHANES survey, encompassing 7,531 men and 6,534 women who underwent dual-energy x-ray absorptiometry (DXA) examination, as formulated by Lee DH et al. (10).

2.5 Physical examination and biochemical analyses

Blood pressure (BP) was taken on all eligible individuals using a mercury sphygmomanometer. The technique used to obtain BP follows the recommendations of American Heart Association Human Blood Pressure Determination by sphygmomanometers (11).

Biochemical analyses were performed with a Hitachi Model 704 multichannel analyzer (Boehringer Mannheim Diagnostics, Indianapolis, IN), which encompassed the measurement of glucose, total cholesterol, triglycerides, creatinine, and urea nitrogen. HbA1c was measured by a fully automated glycohemoglobin analyzer, utilizing the principle of boronated affinity high performance liquid chromatography (HPLC) (Primus Corporation). HDL-cholesterol was measured using a direct immunoassay technique the LDL-cholesterol level was calculated according to the Friedewald equation. Latex-enhanced nephelometry is utilized for quantifying CRP.

2.6 Other covariates

Socio-economic features (such as age, gender, educational attainment, and family income to poverty ratio, PIR), lifestyle behaviors (including smoking habits, alcohol consumption, and level of physical exercise) and medical background of diabetes, hypertension, and hyperlipidemia were obtained through personal interviews (12). The degree of physical activity was assessed using the Czech adaptation of the International Physical Activity Questionnaire long form (IPAQ-L) and quantified as MET-min/week (13). Smoking status was classified into three categories: "never" (individuals who had smoked fewer than 100 cigarettes in their lifetime), "former" (individuals who had smoked more than 100 cigarettes in their lifetime but currently do not smoke at all), and "current" (individuals who had smoked more than 100 cigarettes in their lifetime and currently smoke either some days or every day). Alcohol consumption was classified into five categories: "never" (had <12 drinks per week in lifetime, one standard drink contains 14 grams of pure alcohol), "former" (had ≥12 drinks in 1 year and did not drink last year, or did not drink last year but drank ≥12 drinks in lifetime), "mild" (1 drink per day for female and 2 drinks per day for male), "moderate" (2 drinks per day for female and 3 drinks per day for male; or binge ≥2 and binge <5 per month), "heavy" (3 drinks per day for female and 4 drinks per for male; or binge ≥5 per month). Hypertension was characterized by a blood pressure measurement of 140/90 mmHg or higher, a previous diagnosis of hypertension, or the use of antihypertensive medication. Hyperlipidemia was determined by the presence of total cholesterol levels equal to or exceeding 5.0 mmol/L, LDL-cholesterol levels equal to or exceeding 3 mmol/L, triglyceride levels equal to or exceeding 1.7 mmol/L, or the use of lipid-lowering medication. Diabetes mellitus was identified by a previous diagnosis of diabetes, a fasting glucose level equal to or exceeding 7 mmol/L, or the use of antidiabetic medication.

2.7 Outcomes

All-cause mortality, referring to death from any cause until 31 December 2018, was the primary outcome. Mortality data were extracted from the 1999–2004 NHANES public-use linked mortality files. We analyzed the period from enrollment, marked by the date of the interview, to mortality for the purpose of censoring. The International Classification of Diseases, Tenth Revision codes (I00-I09, I11, I13, I20-I51, I60-I69) were used to define cardiovascular deaths. Any participant not matched with any death records was considered alive throughout the follow-up period.

2.8 Statistics

The characteristics of the study participants were depicted using percentages (%) or median along with interquartile range (IQR). Prior to subsequent analyses, the normal distribution of continuous variables was examined using the Kolmogorov–Smirnov test. For continuous variables exhibiting skewed distribution, the Mann–Whitney *U*-test (for comparisons between two groups) or the Kruskal–Wallis test (for comparisons involving three or more groups) was utilized. The χ^2 test was employed for contrasting categorical variables. Spearman tests were carried out for correlation analyses. To explore the relationships between dietary antioxidant intakes and ABPI, linear regression models were employed. Age, previous medical history, diabetes, hypertension, hyperlipidemia, income, education, smoking, alcohol consumption, physical activity, and total calorie intake were considered in Model 1. Model 2 included additional adjustments for body mass index (BMI), body fat mass (BFM), blood pressure (both systolic and diastolic), triglycerides, LDL-cholesterol, HDL-cholesterol, and CRP. Furthermore, we examined potential gender-related interactions in the relationship between antioxidant intake, CDAI, and ABPI. For female participants, an extra adjustment was made in Model 3 to account for menopause status. To address multiple comparisons, the Bonferroni method was employed, and adjusted *p*-values were obtained by multiplying the original *p*-value by the number of comparisons. The relationships among antioxidant intake, CDAI, ABPI, gender, and outcomes are examined through mediation and moderation effect analyses. The risk of outcomes occurrence influenced by various factors is analyzed using Cox regression analysis. All statistical tests were performed as two-tailed tests, and a *p*-value less than 0.05 was considered statistically significant. SPSS software (version 24.0) was employed for all statistical analyses.

3 Results

The study included 1,049 individuals (median age 57.00 years, IQR = 20.00; 53.48% of men), who satisfied the inclusion and exclusion criteria, with 46.52% being females and 67.83% being menopausal women (Table 1). While women exhibited a lower BMI ($p = 0.001$), men displayed a lower body fat mass ($p < 0.001$). In addition, men reported higher glucose and HbA1c levels compared to women ($p < 0.001$ and $p = 0.006$, respectively), and a greater prevalence of previous diabetes history ($p = 0.003$). Men also showed higher levels of creatinine ($p < 0.001$), urea nitrogen ($p < 0.001$), and triglycerides ($p = 0.009$), as well as lower levels of HDL-cholesterol ($p < 0.001$) than women. Age, educational level, PIR, use of lipid-lowering or antihypertensive medications did not differ between the two groups.

In our study, we observed that women had a decreased total energy intake compared to men, leading to a lower consumption of zinc ($p < 0.001$), selenium ($p < 0.001$), and vitamin E ($p < 0.001$), but not affecting the intake of vitamin A, vitamin C, and carotenoids. We investigated the relationships between dietary antioxidant intake, metabolic risk factors, and ABPI (Figure 2). Our findings revealed positive correlations between ABPI and the intake of zinc, selenium, vitamin A, vitamin C, and vitamin E. Additionally, we found significant interactions

TABLE 1 Characteristics of study participants in general and stratified by gender.

Characteristics median (IQR) or %	Total ($N = 1,049$)	Male ($N = 561$)	Female ($N = 488$)	Z/χ^2	p-value
Age, year	57.00 (20.00)	57.00 (21.00)	58.00 (20.00)	−0.658	0.511
High educational level	57.9%	57.0%	58.8%	0.336	0.562
PIR	3.47 (3.16)	3.68 (3.09)	3.32 (3.27)	−1.515	0.130
Smoking status				35.435	<0.001
Never	47.2%	38.9%	56.8%		
Former	37.4%	44.6%	29.1%		
Current	15.4%	16.6%	14.1%		
Alcohol drinking	88.5%	93.2%	83.1%	73.269	<0.001
Never	11.3%	5.9%	17.6%		
Former	21.1%	21.9%	20.1%		
Mild	44.2%	51.2%	36.3%		
Moderate	12.8%	8.0%	18.2%		
Heavy	10.6%	13.6%	7.8%		
Physical activity, MET-min/week	3,710.00 (5,527.50)	4,030.00 (6,055.00)	3,150.00 (4,983.80)	−3.327	0.001
BMI, kg/m^2	27.37 (6.38)	27.77 (5.83)	26.91 (7.06)	−3.286	0.001
BFM, kg	27.00 (11.50)	25.21 (10.63)	28.98 (11.29)	−6.918	<0.001
Systolic pressure, mmHg	125.00 (25.00)	124.00 (22.00)	126.00 (29.00)	−2.168	0.030
Diastolic pressure, mmHg	73.00 (15.00)	74.00 (15.00)	73.00 (15.00)	−1.627	0.104
ABPI	1.10 (0.15)	1.13 (0.13)	1.08 (0.13)	−6.915	<0.001
HbA1c, %	5.40 (0.50)	5.50 (0.50)	5.40 (0.50)	−2.741	0.006
Glucose, mmol/L	5.22 (0.84)	5.38 (0.88)	5.12 (0.78)	−7.336	<0.001
Creatinine, umol/L	79.56 (17.68)	88.40 (17.68)	70.72 (17.68)	−19.199	<0.001
Urea nitrogen, mmol/L	5.00 (2.14)	5.36 (2.15)	4.64 (2.14)	−5.957	<0.001
Triglycerides, mmol/L	1.26 (0.96)	1.33 (0.97)	1.21 (0.90)	−2.608	0.009
Total cholesterol, mmol/L	5.30 (1.31)	5.20 (1.27)	5.43 (1.27)	−4.415	<0.001
HDL-cholesterol, mmol/L	1.34 (0.55)	1.19 (0.39)	1.55 (0.62)	−14.034	<0.001
LDL-cholesterol, mmol/L	3.18 (1.14)	3.21 (1.18)	3.15 (1.11)	−0.774	0.439
CRP, mg/dl	0.21 (0.33)	0.17 (0.28)	0.25 (0.37)	−4.594	<0.001
Diabetes mellitus	13.6%	16.6%	10.2%	8.886	0.003
Hypertension	54.2%	52.4%	56.4%	1.637	0.201
Hyperlipidemia	80.9%	80.4%	81.6%	0.230	0.632
Use of antihypertensive drugs	23.0%	21.7%	24.4%	1.027	0.311
Use of antidiabetic drugs	7.4%	9.3%	5.3%	5.890	0.015
Use of lipid lowering drugs	20.1%	21.7%	18.2%	2.000	0.157
Total energy intake, kcal	1,908.00 (1,057.00)	2,191.00 (1,203.00)	1,641.00 (804.00)	−11.279	<0.001
Zinc, mg	9.86 (7.72)	12.26 (8.40)	8.29 (6.00)	−8.761	<0.001
Selenium, μg	94.90 (61.20)	109.20 (67.20)	80.95 (51.10)	−9.545	<0.001
Vitamin A, μg	490.00 (497.00)	508.00 (498.00)	461.00 (503.00)	−1.176	0.239
Vitamin C, mg	61.60 (99.80)	60.20 (99.70)	62.80 (98.90)	−0.566	0.571
Vitamin E, mg	5.73 (5.08)	6.47 (5.40)	5.29 (4.65)	−4.426	<0.001
Carotenoids, μg	5,685.00 (11,258.00)	5,360.00 (11,254.00)	6,177.00 (11,651.00)	−1.528	0.127
CDAI	−0.35 (3.54)	−0.54 (3.19)	−0.19 (3.90)	−0.823	0.410
All-cause death	29.8%	33.9%	25.2%	9.356	0.002
Cardiovascular death	8.8%	10.9%	6.4%	6.667	0.010

($p < 0.05$) between the intake of these six antioxidants and gender, suggesting that their effects on ABPI varied between men and women. After adjusting for various factors such as age, medical history, and lifestyle factors, the association between higher ABPI in men and dietary intake of zinc, selenium, and vitamin A remained significant (Table 2 and Figure 3). Furthermore, when looking at quantile regression, the regression coefficients of these dietary antioxidants at different quantiles of ABPI increase as the quantile values increase. This indicates that as antioxidant intake increases, the impact on ABPI becomes greater (Table 3). In contrast, there were no significant associations observed between ABPI and dietary intakes of vitamin C, vitamin E, or carotenoids, whether considering the entire study cohort or when stratifying by gender.

Although women displayed lower dietary intake of certain antioxidants, our analysis of individual intake in relation to population norms revealed no significant difference between men and women in CDAI ($p = 0.410$). Overall, CDAI exhibited negative correlations with BMI, SBP, DBP, HbA1c, and creatinine, while showing a positive correlation with HDL-cholesterol (Figure 2). In a similar vein, we detected a noteworthy gender interaction in the association between CDAI and ABPI. This observation implies that CDAI may exert a significant impact on ABPI in men, while such an effect may not

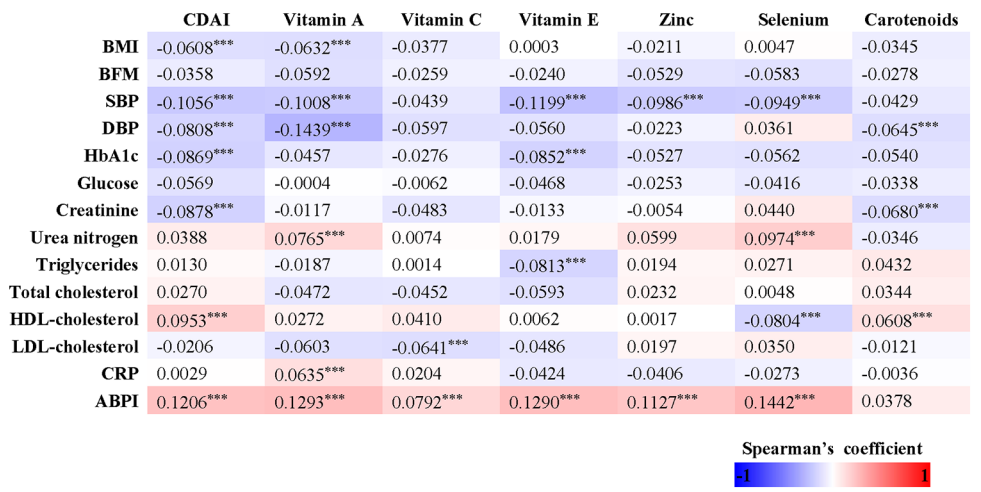

FIGURE 2
Heatmap representation of the spearman's correlation coefficients of dietary antioxidant intake and composite dietary antioxidant index with anthropometric measures (BMI, body mass index; and BFM, body fat mass), cardio-metabolic parameters (SBP, systolic blood pressure; DBP, diastolic blood pressure; glucose; HbA1c; creatinine; urea nitrogen; total cholesterol; triglycerides; LDL; HDL; CRP) and ABPI.

TABLE 2 Hierarchical multiple regression between dietary antioxidant intake and ABPI.

Variates	Model 1		Model 2	
	Coefficients	Standardized coefficients	Coefficients	Standardized coefficients
Zinc	0.002*	0.106	0.001*	0.102
R^2	0.131		0.179	
F	5.858**		4.317**	
ΔR^2	0.131		0.049	
ΔF	5.858**		2.441*	
Selenium	1.360×10^{-4}	0.085	1.650×10^{-4}*	0.103
R^2	0.113		0.162	
F	4.885**		3.776**	
ΔR^2	0.113		0.050	
ΔF	4.885**		2.404*	
Vitamin A	3.307×10^{-5}*	0.144	3.257×10^{-5}*	0.142
R^2	0.140		0.188	
F	6.351**		4.585**	
ΔR^2	0.140		0.048	
ΔF	6.351**		2.449*	

Model 1: The model was adjusted for age, previous history and treatment of diabetes, hypertension and hyperlipidemia, PIR, educational level, smoking status, alcohol usage, physical activity, and total energy intake.
Model 2: The model was adjusted based on model 1, incorporating anthropometric measures and cardio-metabolic parameters.
*$p < 0.05$.
**$p < 0.001$.

be as pronounced in women. Next, we conducted separate data analysis for males and females. For males, we performed adjustments for age, prior medical history, and management of diabetes, hypertension, and hyperlipidemia, in addition to factors such as personal income, educational attainment, smoking habits, alcohol consumption, physical activity, overall calorie intake, body measurements, and cardio-metabolic indicators (mode 2). Using quantile regression, our findings indicate that the regression coefficients of CDAI vary across different percentiles of ABPI. Specifically, these coefficients initially increase and then decrease with higher percentiles, suggesting the presence of a potential U-shaped association between CDAI and ABPI. Detailed results are presented in Table 3 and Figure 3.

Through mediation analysis, we found that the direct effects of CDAI, intake of Vitamin A, Vitamin C, and Vitamin E on all-cause death are statistically significant ($p < 0.05$). However, ABPI, serving as a mediator, does not statistically significantly mediate the indirect effects of antioxidant intake and CDAI on

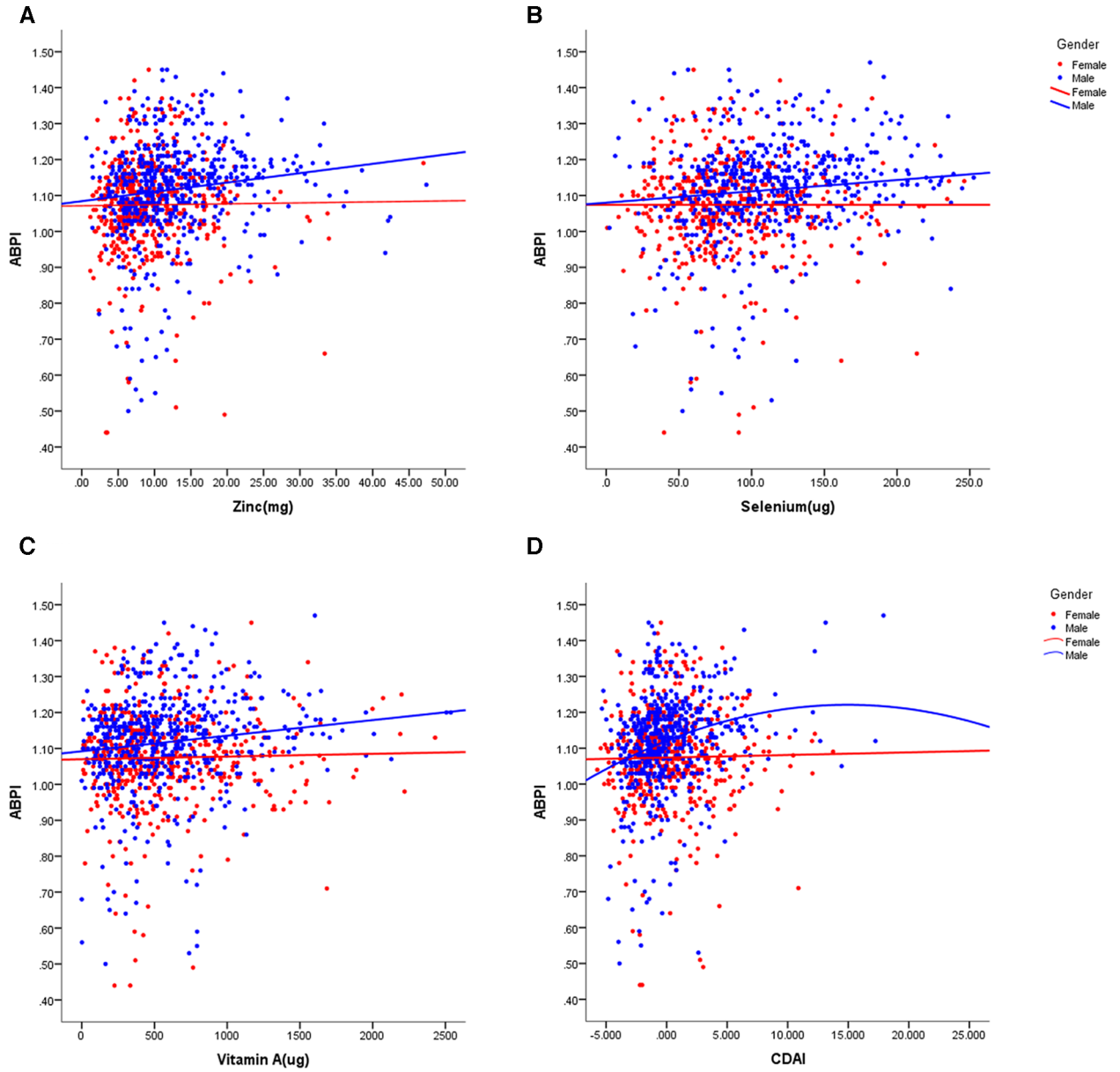

FIGURE 3
Scatter plots of the linear regression of dietary intake of zinc (A), selenium (B), vitamin A (C), and CDAI (D) with ABPI, stratified by gender. The models were adjusted for age, previous history and treatment of diabetes, hypertension and hyperlipidemia, PIR, educational level, smoking status, alcohol usage, physical activity, and total energy intake, anthropometric measures and cardio-metabolic parameters.

all-cause death ($p > 0.05$). Surprisingly, both the direct and indirect effects of antioxidant intake, CDAI, and ABPI on cardiovascular death are not statistically significant (Table 4). Moderation analysis shows a significant interaction between Vitamin A intake and gender in influencing all-cause death. Moderation analysis reveals a significant interaction between Vitamin A intake and gender in influencing all-cause death, while no significant interaction is observed between gender and other antioxidant intakes or CDAI (Table 5). Furthermore, the Cox regression of all-cause mortality with Vitamin A, stratified by gender, shows that a daily intake of Vitamin A more than 776 ug is especially beneficial for women (Figure 4).

4 Discussion

The current study evaluated the association between antioxidant intake, both at individual and cumulative levels, and ABPI. We discovered that dietary zinc, selenium, and Vitamin A are positively linked to ABPI in men, but not in women. Additionally, at the cumulative level, we observed a U-shaped association between CDAI and ABPI. ABPI is a measure of blood pressure in the legs compared to the arms. ABPI is typically used to assess the health of the arteries in the legs and to diagnose Peripheral Artery Disease (PAD). PAD is a condition where the arteries in the legs become narrowed or blocked,

TABLE 3 Stratified regression analysis based on quartiles of the composite dietary antioxidant index in men and women using model 2.

Model 2	Character	Q1	Q2	Q3	Q4	*p* for trend	*p* for interaction
CDAI	Gender						0.001
	Male	ref	0.049 (0.018, 0.081)	0.056 (0.022, 0.091)	0.051 (0.012, 0.089)	0.019***	
	Female	ref	0.048 (0.013, 0.083)	0.037 (0.002, 0.071)	0.011 (−0.027, 0.049)	0.755	
Vitamin A	Gender						< 0.001
	Male	ref	0.043 (0.011, 0.074)	0.042 (0.009, 0.075)	0.049 (0.016, 0.082)	0.009***	
	Female	ref	−0.018 (−0.051, 0.015)	−0.017 (−0.051, 0.017)	−0.015 (−0.051, 0.021)	0.475	
Vitamin C	Gender						0.014
	Male	ref	0.029 (−0.002, 0.059)	0.045 (0.014, 0.076)	0.015 (−0.018, 0.047)	0.242	
	Female	ref	0.019 (−0.014, 0.053)	0.005 (−0.030, 0.040)	0.007 (−0.027, 0.042)	0.943	
Vitamin E	Gender						0.013
	Male	ref	0.023 (−0.011, 0.057)	0.019 (−0.015, 0.053)	0.028 (−0.010, 0.065)	0.224	
	Female	ref	−0.008 (−0.039, 0.024)	0 (−0.035, 0.036)	−0.006 (−0.046, 0.034)	0.896	
Zinc	Gender						< 0.001
	Male	ref	0.009 (−0.026, 0.044)	0.048 (0.012, 0.084)	0.053 (0.014, 0.092)	0.002***	
	Female	ref	0.039 (0.008, 0.070)	0.036 (0.001, 0.071)	0.011 (−0.032, 0.054)	0.447	
Selenium	Gender						< 0.001
	Male	ref	0.028 (−0.008, 0.064)	0.034 (−0.001, 0.070)	0.049 (0.010, 0.089)	0.020***	
	Female	ref	0.01 (−0.021, 0.041)	0.006 (−0.029, 0.041)	−0.004 (−0.047, 0.038)	0.917	
Carotenoids	Gender						0.019
	Male	ref	0.019 (−0.011, 0.049)	0.027 (−0.005, 0.058)	0.015 (−0.017, 0.046)	0.318	
	Female	ref	0.006 (−0.028, 0.040)	0.001 (−0.032, 0.033)	−0.007 (−0.042, 0.028)	0.618	

***$p < 0.05$.

TABLE 4 Mediation.

Path (X→M→Y)	Effects	Coefficients	95% CI	*p*-value
CDAI→ABPI→All-cause death	Total	−0.053	(−0.085, −0.020)	<0.001**
	Direct	−0.052	(−0.084, −0.020)	<0.001**
	Indirect	−0.001	(−0.003, 0)	0.250
CDAI→ABPI→Cardiovascular death	Total	0.004	(−0.015, 0.020)	0.510
	Direct	0.004	(−0.015, 0.020)	0.520
	Indirect	0	(−0.001, 0)	0.680
Vitamin A→ABPI→All-cause death	Total	−0.040	(−0.066, −0.010)	0.002*
	Direct	−0.039	(−0.065, −0.010)	0.002*
	Indirect	−0.001	(−0.003, 0)	0.296
Vitamin A→ABPI→Cardiovascular death	Total	0.005	(−0.011, 0.020)	0.410
	Direct	0.005	(−0.011, 0.020)	0.430
	Indirect	0	(−0.001, 0)	0.670
Vitamin C→ABPI→All-cause death	Total	−0.038	(−0.063, −0.010)	0.002*
	Direct	−0.037	(−0.062, −0.010)	0.002*
	Indirect	−0.001	(−0.002, 0)	0.414
Vitamin C→ABPI→Cardiovascular death	Total	0.001	(−0.018, 0.010)	0.810
	Direct	0.001	(−0.018,0.010)	0.820
	Indirect	0	(0, 0)	0.740
Vitamin E→ABPI→All-cause death	Total	−0.070	(−0.096, −0.040)	<0.001**
	Direct	−0.070	(−0.096, −0.040)	<0.001**
	Indirect	0	(−0.002, 0)	0.620
Vitamin E→ABPI→Cardiovascular death	Total	-8.090×10^{-3}	$(-3.840 \times 10^{-2}, 0.010)$	0.470
	Direct	-8.970×10^{-3}	$(-3.830 \times 10^{-2}, 0.010)$	0.480
	Indirect	8.480×10^{-5}	$(-5.860 \times 10^{-4}, 0)$	0.850
Zinc→ABPI→All-cause death	Total	−0.009	(−0.038, 0.020)	0.570
	Direct	−0.007	(−0.037, 0.020)	0.630
	Indirect	−0.001	(−0.004, 0)	0.150
Zinc→ABPI→Cardiovascular death	Total	0.009	(−0.008, 0.020)	0.240
	Direct	0.008	(−0.009, 0.020)	0.260
	Indirect	0	(−0.001, 0)	0.680
Selenium→ABPI→All-cause death	Total	−0.029	(−0.061, 0)	0.063
	Direct	−0.029	(−0.060, 0)	0.072
	Indirect	−0.001	(−0.003, 0)	0.336

(*Continued*)

TABLE 4 Continued

Path (X→M→Y)	Effects	Coefficients	95% CI	*p*-value
Selenium→ABPI→Cardiovascular death	Total	0.002	(−0.020, 0.020)	0.750
	Direct	0.001	(−0.020, 0.020)	0.770
	Indirect	0	(−0.001, 0)	0.720
Carotenoids→ABPI→All-cause death	Total	−0.019	(−0.043, 0.010)	0.150
	Direct	−0.018	(−0.043, 0.010)	0.150
	Indirect	0	(−0.002, 0)	0.710
Carotenoids→ABPI→Cardiovascular death	Total	-5.680×10^{-3}	$(-2.820 \times 10^{-2}, 0.010)$	0.600
	Direct	-5.740×10^{-3}	$(-2.820 \times 10^{-2}, 0.010)$	0.600
	Indirect	5.200×10^{-5}	$(-5.930 \times 10^{-4}, 0)$	0.890

X, independent variable; M, mediator variable; Y, dependent variable.
Model (M→Y): The model was adjusted for age, gender, previous history and treatment of diabetes, hypertension and hyperlipidemia, PIR, educational level, smoking status, alcohol usage, physical activity, and total energy intake, anthropometric measures and cardio-metabolic parameters.
Model (X + M→Y): The model was adjusted for Quartile of X, M, age, gender, previous history and treatment of diabetes, hypertension and hyperlipidemia, PIR, educational level, smoking status, alcohol usage, physical activity, and total energy intake, anthropometric measures and cardio-metabolic parameters.
*$p < 0.05$.
**$p < 0.001$.

TABLE 5 Moderation.

Interaction	Coefficients	SE	*t*	*p*-value
CDAI × gender	-5.438×10^{-4}	6.989×10^{-3}	−0.078	0.938
Vitamin A × gender	1.102×10^{-4}	4.896×10^{-5}	2.251	0.025*
Vitamin C × gender	-1.463×10^{-4}	2.752×10^{-4}	−0.532	0.595
Vitamin E × gender	1.570×10^{-3}	4.987×10^{-3}	0.315	0.753
Zinc × gender	2.546×10^{-3}	2.122×10^{-3}	1.200	0.230
Selenium × gender	2.505×10^{-5}	3.875×10^{-4}	0.065	0.948
Carotenoid × gender	2.026×10^{-7}	1.905×10^{-6}	0.106	0.915

X, independent variable; Moderator (M): gender; Y, all-cause death.
Model (X × M→Y): The model was adjusted for X, M, X × M, age, gender, previous history and treatment of diabetes, hypertension and hyperlipidemia, PIR, educational level, smoking status, alcohol usage, physical activity, and total energy intake, anthropometric measures and cardio-metabolic parameters.
*$p < 0.05$.

causing pain, numbness, or problems with walking (14). ABPI is typically measured using Doppler ultrasound, which uses sound waves to measure blood flow through the arteries. A lower ABPI may indicate PAD or other leg artery disease, while a normal ABPI is typically greater than 0.9. Some studies have shown that ABPI is associated with an increased risk of cardiovascular events such as heart attack, stroke, and death. ABPI may be an independent risk factor for cardiovascular disease and can predict the risk of future cardiovascular events (15–18). In general, men may have slightly higher ABPI values than women (19). This is partly because men typically have larger body mass and muscle mass, which can potentially influence the measurement results. This difference is usually within the normal range and should not lead to significant clinical disparities. It's important to note that ABPI depends on various factors, including age, genetics, and physiological condition. However, there is still debate about whether traditional cardiovascular risk factors such as high blood pressure, high cholesterol levels, smoking, and diabetes have gender differences in their impact on ABPI. In our cohort, men exhibit higher BMI, a greater history of smoking, a higher history of alcohol consumption, and elevated levels of fasting glucose and triglycerides, yet they show a higher ABPI, age partially explains why. Gender differences in the prevalence and severity of atherosclerosis become more significant as people age (20, 21). The changes in female hormone levels may potentially accelerate the development of atherosclerosis after menopause. Hormone-related effects on vascular oxidative stress partially account for these differences (22). Furthermore, in our study, men also demonstrate higher intake of zinc, selenium, and vitamin E, despite there being no gender differences in CDAI. Interestingly, in our study, although men can normalize ABPI through the intake of antioxidants, this seems to be unrelated to the final all-cause death and cardiovascular death. In fact, with the same intake, women seem to benefit more, resulting in lower all-cause death.

An adequate dietary intake of antioxidants may contribute to the prevention of atherosclerosis. A prospective study involving 4,564 healthy adults indicated a negative correlation between dietary zinc intake and subclinical atherosclerosis as measured by carotid intima-media thickness (23). The benefits of zinc in the context of atherogenesis suggest the inhibition of both LDL-cholesterol oxidation and caspases that are involved in several apoptotic pathways (24). As for selenium, an essential trace element in the human body, it is closely associated with the development of atherosclerosis. On one hand, selenium deficiency affects the synthesis of selenium-containing enzymes, leading to an increase in oxidative stress levels within the body. On the other hand, selenium can participate in various signaling pathways, inhibiting vascular calcification (25–27). Another randomized clinical trial has shown that supplementation with vitamin E alone can significantly reduce the progression of atherosclerosis in men, but not in women (28), which is corroborated by our findings. Overall, men are more prone to myocardial infarction and heart failure than women. However, women experience an increased risk after menopause, suggesting a cardiovascular protective role of estrogen. The latter exerts its cardiac protection through its antioxidant effect (29). Besides estrogen, other mechanisms may also contribute to cardiovascular risk, as hormone replacement therapy has not

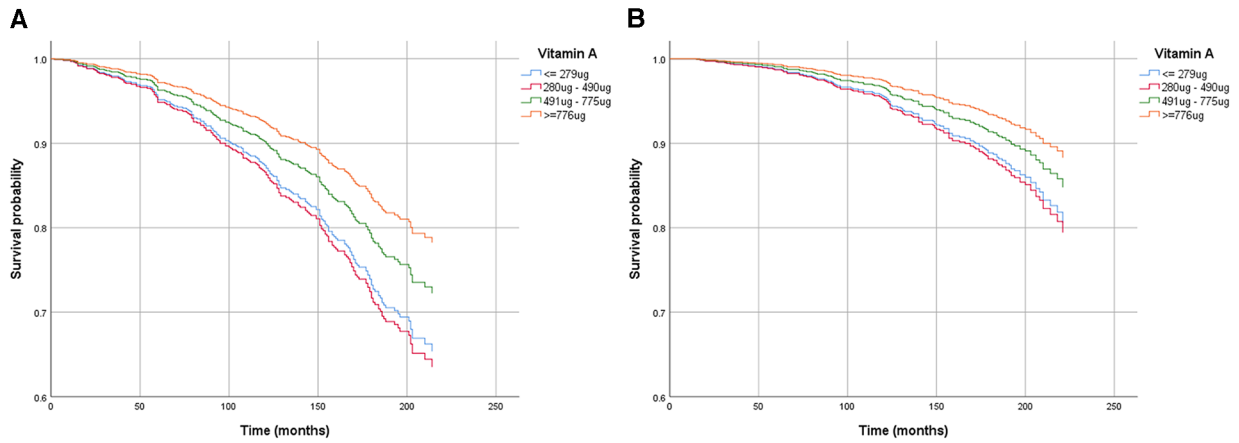

FIGURE 4
Cox regression of all-cause death mortality with vitamin A, stratified by gender [(A) for male, (B) for female]. The models were adjusted for age, previous history and treatment of diabetes, hypertension and hyperlipidemia, PIR, educational level, smoking status, alcohol usage, physical activity, and total energy intake, anthropometric measures and cardio-metabolic parameters.

reduced cardiovascular risk in postmenopausal women (30). It is worth noting that in our study, CRP levels were higher in women compared to men, which aligns with previous research on peripheral artery diseases (31), suggesting that oxidative stress and pro-inflammatory biomarkers may be higher in women than in men. A partial explanation for these differences may be the varying expression and/or activity of antioxidant enzymes (namely, superoxide dismutase, glutathione peroxidase, and NADPH oxidase) between men and women (29).

It is challenging to accurately determine the independent impact of individual antioxidants on atherosclerosis because the dietary intake of these nutrients is highly correlated with each other. While specific antioxidants may play a role in atherosclerosis, potential biological interactions between dietary antioxidants and other nutrients should be taken into consideration. Therefore, our study utilizes a dietary antioxidant index (CDAI) to assess the effects of a combination of dietary antioxidants on atherosclerosis measured by ABPI. We found a potential U-shaped, rather than linear, association between CDAI and ABPI in men, but this pattern was not observed in women. This finding implies the possibility of potential biological interactions among dietary antioxidants, which could either synergize or offset the independent effects of individual antioxidants on atherosclerosis. Jaouad Bouayed and Torsten Bohn (32) have stated that the administration of high doses of isolated antioxidants may carry potential toxicity risks, primarily due to their prooxidative effects when present at elevated concentrations. Furthermore, these antioxidants have the capacity to interact with the beneficial concentrations of Reactive Oxygen Species (ROS) that are typically found in physiological conditions, which are essential for optimal cellular function. As such, caution is warranted when considering high-dose supplementation of isolated antioxidants, as it may disrupt the delicate balance required for normal cellular functioning. Further research is now needed to understand the mechanistic basis of these gender differences in cardiovascular disease risk.

There are some limitations to consider in our study. Despite analyzing a large sample size, our study does not have enough evidence to fully explain why there are gender differences in the effects of dietary antioxidants on atherosclerosis. Although we took into account several variables, there is still a possibility of other unknown factors that may affect the intake, metabolism, and ABPI related to antioxidants. Thus, it is crucial to conduct more thorough and extensive prospective studies on a larger scale to evaluate the impact of dietary antioxidant intake on the progression of atherosclerosis.

In conclusion, the findings from our research suggest that dietary antioxidants may have a gender-specific role in preventing arterial lesions and cardiovascular events and influence the outcome. However, further investigation is necessary to better comprehend the underlying mechanisms responsible for the gender disparities in the progression of atherosclerosis.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by NCHS Ethics Review Board Approval. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JW: Writing – original draft, Data curation, Formal Analysis, Software. YW: Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Silicon in prevention of atherosclerosis and other age-related diseases

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Researchers' interest in silicon as an element important for the functioning of the animal and human body began in the 1970s. Soluble compounds of silicon bioavailable from water and food seem to have important meaning for life processes occurring in the body. So far, researchers have focused on the significance of silicon for the development of bones and connective tissue, and its role in preventing neurodegenerative diseases and atherosclerosis. Despite numerous studies, the role of silicon as an active element in the human body is poorly understood. Since the involvement of lipid oxidation and inflammatory processes in the pathogenesis of atherosclerosis is well known, this article summarizes and discusses the current research and scientific observations regarding silicon, primarily in terms of its beneficial influence on redox and anti-inflammatory reactions and the lipid profile. The association of silicon with the stabilization of the structure of glycosaminoglycans and their protein complexes may also support the anti-atherosclerotic effect. The authors attempted to collect and present existing publications that could confirm the beneficial role of dietary silicon in the prevention of age-related diseases and explain the potential mechanisms of its action.

KEYWORDS

silicon, orthosilicic acid, atherosclerosis, glycosaminoglycan, inflammation

1 Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall, the complications of which (heart attack and cerebral stroke) are the most common cause of death in developed countries (1). Despite the relatively well-understood pathomechanism and the availability of drugs that slow down the formation of atherosclerotic plaques, there are still many questions regarding its development and new agents with anti-atherosclerotic potential are constantly being searched for. For several decades, the role of silicon in preventing the development of some chronic diseases has been discussed in scientific literature, and reports about the possible role of the element in preventing atherosclerosis date back to the 1970s (2, 3). Researchers indicate the possible role of silicon for the proper functioning of the cardiovascular, skeletal and central nervous systems (4, 5). Taking into account the prevalence of the element in the environment, we reviewed existing scientific work with the aim of answering the question whether silicon as a molecular component can modify/influence the course of atherosclerosis and other age-related diseases.

2 Sources of silicon for the human body and its bioavailability

The main source of silicon for the human body is diet based on natural products containing the bioavailable forms of Si. Although the element is widespread in nature as a component of earth's minerals, rocks, sands, clays, dust and also widely used in industry (inter alia cosmetics, medical implants, medical devices, electronics, computer devices, metallurgy), environmental exposure to humans is quite limited due to the difficult absorption of present in nature or industrially used chemical forms of silicon. However, forms of the element commonly used in the food industry, such as amorphous silica (SiO_2), silicates or polymethylsiloxane occurring as food additives, clarifying agents in drinks, ingredients of medicines and vitamins, can be significant source of the silicon for the human body (6, 7). Silicon is also present in the human diet in the form of highly soluble forms derived from water and natural products.

To date, no minimum daily requirement for silicon has been established. However, the recommended daily intake of the element was estimated at 10–25 mg/day taking into account the daily urinary excretion of silicon (8).

Silicon the most often occurs as poorly soluble mineral silica (SiO_2) in a polymerized form (9). When exposed to water, silicates are formed, which release orthosilicic acid Si(OH)4' to a concentration of 1–15 mg/L SiO_2. In this form silicon is bioavailable and easily absorbed in the digestive tract. The bioavailability of silicon varies depending on the food: orthosilicic acid and water-soluble silicates from beverages are easily absorbed, while phytolytic silica, present in solid plant foods, is less digestible (10).

The high silicon excretion after beer consumption indicates that it is a particularly important source of easily absorbable silicon (11). The beer is rich in bioavailable silicon from hot mashing techniques that extract orthosilicic acid from the phytolitic silica of barley. Other beverages are also indicated as sources of bioavailable silicon, especially tap water, mineral and spring waters (7).

Silicon levels are usually higher in plant-based foods than in animal-based foods. Foods with the highest silicon content are cereals, especially oats, barley and some rice fractions (12). Cereal grains, along with beverages (especially beer) and some vegetables and fruits, contribute the most to dietary silicon intake (8).

3 The importance of silicon in cardiovascular diseases

Silicon is an essential trace element that occurs in the human body in small amounts. In serum, the element occurs almost entirely as orthosilicic acid. Concentrations of Si in plasma in fasting state are 2–10 μM, growing to 20–30 uM after meals, and approximately 700 μmol/day is normally excreted in urine (13). Serum silicon levels in adults shows statistically significant dependency on age and sex. According to Bisse et al. (14) the decrease of serum silicon with age is clearly visible over 74 years, especially in women.

Some reports have observed also age-related lowering the silicon content in the hair of healthy people (15).

3.1 The effect of silicon on the structure of arteries and glycosaminoglycans

Connective tissues, including the aorta and trachea are extremely rich in silicon, as has been shown by studies on several animal species (16). The high content of silicon in connective tissue is mainly due to its participation in the formation of glycosaminoglycans, as well as the presence of the element as an integral element of glycosaminoglycans and their protein complexes. Proteoglycans are components of the extracellular amorphous ground substance surrounding cells, collagen and elastic fibers, forming the structure of this tissue. The structural role of silicon in the extracellular matrix, which involves cross-linking proteoglycans and protein, increases the strength and reduces the permeability of the matrix (17), thus strengthening the arterial walls. Interaction of proteoglycans with LDL leading to subendothelial retention of LDLs has been proposed to be a key process in the pathogenesis of atherosclerosis (18, 19). It cannot be ruled out that strengthening the proteoglycans structure can limit the availability of the negative sulfate groups of glycosaminoglycans for binding sites of apoB protein in LDL. Protection of the proteoglycan structure by silicon may contribute to the prevention of atherosclerosis not only by improvement the function of the arterial wall, but also by the action of the proteoglycans themselves. The results of current scientific research indicate that certain types of heteroglycans have a beneficial effect in the prevention of metabolic syndrome. Administration of medium and high doses of heteropolysaccharides to rats with metabolic syndrome (MetS) significantly reduced both systolic and diastolic blood pressure ($p < 0.05$), improved cardiac ejection fraction ($p < 0.05$) and contractility ($p < 0.05$) compared to untreated MetS rats ($p < 0.05$). Supplemented MetS rats also demonstrated amelioration of the lipid status with statistically significant decrease in triglycerides, LDL cholesterol, total cholesterol and increase in HDL cholesterol compared to untreated MetS group. An improvement in glucose homeostasis in the OGTT test and decrease in insulin levels ($p < 0.05$) were also observed (20).

Research results have confirmed that aging causes a marked reduction in the level of this element also in the normal human aorta (2), which may have an adverse effect on the structure of arterial blood vessels in older people.

3.2 Silicon and the development of atherosclerosis

Loeper et al. (2), found that the level of silicon in the wall of the human aorta decreases with the development of atherosclerosis. The silicon content in the aorta with moderate atherosclerosis (1–2

atherosclerotic plaques present) was approximately 58%, and in the aorta with severe atherosclerosis (numerous atherosclerotic plaques and massive lipid infiltrates present) was approximately 35% of the content in the aorta without atherosclerosis. The decrease in silicon content paralleled lipid infiltration, changes in elastic fibers, and changes in the ground substance. A very significant difference was found between group 0 (aorta without atherosclerosis, 0 atherosclerotic plaque) and group + (aorta with moderate atherosclerosis, 1–2 atherosclerotic plaques), as well as between group + and group ++ (aorta with severe atherosclerosis, numerous atherosclerotic plaques and massive lipid infiltrates), with $p < 0.001$. Similar results were obtained in study of rabbit aortas (21) (Table 1).

Human study assessed the silicon content also in the hair of patients with atherosclerosis (15). Silicon levels were compared in the same age groups of healthy patients and those suffering from atherosclerosis. Statistically, no major differences were found, but the mean silicon hair content in patients with atherosclerosis was much lower and ranged from 14.0 ± 6.7 to 7.9 ± 4.9 μg/g, depending on the age range. A wide range of silicon values was observed in each age group, even in the healthy military students' group living for many years in the same conditions and using the same diet. However, among patients with atherosclerosis, a group was found with very low levels of silicon in the hair, below 10 μg/g. Due to the wide range of concentrations in the samples, it is risky to consider this parameter as a marker of the disease, but a very low silicon hair content below 10 μg/g may be a clear indicator of the disease.

The potential contribution of silicon deficiency to atherosclerosis was also indicated by other researchers (3, 23, 24). The authors suggest that poorly soluble forms of polymeric silicic acid or silica may reduce the development of atherosclerosis by binding bile acids in the gastrointestinal tract, which would improve the elimination of end products of cholesterol metabolism; the binding of cholesterol itself may also play a role. The proposed concept may be confirmed by studies that have shown that the addition of silicic acid to drinking water increases the excretion of tritium-labeled cholesterol and its transformation products in the feces and reduces the absorption of labeled cholesterol in the liver, spleen and kidneys (3). The results of other study suggest that silicon among other metal ions (calcium, magnesium, lithium, strontium) may protect against cardiovascular mortality; possibly, by competing with sodium and potassium for transport in the gastrointestinal tract (23).

Epidemiological studies have revealed many genetic and environmental risk factors for atherosclerosis (25). Genetic factors include: dyslipidemia, increased LDL/VLDL levels, decreased HDL, increased blood pressure, increased homocysteine levels, diabetes and obesity, systemic inflammation (elevated CRP), metabolic syndrome with insulin resistance, male gender. Environmental factors include primarily a high-fat diet, smoking, low levels of antioxidants, low physical activity, and some infectious factors.

3.3 Silicon and the lipid profile

The effect on lipids is considered as one of the mechanisms of the anti-atherogenic effect of silicon. In studies on rabbits (21), it was found that 88% of rabbits on an atherogenic diet developed atherosclerosis, while on an atherogenic diet with intravenous

TABLE 1 Summary of works on the assessment of silicon in atherosclerosis based on pathology.

Authors	Materials	Groups	Results		
			Si concentration in the aorta (Silicon in μg/100 mg nitrogen)	*p*	Others
Loeper et al. (2)	human aorta	Group 0 aorta without atherosclerosis, 0 atherosclerotic plaque	180 μg ± 21	<0.001 (group 0 vs. group +) (group + vs. group + +)	–
		Group (+) aorta with moderate atherosclerosis, 1–2 atherosclerotic plaques	105 μg ± 12		
		Group (++) aorta with severe atherosclerosis, numerous atherosclerotic plaques and massive lipid infiltrates	63 μg ± 8		
Loeper et al. (21)	rabbit aorta	Group 1 normal aorta from rabbits on standard diet	490 μg ± 40	<0.05 (group 1 vs. group 2)	Among all rabbits fed a high-cholesterol diet (Group 2 + Group 3), atherosclerotic lesions occurred in 30% of rabbits treated with silicon, while in 88% of untreated rabbits.
		Group 2 aorta with experimental atherosclerosis induced by high cholesterol diet	398 μg ± 66		
		Group 3 aorta with experimental atherosclerosis induced by high cholesterol diet with addition of silicon i.v. (monomethyltrisilanol 10 mg every other day)	470 μg ± 53		
Gangapatnam et al. (22)	rats	Group I normal control rats	–	–	Histopathological studies were estimated. Silicon at 20 mg/ml concentration showed the regression of atherosclerosis in high fat diet induced rats compared to 10 mg/ml concentration.
		Group II high fat diet rats			
		Group III statins as positive control			
		Group IV high fat diet + silicon treatment with 10 mg/ml			
		Group V high fat diet + silicon treatment with 20 mg/ml concentration.			

silicon addition, only 30% of rabbits showed atherosclerotic plaques. In the group of animals receiving silicon, the increase in the plasma concentration of mono- and polyunsaturated fatty acids was lower compared to the group not treated with silicon. It seems that silicon may have a beneficial effect on atherosclerosis by reducing mono- and polyunsaturated fatty acids in lipids, which in the peroxidation process release toxic peroxides that cause damage to arteries. A study by Najda et al. (26) showed that the concentration of HDL-cholesterol and HDL-phospholipids increased and the concentration of triglycerides and LDL-cholesterol decreased in the blood serum of rats supplemented with an aqueous silica solution. However, there was no effect of silicon supplementation on the plasma concentration of total cholesterol or phospholipids. However, Garcimartin et al. (27) showed in animal studies that in the group receiving the hypercholesterolemic + silicon diet, the levels of cholesterol (C), phospholipids (PL) and triglycerides (TG) were significantly lower compared to the group receiving only the hypercholesterolemic diet. Cholesterol supplementation increased C and PL in very low-density lipoprotein (VLDL) and intermediate-density lipoprotein + low-density lipoprotein (IDL + LDL). In the group on the hypercholesterolemic + silicon diet a reduction in the total VLDL mass ($p < 0.001$) to the value of the control group receiving a normal diet, a decrease in the atherogenic index (AI) and a decrease in the share of TG in all lipoproteins in relation to the other two groups were observed.

3.4 Silicon and blood pressure

Arterial hypertension is a known risk factor for the development of atherosclerosis. In a study conducted by Maehira et al. (28) using soluble silica and coral sand as a natural material containing silicon, the effect of silicon on hypertension in spontaneously hypertensive rats was assessed. In rats fed with soluble silica (at a dose of 50 mg/kg body weight) or coral sand for 8 weeks, systolic blood pressure was significantly reduced respectively by 18 mmHg or 16 mmHg compared to control group. Moreover, providing both dietary soluble silica and coral sand inhibited the expression of angiotensinogen and aortic growth factor genes associated with vascular remodeling. Soluble silica also stimulated the expression of peroxisome proliferator-activated receptor γ, which has anti-inflammatory and antihypertensive effects on blood vessel cells.

3.5 Possible effect of silicon on the functions of endothelial cells

Atherosclerosis is a chronic inflammatory process taking place in the intima of large and medium-sized arteries. Some authors suggest that dietary silicon may influence endothelial cells and maintain the proper inner lining of arteries. This is related to abundant evidence for the physiologically important role of heparan sulfate-containing proteoglycans (HSPG) produced by vascular endothelial cells in inhibiting the morphological transformation, migration and proliferation of vascular smooth muscle cells, i.e., processes that are crucial for the intimal hyperplasia observed in atherosclerosis and restenosis (29). It has been suggested that oral administration of glucosamine may stimulate the production of heparan sulfate-containing proteoglycans and thus exert antiatherosclerotic effects. Similarly, the authors suggest that the intake of bioavailable silicon may also increase the production of heparan sulfate-containing proteoglycans by endothelium and that this phenomenon, at least partially, underlies the beneficial effect of silicon administration on the inhibition of intimal hyperplasia in cholesterol-fed rabbits and may also explain the lower cardiovascular risk associated with increased silicon intake in epidemiological studies. To evaluate this hypothesis it is suggested to study the effect of soluble silicates on production of HSPG by cultured cells of vascular endothelium. But also reports with contrary results showing potentially adverse effects of Si for function of endothelial cells and decreased endothelial NO synthase expression are present in scientific literature (30). The experimental study has shown that contractility induced by norepinephrine and dilation response to acetylcholine were significantly higher in the aortic rings of Si-treated rats compared to controls. The study demonstrated that Si modified the characteristics of endothelial relaxants and attenuated smooth muscle cell responsiveness to NO caused by decrease of production of basal NO generation (31).

3.6 Silicon and inflammatory processes/the function of the immune system

The formation of atherosclerotic plaque is a complex process of oxidative, inflammatory and immunological origin, which involves both activated macrophages and T lymphocytes, as well as the production of numerous cytokines and growth factors (32). Results of studies on silicon-deprived animals suggest the element's involvement in immune and inflammatory reactions (33, 34). The role of silicon in enhancing the anty-inflammatory response and modifying the immune response has so far been observed in a study on animals with collagen-induced arthritis (33). During inflammation, the number of lymphocytes decreased and the number of neutrophils increased significantly in rats whose diet was supplemented with silicon, compared to rats on the basic diet. The importance of these changes in the context of the pathogenesis of atherosclerosis is ambiguous, but the lack of significant increase in monocyte levels in the group with silicone supplementation seems to be beneficial. There was also increased release of prostaglandin E2 (PGE2) in the tibia and higher plasma osteopontin levels in rats supplemented with silicon, suggesting a weaker anti-inflammatory response in rats fed a diet without added silicon.

No differences were found in cellular parameters and levels of pro-inflammatory cytokines in the initial acute phase (2 h) of the inflammatory process induced by lipopolysaccharide endotoxin in rats deprived of silicon compared to those supplemented. However, it was observed that after exposure to endotoxin, rats

deprived of silicon accumulated more silicon in the liver and bones than rats receiving silicon supplements, which may suggest its involvement in the response to the chronic inflammatory process. Moreover, in the control group of rats deprived of silicon, there was an increase in the number of monocytes (35), cells of key importance in the process of initiating atherosclerosis.

In mouse induced macrophage cells, the inhibitory effects of silicon on nitric oxygen generation and on IL-6 secretion were observed at addition respectively of 100 μM and of 50 μM of Si in sodium metasilicate form, significantly exceeding the concentration of Si found in human blood. But significant decrease in mRNA expression of tumor necrosis factor TNF-α and inducible nitric oxide synthase level by 1 μM, 5 μM and 10 μM of Si in sodium metasilicate form and also suppression of cyclooxygenase-2 mRNA expression by 1 μM, 5 μM and 25 μM of Si in the same chemical form suggest ability of silicon to regulate the inflammatory process (36). Similarly to neurodegenerative diseases, demonstrated by Garcimartin et al. (37) in *in vitro* studies, a reduction in the level of tumor necrosis factor TNF-α in response to low concentrations of silicon and found by Gonzalez-Munoz et al. (38) in a study on mice, normalization of TNF-α mRNA expression after the use of silicic acid may be important in atherosclerosis.

In 2015, Vide et al. (39) studied the effect of dietary silicon-enriched spirulina (SES) on atherosclerosis. Spirulina are cyanobacteria that live in highly saline tropical and subtropical waters. These microalgae are one of the richest plant sources of proteins (60%–70%), lipids (7%), carbohydrates (20%) and good source of vitamins and minerals such as calcium, magnesium, phosphorus, potassium, sodium, zinc (40). Spirulina is used in biotechnology to produce and accumulate specific bioactive compounds and nutrients. In this way, a new type of dietary supplement was developed that can serve as a rich source of trace elements (41). In the study, hamsters on a high-fat diet received either SES or unfortified spirulina (both at a dose of 57 mg/kg body weight) daily. This corresponded to a daily dose of 0.57 mg of silicon/kg body weight. The high-fat diet induced dyslipidemia, insulin resistance, oxidative stress, and vascular dysfunction. Compared with the high-fat diet group, SES attenuated the increase in lipemia and prevented insulin resistance ($p = 0.001$), which was not observed in the unsupplemented spirulina group. Another significant effect seen only in the SES group was the reduction of inflammation by lowering the levels of TNF-α ($p = 0.0006$) and interleukin-6 ($p = 0.0112$), reducing the number of polymorphonuclear cells and preventing the increase in the activity of nuclear factor kB ($p = 0.0259$). SES more significantly than spirulina alone corrected the increased plasma levels of the monocyte chemotaxis protein MCP-1 by the high-fat diet ($p = 0.0380$). Furthermore, only SES supplementation prevented the attenuation of aortic vascular and endothelial responses induced by a high-fat diet, as assessed by the contractile response to the phenylephrine agonist and the relaxation response to acetylcholine, respectively. However, both SES and spirulina itself similarly protected against oxidative stress by reducing the activity of nicotinamide adenine dinucleotide phosphate oxidase in the heart and liver and maintaining the activity of the antioxidant enzymes superoxide dismutase and glutathione peroxidase.

3.7 Silicone effects on redox status

Observed by Gonzalez-Munoz et al. (38) the effect of silicon on the redox system in mice receiving silicic acid expressed by inhibiting the aluminum-induced decrease in mRNA expression of endogenous antioxidant enzymes, normalizing the reduced levels of the antioxidant enzymes catalase and superoxide dismutase in brain tissue homogenates and reducing the level of free oxygen radicals (TBARS, thiobarbituric acid reactive substances) was confirmed in further studies. Other researchers similarly found that the redox state in rats with nonalcoholic steatohepatitis improved with silicon administration. Increased gene expression of liver antioxidant enzymes and decreased levels of glutathione persulfide were observed (42).

In 2018, Gangapatnam et al. (22) demonstrated regression of atherosclerosis in experimental animals. The research was carried out in the following five groups: group I - normal diet, control group; group II - high-fat diet; group III - statins as a positive control; group IV – high-fat diet + silicon treatment at a concentration of 10 mg/ml; group V - high-fat diet + silicon treatment at a concentration of 20 mg/ml. Levels of physical, biochemical and serum marker enzymes were assessed. A significant difference was found between rabbits on the high-fat diet and the other treatment and control groups. Histopathological examinations were then performed and the levels of antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase in the liver tissue were assessed. The results showed that there were significant differences, with silicon at a concentration of 20 mg/ml showing regression of atherosclerosis in rabbits induced with a high-fat diet compared to a concentration of 10 mg/ml.

The beneficial effect of silicon on the antioxidant system may also be strengthened through its protective effect on the structure of proteoglycans, which themselves have an antioxidant properties (43). The polysaccharides extracts of four of the most widely known mushrooms often used in medicinal applications named Ganoderma applanatum, Ganoderma lucidum, Lentinus edodes and Trametes versicolo showed an increase in the antioxidant activities and inhibition of lipid peroxidation with increasing concentrations (44). The heteropolysaccharides supplemented in medium and high doses to rats with MetS significantly reduced the level of prooxidants (superoxide anion radical O_2^-, hydrogen peroxide H_2O_2, TBARS; $p < 0.05$) and increased antioxidants activity (superoxide dismutase, catalase, reduced glutathione; $p < 0.05$) compared to untreated MetS animals (20). Mushroom polysaccharides have been shown to mitigate oxidative stress also in type 2 diabetes mellitus by the decreased lipid peroxidation and the increased activity of superoxide dismutase in the plasma, and by the elevated glutathione levels in the plasma and erythrocytes (45).

It is also worth mentioning that the role of silicon in enhancing the antioxidant capacity, including the activity of antioxidant enzymes, has been confirmed for plants exposed to abiotic stress (46).

The few studies on the effect of silicon on the redox system in humans have been conducted in patients with rheumatoid arthritis (RA) (10). Increased dietary silicon intake and higher serum levels have been observed in patients with RA. Under conditions of intense oxidative stress in patients with RA, a correlation was found between silicon in the diet and serum and serum markers of the redox state, which was expressed by lower levels of hydrogen peroxide and lipid peroxide measured in the total oxidant status (TOS) test and was more pronounced in women with an increased content of silicon in their diet. Moreover, the ratio of oxidant and antioxidant status decreased as silicon intake increased in these individuals, indicating a role for Si in managing RA-related oxidant overproduction. In RA patients, these correlations were found only in people without factors affecting the redox state, i.e., lower albumin concentration and cigarette smoking. The observed relationship could be the result of the composition of foods providing silicon, which are mainly of plant origin and contain a wealth of antioxidants with proven effectiveness in neutralizing reactive oxygen species. However, in the light of the studies on animal models and cell lines discussed above showing that silicon has the ability to remove hydrogen peroxide or other reactive oxygen species (36, 37, 47), its protective effect against peroxide toxicity seems likely also in humans. A study of RA patients also demonstrated a negative correlation between silicon and IL-6 in the RA group, indicating its role in preventing bone, cartilage and joint destruction in RA. In patients with RA, a negative correlation was also found between silicon in plasma and the number of swollen joints. Moreover, there was a non-significant positive trend in the duration of the disease among non-smokers. These findings may support a role for silicon in the disease response in RA patients.

The role of silicon in the inflammatory process in humans is also suggested by observations of patients with chronic osteoarthritis (48), which showed significantly higher plasma silicon concentrations in the study group compared to the control group of healthy people. These differences were particularly pronounced in men. In males, symptoms of osteoarthritis decreased and were accompanied by significant reductions of cartilage degradation markers. No such effect was observed in women, most of whom were postmenopausal in this study. The influence of hormones on silicon metabolism is still an unexplained issue.

3.8 Silicon and diabetes mellitus

In animal model, silicon demonstrated novel antidiabetic effects by lowering blood glucose levels and improving tolerance to insulin, leptin and adiponectin (49). Strengthening the structure of proteoglycans with silicon may have additional beneficial anti-atherosclerotic effect, as heteropolysaccharides also has been shown to regulate glucose metabolism (20, 45, 50).

The potential beneficial effects of silicon in atherosclerosis pathogenesis and possible sites of action have been summarized in Figure 1.

4 The relationship of silicon with diseases of the connective tissue, osteoarticular and nervous systems

4.1 Silicon and the function of connective tissue and the osteoarticular system

Silicon plays an important role in the formation and maintenance of connective tissue, including collagen and elastin.

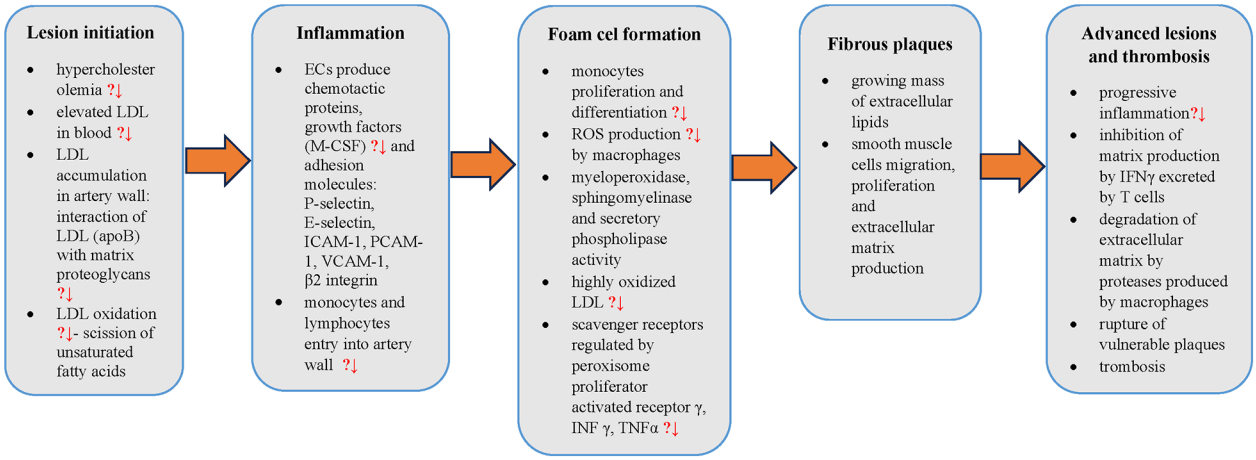

FIGURE 1
Steps in atherosclerosis pathogenesis with points to potential silicon preventive action (?↓). LDL, low density lipoprotein cholesterol; apoB, apolipoprotein B; ECs, endothelial cells; M-CSF, macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule 1; PCAM-1, platelet cell adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; ROS, reactive oxygen species; INFγ, interferon γ; TNFα, tumor necrosis factor-α,?↓ - potential decrease.

This element occurs in various organs, including: skin, bones, hair and nails, arteries. The role of silicon in bone formation was already demonstrated in the 1970s in animal studies (51, 52). Soon, *in vitro* studies (17) showed that Si binds to glycosaminoglycan macromolecules and plays a role in creating cross-links between collagen and proteoglycans, which results in the stabilization of extracellular bone matrix molecules and the prevention of their enzymatic degradation. Studies on cell cultures (53) also showed that silicon is necessary for the formation of glycosaminoglycans; on day 8 of observation, the increase in hexosamine content in silicone-supplemented bones was almost 200% greater than in bones with low silicon content. In animal studies (54), the effect of silicon on bone formation was found to be essentially independent of the effects of vitamin D. All chicks on the silicon-deficient diet, regardless of dietary vitamin D levels, had severe abnormalities in skull architecture (the bone matrix was completely devoid of the normal striated trabecular pattern, less calcified, and more transparent); furthermore, silicon-deficient skulls showed significantly less collagen at each level of vitamin D. The above studies suggested that the main effect of silicon on bone involves the formation of both glycosaminoglycans and collagen in the connective tissue matrix and is independent of vitamin D.

There are many reports in the scientific literature regarding the active role of silicon in bone mineralization and the prevention of osteoporosis (4, 55, 56). The first large population-based study on 1,251 men and 1,596 pre- and post-menopausal women from the Framingham Offspring cohort (age 30–87 years) was conducted by Jugdaohsingh et al. (4), in which they showed a positive correlation between silicon intake and adjusted bone mineral density (BMD) marked in four hip sites in men and women of premenopausal age. No such correlation was found in the group of postmenopausal women or in any of the groups with respect to the BMD of the lumbar spine. Categorical analysis by Si intake or energy-adjusted Si intake confirmed these observations and demonstrated extensive differences in BMD (up to 10%) between the highest (>40 mg Si/day) and lowest (<14 mg Si/day) silicon intake quintiles.

Similar results were obtained by Macdonald et al. (56) - a study on a cohort of 3,198 women aged 50–62 years showed a significant relationship between dietary silicon intake and hip bone mineral density in women currently using hormone replacement therapy (HRT) and for premenopausal women, but not for women with estrogen deficiency (postmenopausal women not taking HRT). This may suggest that the correct level of estrogen, especially estradiol, is necessary for the proper functioning of silicon in the skeletal system.

Also a 2-year follow up pilot study (57) showed that beer consumption did not cause a difference in bone mineral density in early postmenopausal women compared to the control group not consuming beer, although bone formation markers (bone alkaline phosphatase and type I collagen N-propeptide) increased in relation to the control group.

The beneficial effect of silicon on the skeletal system may be due to the effect of orthosilicic acid on osteoblasts. An *in vitro* study (13) demonstrated that orthosilicic acid at physiological concentrations stimulates the synthesis of type 1 collagen in human osteoblast-like cells and fibroblasts of skin and also strengthens osteoblast differentiation. Expression of the type 1 collagen gene was not changed by the orthosilicic acid, but the results suggested that the activity of prolyl hydroxylase, a key enzyme for the synthesis of type 1 collagen may be modulated by this silicon compound. The mechanism of the interaction between soluble silicon and prolyl hydroxylase is unclear.

4.2 Silicon and the nervous system

Silicon may have a protective effect against the development of neurodegenerative diseases. In recent years, many studies have appeared which indicate that aluminum is one of the risk factors for Alzheimer's disease. Experimental studies in rats and mice have shown that aluminum accumulates in the cerebral cortex, hippocampus and cerebellum (58), subsequently promoting the aggregation of highly phosphorylated proteins such as tau (1). According to Kawahara (59), this metal induces neuronal apoptosis both *in vivo* and *in vitro*. The mechanism of the toxic effect of aluminum on the brain has not been finally elucidated, but the following factors are considered: cross-linking of hyperphosphorylated proteins, leading to the formation of tau protein (60); promoting the expression of amyloid precursor protein (APP) and increasing the level of fragments β-40 and β-42 in the brain, which may lead to the formation of neurofibrillary tangles characteristic of the disease (61); increase in oxidative stress and inflammatory reaction due to decreased activity of antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase) (62); damage to the cholinergic system (63). Silicon and silicic acid reduce the bioavailability of aluminum by partially blocking its absorption in the gastrointestinal tract (64, 65) and hindering its reabsorption (66) (Figure 2). A controlled clinical study of Alzheimer's disease showed that drinking up to 1 liter of silicon-rich mineral water daily for 12 weeks increased urinary aluminum excretion in both patients and controls, without any concomitant effect on urinary excretion of essential metals, iron and copper (67).

The effectiveness of silicon in the prevention of Alzheimer's disease can also be attributed to its interaction with aluminum through the formation of non-toxic aluminosilicate complexes, which reduce the availability of free aluminum not only during absorption in the gastrointestinal tract, but also in the cells. A number of biological sites have been identified where silicon and aluminum are colocalized. The enlarged Si content (3–50 times) has been revealed in the cores and rims of senile plaques in the cerebral cortex of patients suffering from senile dementia/Alzheimer's disease type (68). Measurements in the central areas of senile plaques have shown that silicon and aluminum are present in the form of aluminosilicates (69), what possibly can be a way to curtailing of the toxicity of aluminum (38). Senile plaque structures have also been observed in mentally healthy elderly patients and the use of dietary silicon supplements has been suggested as a preventive measure for Alzheimer's disease (9).

Other authors (5) have examined correlation between exposure to aluminum or silica from drinking water and the risk of

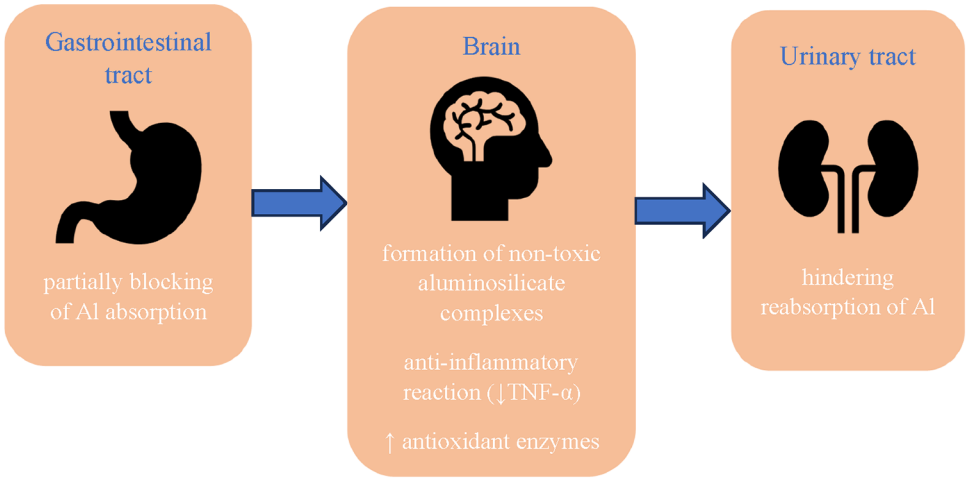

FIGURE 2
Protective effect of silicon against the development of neurodegenerative diseases. Al, aluminum; TNF-α, tumor necrosis factor-α.

dementia, cognitive decline and Alzheimer's disease among older adults. They analyzed 1,925 people who were followed for 15 years (1988–2003). Using random models, the authors demonstrated that cognitive decline over time was larger in individuals with greater daily intake of aluminum from drinking water (≥0.1 mg/day, $p = 0.005$) or greater geographic exposure to this element. On the contrary, increasing silicon intake by 10 mg/day was related to reduced risk of dementia (adjusted relative risk = 0.89, $p = 0.036$) and especially Alzheimer's disease.

Single *in vitro* study (37) reports a neuroprotective action of low silicon concentrations on human SH SY5Y neuroblastoma cell lines by inducing an anti-apoptotic effect and anti-inflammatory reaction as a result of diminishing the level of tumor necrosis factor TNF-α. Also animal studies (38) showed that beer and silicic acid inhibit the aluminum-induced decrease in mRNA expression of endogenous antioxidant enzymes and normalize the expression of TNF-α mRNA. Beer also significantly prevented the accumulation of lipid damage that resulted from aluminum consumption. This finding is important because increased levels of oxidative stress and lipid peroxidation products in brain tissue are major factors contributing to the development of neurodegenerative diseases. In subgroups of animals receiving silicic acid and beer, decreased levels of the antioxidant enzymes catalase and superoxide dismutase in brain tissue homogenates normalized, while levels of thiobarbituric acid reactive substances (TBARS) decreased.

5 Conclusions

The role of silicon in the pathogenesis of atherosclerosis and its complications remains controversial (60). Most of the works indicating the involvement of this element in atherogenesis processes come from animal studies. Currently, the number of studies in humans regarding the relationship between silicon and established risk factors for atherosclerosis, as well as the possible impact on individual cells involved in the process of initiation, progression and destabilization of atherosclerotic plaque is very limited. However, taking into account the above-mentioned results of animal studies indicating beneficial changes in the lipid profile after the use of silicon, adequate dietary intake of the element may contribute to inhibiting the progression of atherosclerosis. Moreover, the beneficial immunomodulatory effect of the element demonstrated in an animal model, and in particular the observation of a decrease in the number of monocytes accompanying a higher intake of silicon, may have a significant impact on the course of the disease caused by chronic inflammation. The role of Si is also supported by the relationship observed in patients with rheumatoid arthritis with both the function of the redox system and the value/titer of inflammatory markers. Due to the existing research conducted mainly on cell lines or animal models and the small number of studies in humans, the importance of silicon in preventing atherosclerosis and other age-related diseases requires further in-depth research.

Author contributions

ŁD: Project administration, Writing – original draft. WK: Conceptualization, Supervision, Writing – review & editing. ED: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

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New light on changes in the number and function of blood platelets stimulated by cocoa and its products

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Hyperactivation of blood platelets, one of the causes of heart attack, and other cardiovascular diseases (CVDs), is influenced by various dietary components, including phenolic compounds from vegetables, fruits, teas, wines, cocoa and its products, including chocolate. The present paper sheds new light on the effect of cocoa and its products, especially dark chocolate, on the number and function of blood platelets, and the anti-platelet activity of their constituent phenolic compounds. A review was performed of papers identified in various electronic databases, including PubMed, Science Direct, Scopus, Web of Knowledge, and Google Scholar, with the aim of determining whether their anti-platelet activity may serve as part of a sweet strategy in countering CVDs. Various studies demonstrate that cocoa consumption, especially in the form of dark chocolate, with a high flavanol concentration, has anti-platelet activity and may play a significant role in cardioprotection; they also note that cocoa consumption may be a good strategy in diminishing cardiovascular risk, including hyperactivation of blood platelets.

KEYWORDS

blood platelets, cocoa, flavanols, flavonoids, phenolic compounds

Introduction

Cardiovascular diseases (CVDs), disorders related to the heart and circulatory system, include various disease units (Zhang et al., 2019; Desai et al., 2023). Among them, coronary artery disease and cerebrovascular artery disease are two of the most common causes of death globally (Zhang et al., 2019; Desai et al., 2023). For many of these conditions, especially stroke and ischemic heart disease, the etiology is based around atherosclerosis and blood platelet activation.

Blood platelets are small, discoid-shaped circulatory cells. Their cell membrane contains various receptors (including glycoprotein GPIIb/IIIa, also known as integrin $\alpha_{IIb}\beta_3$), that regulate the interaction between blood platelets, leukocytes, and intracellular matrix. Upon

Abbreviations: ARU, aspirin reaction unit; CK, creatine kinase; COX-1, cyclooxygenase-1; CVDs, cardiovascular diseases; eNOS, endothelial nitric oxide synthase; GAE, gallic acid equivalent; GP, glycoprotein; ICAM-1, intracellular adhesion molecule 1; IL, interleukin; LDL, low-density lipoprotein; NOX2, NADPH oxidase 2; PFA100, platelet function analyzer; PRU, P2Y2 reaction unit; ROS, reactive oxygen species; TG, triglycerides; TRAP, thrombin receptor activating peptide; VCAM-1, vascular cell adhesion molecule 1.

activation (inducted by various agonists, including thrombin), blood platelets change their shape and release more adhesion receptors, exposing them on their surface (Sang et al., 2021). Although blood platelets are known to play an important role in developing atherothrombosis, its occurrence is also associated with hypertension, smoking, abnormal cholesterol levels, physical inactivity, diabetes, obesity, and a family history of CVD (Teissedre et al., 2018; Bittner, 2020). However, it has been found that blood platelet activation can also be modulated by certain dietary components, including phenolic compounds from herbs, vegetables, fruits, teas and wines, as well as cocoa and its products, including chocolate (Kerimi and Williamson, 2015; Sperkowska et al., 2021).

A number of *in vitro*, *in vivo* and epidemiological studies indicate that diets rich in flavonoids, a subgroup of phenolic compounds, may protect against CVDs by inhibiting blood platelet function (Mozaffarian, 2016). A number of epidemiological studies (Almoosawi et al., 2012; Arranz et al., 2013; Ferri et al., 2015; Vlachojannis et al., 2016; Dong et al., 2017; Lee et al., 2017; Loffredo et al., 2017; Barrios et al., 2018; Garcia et al., 2018; Rees et al., 2018; Zięba et al., 2019; Latif and Majeed, 2020; Tyc et al., 2021; Seecheran et al., 2022), highlight a relationship between the consumption of flavanol-rich cocoa products and a lower incidence of CVDs, including ischemic heart disease and stroke. These studies included consumption of various cocoa products, including dark chocolate, milk chocolate, cocoa powder, and drinks with theobromine. Most importantly, consumption of cocoa and its products was often associated with positive modulation of blood platelet-mediated hemostasis and CVDs (Seecheran et al., 2022). Although a number of review papers indicate that cocoa and its products have cardioprotective potential, these present little information about their anti-platelet action (Fernandez-Murga et al., 2011; Andjuar et al., 2012; Senturk and Gunay, 2015; Loffredo et al., 2017; Gammone et al., 2018; Santos and Macedo, 2018; Zięba et al., 2019; Ghaffari and Roshanravan, 2020). While other studies have found the phenolic compounds from cocoa and its products to have anti-platelet activity, little is known of the mechanisms behind it (Almoosawi et al., 2012; Dong et al., 2017; Lee et al., 2017; Loffredo et al., 2017; Barrios et al., 2018; Latif and Majeed, 2020; Otreba et al., 2021; Seecheran et al., 2022).

The aim of the present study was to review the most recent literature concerning the possible mechanisms behind the anti-platelet activity of the phenolic compounds in cocoa and its products, especially dark chocolate. It sheds new light on the effect of cocoa and its products on blood platelet number and function. It presents a review of 70 review papers and 254 research papers published over the last 20 years. The papers were drawn from various electronic databases, including PubMed, Science Direct, Scopus, Web of Knowledge, and Google Scholar, with extra papers identified by manually reviewing the references. The search was restricted to English language publications (reviews and articles). The following terms were used: *cocoa, cocoa product, blood platelet, platelet, flavonoid,* and *cardiovascular disease*. The last search was run on 8 February 2024.

Cocoa and chocolate–chemical content

Although cocoa and chocolate are two different terms, their main component is the cocoa bean (*Theobroma cocoa*). The term *cocoa* refers to the natural products, i.e., the non-fat component of cocoa liquor, the pure extract of cocoa beans. Although chocolate is a processed food, in some countries such as Spain, it is also taken as a beverage (Fernandez-Murga et al., 2011; Andjuar et al., 2012; Senturk and Gunay, 2015; Tyc et al., 2021). Cocoa contains about 380 known chemical compounds (Fernandez-Murga et al., 2011; Andjuar et al., 2012).

Cocoa and its products are widely consumed throughout the world. Of these products, chocolate is the most popular, and due to its unique texture and teste, it is usually consumed for pleasure. Chocolate is a confectionery product made from cocoa beans, cocoa lipids, and sugar; however, it often contains other ingredients, including nuts, milk, coffee, alcohol and fruits, typically raisins. The type of chocolate depends on the content of cocoa beans, added sugar and other ingredients. Dark chocolate contains the most cocoa beans (50%–85% of total weight), followed by dessert chocolate (30%–70%) and milk chocolate (20%–30%). Some regional differences exist: commercially-produced dark chocolate has about 15% cocoa in the USA and 35%–50% in Europe (Senturk and Gunay, 2015). White chocolate does not contain cocoa beans, but only cocoa butter, fat, milk and sugar.

Cocoa and its products are rich sources of bioactive compounds. For example, cocoa contains about 30%–50% lipids, with this value consisting of approximately 30% stearic acid (saturated fatty acid) and 25% palmitic acid (saturated fatty acid), as well as 30% oleic acid: an unsaturated fatty acid which may play an important role in the prevention and treatment of CVDs through different mechanisms, including inhibition of blood platelet activation (Olas, 2020). Moreover, stearic acid exerts a neutral cholesterolemic response in humans (Fernandez-Murga et al., 2011).

Another important group of ingredients in chocolate, representing up to 10% of the dry weight of the bean, comprises phenolic compounds. However, their precise content depends on the manufacturing process (Andjuar et al., 2012; Senturk and Gunay, 2015; Sperkowska et al., 2021) and its place of origin, for example, the concentration of catechin is 16.52 mg/g in Costa Rican cocoa, and 2.66 mg/g in cocoa grown in Jamaica (Andjuar et al., 2012). The phenolic compound content is also reduced by several production processes, including fermentation, storage, drying and roasting (Loffredo et al., 2017).

Phenolic compounds can be classified according to their chemical structure, biological properties and source of origin. For example, according to Vermerris and Nicholson (Vermerris and Nicholson, 2006), these compounds can be classified into three groups: 1) simple phenols and phenolic acids, 2) flavonoids (including flavanones, flavonols, flavanols, anthocyanins, flavones, and isoflavones), and 3) other polyphenols. Flavonoids are made up of three rings A, B, and C; they usually occur in nature in a bound form, most often as glycosides. An important subclass of flavonoids are the flavanols, which differ from the other flavonoids by the presence of an OH group attached to the C_3 carbon in the heterocyclic C ring, and the lack of double bonds and carbonyl groups.

In addition, chocolate is also recommended as a valuable source of other compounds, including vitamins, minerals (especially magnesium, and phosphorus), trace elements and low concentrations of theobromine (Senturk and Gunay, 2015; Davinelli et al., 2018; Montagna et al., 2019; Febrianto et al., 2021). The chemical composition of dark chocolate is presented in Table 1. It important to note that cocoa also contains small amounts of fiber and plant sterols, which may contribute to lower serum lipids (Fernandez-Murga et al., 2011).

TABLE 1 Chemical content of dark chocolate (Sperkowska et al., 2021).

Chemical compounds	Chocolate
Water (g)	0.6
Protein (g)	6.7
Lipid (g)	34.3
Stearic acid (mg/g cocoa fat)	0.9
Palmitic acid (mg/g cocoa fat)	203.8
Oleic acid (mg/g cocoa fat)	304
Linoleic acid (mg/g cocoa fat)	27.8
Carbohydrate (g)	56.6
Sugar (g)	38.3
Total fiber (g)	1.7
Theobromine (g/kg)	10
Sodium (μg)	4,000
Potassium (μg)	581,000
Iron (μg)	21,000
Calcium (μg)	42,000
Phosphorum (μg)	244,000
Thiamin (μg)	40
Riboflavin (μg)	10
Total phenolic compounds (ng/gallic acid equivalent (GAE)/L)	11.7–14.8
Catechin (mg/kg)	107–500
Epicatechin (mg/kg)	32,7–125
Quercetin (mg/kg)	250
Ferulic acid (mg/kg)	240
Resveratrol (mg/kg)	0.4

Intake of phenolic compounds and their bioavailability

The intake of phenolic compounds, including flavonoids, varies greatly from population to population, and the daily intake of flavonoids is believed to range from 20 to 650 mg per day in terms of aglycan content. In addition, it has been suggested that the mean flavonoid consumption is 189.7 mg per day, of which as much as 83.5% are flavanols (catechins), 7.6% flavanones, 6.8% flavonols and 1.6% anthocyanins (Williamson and Holst, 2008; Loffredo et al., 2017; Azad et al., 2021). For example, Williamson and Holst (Williamson and Holst, 2008) note that >500 mg of phenolic compounds daily can be obtained from "five-a-day," i.e., consuming five portions of fruit or vegetables each day. Consumption of cocoa could easily increase this by 500–1,000 mg. Recently, Crowe-White et al. (Crowe-White et al., 2022) have described that consumption of 400–600 mg/day flavan-3-ols can reduce risk associated with CVDs.

Flavonoids, including flavanols (catechins), are generally considered to be the main phenolic compounds in cocoa and chocolate. Catechin, epicatechin, and their analogs (gallocatechins) are abundant in cocoa beans (1.4 g/kg). Flavanols are also found in many other plant foods, like grape seeds, grapes, apples, apricots, green tea, black tea, and red wines (Murkovic, 2016; Prasain et al., 2018) (Table 2). According to the USA Department database (USDA), 100 g of blueberries yields 3 mg of flavanols while 100 g of apple yields 9 mg (Williamson and Holst, 2008). After oral ingestion, they are processed by the gut microflora, resulting in the generation of a large variety of metabolites. Flavanols reach their peak concentration within two to 3 hours after intake; the final concentration is dose dependent, and the products are detectable in the plasma even after 8 hours (Dugo et al., 2018). For example, Li et al. (Li et al., 2000) detected two catechin metabolites in the plasma and urine of human volunteers: 5-(3′,4′,5′-trihydroxyphenyl)-gamma-valerolactone and 5-(3′,4′-dihydroxyphenyl)-gamma-valerolactone. These metabolites have also been also monitored in other studies. Figure 1 demonstrates the chemical structure of the main flavanols (catechin and epicatechin) in cocoa and their metabolites.

TABLE 2 The concentration of flavanols in selected plant foods (Fernandez-Murga et al., 2011).

Plant food	Concentration of flavanols
Cocoa beans	1.4 g/kg
Chocolate	0.46–0.61 g/kg
Grape seeds	1.7 g/kg
Grapes	0.01 g/kg
Apples	0.03–0.1 g/kg
Apricots	0.01 g/kg
Green tea (200 mL)	100–800 mg/L
Black tea (200 mL)	60–500 mg/L
Red wine (100 mL)	80–300 mg/L

However, the bioavailability of flavanols is low, with a maximum plasma concentration rarely exceeding 1 μM (Andjuar et al., 2012). In addition, the degree of absorption varies between flavanols. For example, the concentration of the catechin monomer is less than 10% of that of epicatechin. However, different interactions with foods may change bioavailability and biological activity. For example, milk chocolate demonstrates lower antioxidant capacity than dark chocolate, which has been attributed to the presence of milk proteins. In addition, if a meal is rich in carbohydrates, absorption has been found to increase by up to 40%; however, no such change is observed if the mean is rich in proteins or lipids (Serafini et al., 2003; Fernandez-Murga et al., 2011; Loffredo et al., 2017). The bioavailability of cocoa phenolic compounds has been reviewed by Andjuar et al. (2012).

Effect of cocoa and its products on blood platelet concentration

Although studies indicate that consumption of cocoa products may affect blood platelet count, their observations are sometimes

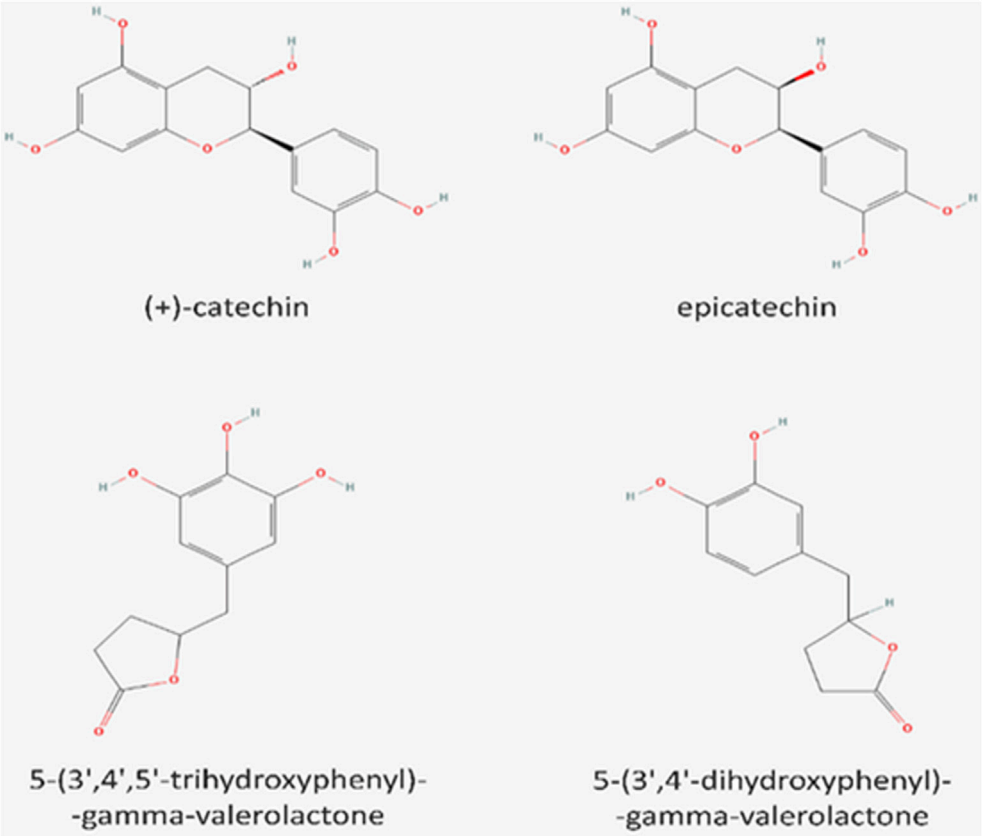

FIGURE 1
The chemical structure of the main flavanols in cocoa: catechin and epicatechin and their metabolites (5-(3′,4′,5′-trihydroxyphenyl)-gamma-valerolactone and 5-(3′,4′-dihydroxyphenyl)-gamma-valerolactone).

controversial. For example, Calderon-Garciduenas et al. (2013) noted that consumption of dark chocolate (30 g with 680 mg of total flavanols, for 9–24 days) increases the number of blood platelets isolated from children who are exposed to air pollution. However, this increase was not statistically significant. Esser et al. (2014) report that consumption of 70 g normal and high flavanol chocolate for 1 month in obese men has no effect on blood platelet number. Another study revealed that four-week consumption of 50 g dark chocolate with low and high flavanols results in no change in blood platelet count in patients with chronic heart failure. Recently, Raguzzini et al. (2019) found no differences in blood platelet count between chocolate consumers (1–3 times/week) and non-consumers; however, the authors did not describe the kind of chocolate or its chemical content.

Cardioprotective mechanisms of cocoa phenolic compounds

Many studies indicate that cocoa phenolic compounds are bioactive and demonstrate cardioprotective properties deriving from several mechanisms. The cardioprotective properties of phenolic compounds, especially flavanols and other flavonoids, are partially attributed to their antioxidant and anti-inflammatory properties (Anger et al., 2016). Cocoa phenolic compounds have been also confirmed to have anti-platelet properties in a number of studies (Holt et al., 2006; Almoosawi et al., 2012; Andjuar et al., 2012; Anger et al., 2016; Vlachojannis et al., 2016; Dong et al., 2017; Lee et al., 2017; Loffredo et al., 2017; Latif and Majeed, 2020; Seecheran et al., 2022). For example, Kim et al. (2017) report that cocoa phenolic compounds exert potent anti-platelet properties both directly and indirectly via endothelial cells, and conclude that they have the potential for lowering the risk of CVD-related hypercoagulation due to hypercholesterolemia.

Murphy et al. (2003) observed that after 28-day exposure to flavanols and procyanidins from cocoa resulted in an increase of catechin and epicatechin concentration in plasma (by 28% and 81%, respectively), and a decrease of blood platelet function.

Another recent paper by D'Amico et al. (2022) studied the relationship between blood platelet activation, oxidative stress and muscular injuries stimulated by physical exercise, as well as the role of cocoa-derived phenolic compounds, in elite athletes. Their results suggest that the cocoa-derived phenolic compounds: catechin and epicatechin significantly reduce oxidative stress and muscle injury in supernatants of human skeletal muscle myoblast cell cultures treated with plasma. They observed downregulation of NADPH oxidase 2 (NOX2) activation, H_2O_2 production and reduction of creatine kinase (CK) and α-actin after cell treatment. However, the authors do not indicate the concentrations of the phenolic compounds.

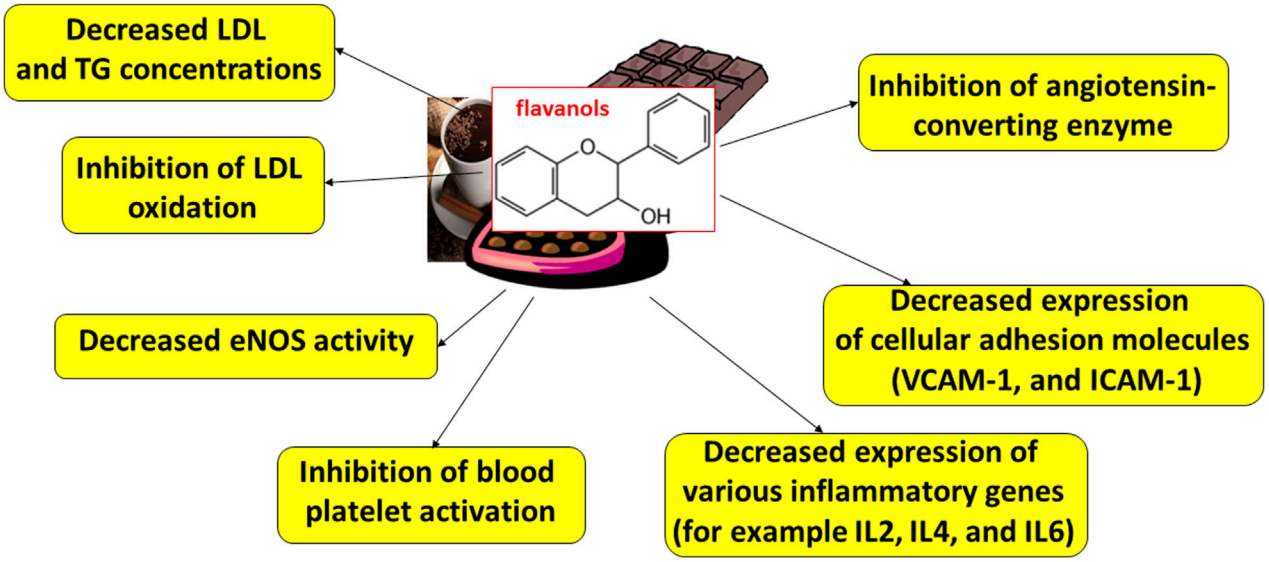

FIGURE 2
Cardioprotective mechanisms of flavanol-rich dark chocolate (Rees et al., 2018). eNOS, endothelial nitric oxide synthase; ICAM-1, intracellular adhesion molecule 1; IL, interleukin; LDL, low-density lipoprotein; TG, triglycerides; VCAM-1, vascular cell adhesion molecule 1. More details in text.

A recent study by Li R. et al. (2022) found that flavonoids exert anti-inflammatory properties in ischemic stroke by acting as modulators of microglia polarization *via* the toll-like receptor (TLR4)/nuclear factor-κB (NF-κB) signaling pathway. Catechin, an important cacao flavonoid, facilitates the cardioprotective role of nuclear factor-erythroid 2-related factor 2 (Nrf2) and its downstream molecules through *inter alia* NF-κB, glutathione peroxidase and kinases (Talebi et al., 2021). Moreover, quercetin, another phenolic compound, can demonstrate cardioprotective potential by affecting molecular pathways such as phosphoinositide 3–kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) (Askrafizadeh et al., 2021; Li Q. et al., 2022).

Proposed cardioprotective mechanism of action of flavanols from cocoa and its products is presented on Figure 2. For example, these compounds may induce a decrease of reactive oxygen species and nitric oxide production. The decrease of intracellular ROS level may be accompanied by the recovery of phosphatase activity. Reactivated phosphatases may inhibit the activity of tyrosine kinases, and thus blood platelet aggregation (by inhibition of GPIIb/IIIa exposition). These compounds may also reduce the activity of cyclooxygenase and reduce arachidonic acid metabolism. Moreover, consumption of flavonol-rich dark chocolate is believed to result in decreased low-density lipoprotein (LDL) and triglyceride (TG) concentrations, inhibition of LDL oxidation, reduced endothelial nitric oxide synthase (eNOS) activity, and reduced expression of several inflammatory genes (interleukins and TNF-α); it also reduces the expression of cellular adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), and inhibition of angiotensin-converting enzyme (Almoosawi et al., 2012; Andjuar et al., 2012; Anger et al., 2016; Vlachojannis et al., 2016; Dong et al., 2017; Kim et al., 2017; Lee et al., 2017; Loffredo et al., 2017; Latif and Majeed, 2020; Seecheran et al., 2022) (Figure 2). However, further studies are needed to clarity the cardioprotective mechanisms of their action.

Anti-platelet properties of cocoa phenolic compounds and cocoa products

Blood platelet functions may be determined by various approaches based on blood platelet aggregation, blood platelet adhesion, ATP release or exposure of the activated conformation of glycoprotein (GP) IIb/IIIa and P selectin. A number of methods can be used to achieve this including blood platelet analysis and flow cytometry based on a range of biological materials, particularly whole blood, platelet-rich plasma, and washed blood platelets (Sang et al., 2021).

About 20 years ago, Rein et al. (2000) observed that consumption of cocoa, containing 897 mg total epicatechin and olimeric procyanidin, decreases platelet aggregation stimulated by various agonists, *viz.* ADP, collagen, and adrenaline. A similar effect was observed after consumption of moderate amounts of cocoa phenolic compound (220 mg). These anti-aggregatory properties were associated with reduced exposure of the activated conformation of GPIIb/IIIa on the blood platelet surface, revealed by a PFA100 platelet function analyzer (*ex vivo* model). Similarly, Pearson et al. (2002) found that flavanol-rich cocoa has an inhibitory effect on the activation of blood platelets stimulated by epinephrine (*ex vivo* model).

Flammer et al. (2007) found blood platelet adhesion to be reduced 2 hours after consumption of dark chocolate containing 0.27 mg/g of catechin an 0.9 mg/g of epicatechin, and a total phenolic compound content of 15.6 mg of epicatechin equivalent per Gram. This effect was observed in 22 heart transplant recipients in a double-blind, randomized study (*ex vivo* model).

TABLE 3 Anti-platelet properties of cocoa and its products in various *ex vivo* and *in vivo* models.

Cocoa and its products	Size group	Time	Subjects	Biological material/method for platelet activation/Parameters of platelet activation	References
Ex vivo					
Cocoa (containing 220 or 897 mg total epicatechin and oligomeric pracyanidin)	30	—	Healthy subjects (blood was obtained before 2 and 6 after ingestion of cocoa)	Blood/Flow cytometry/A platelet function analyzer/Platelet aggregation stimulated by ADP, collagen or adrenaline (inhibition)	Rein et al. (2000)
				GPIIb/IIIa exposure stimulated by these agonists (inhibition)	
Dark chocolate (70% cocoa), 40 g	22	—	Subjects after heart transplant (blood was obtained before 2 h after ingestion of cocoa)	Blood/A platelet function analyzer/Blood platelet adhesion (inhibition)	Flammer et al. (2007)
Flavanol-rich cocoa beverage (18.75 g of flavanol-rich cocoa powder), 300 mL	16	—	Healthy subjects	Blood/An analyzer (the PFA-100)/GPIIb/IIIa exposure stimulated by ADP (inhibition)	Pearson et al. (2002)
Flavan-3-ol-enriched dark chocolate/60 g	42	—	Healthy subjects	Blood/A platelet function analyzer/Platelet aggregation stimulated by ADP (inhibition)	Ostertag et al. (2013)
In vivo					
Tablet (39 mg cocoa flavanols); 6 tablets/day	13	28 days	Healthy (non-smokers)	Blood/Flow cytometry/Platelet aggregation stimulated by ADP or collagen (inhibition)	Murphy et al. (2003)
				P selectin exposure stimulated by ADP (inhibition)	
				ATP release induced by ADP or collagen (inhibition)	
Dark chocolate (85% cocoa) 40 g/day	20	20 days	Healthy subjects (smokers)	Platelet-rich plasma (PRP)/ROS and eicosanoid generation (inhibition)	Carnevale et al. (2012)
Dark chocolate, 16 g/day	23	28 days	Healthy subjects	Urine/Arachidonic acid metabolism (no effect)	Wan et al. (2001)
Milk chocolate, 96 g/day	9	21 days	Healthy subjects	Blood/A platelet function analyzer/Platelet aggregation stimulated by ADP (inhibition)	Kelly et al. (2002)
Dark chocolate (75% cocoa), 100 g/day	9	14 days	Healthy subjects	Platelet-rich plasma (PRP)/Platelet aggregation stimulated by collagen (inhibition)	Innes et al. (2003)
Cocoa (240 mg/day)	8	7 days	Healthy athlete	Blood/Flow cytometry/P selectin exposure stimulated by ADP (inhibition)	Singh et al. (2006)
High-cocoa flavanol (750 mg/day)	16	28 days	Coronary artery disease patients	Blood/Flow cytometry/Platelet microparticles formation (no effect)	Horn et al. (2014)
High flavanol dark chocolate (1,064 mg flavanols), 50 g/day	26	42 days	Subjects with mild hypertension	Platelet-rich plasma (PRP)/Platelet aggregation stimulated by ADP and thrombin receptor activating peptide; (TRAP) (inhibition)	Rull et al. (2015)

Carnevale et al. (2012) found that dark chocolate reduces blood platelet activation by lowering oxidative stress in smokers; however, no such effect was observed in healthy subjects. Twenty healthy subjects and smokers received 40 g of dark chocolate (cocoa >85%) or milk chocolate (cocoa <35%). The level of oxidative stress was measured by different biomarkers, including generation of reactive oxygen species (ROS) and eicosanoids (*in vivo* model).

A recent meta-analysis of randomized clinical trials by Azad et al. (2021) found consumption of cocoa products to have beneficial effects on blood platelet functions in healthy adults regardless of age; this was especially true in men and when consumption was for 4 weeks or longer. This review paper included 21 articles, and 388 participants, including both non-smokers and smokers, as well as individuals with mild hypertension, chronic heart failure, and postmenopausal hypercholesterolemia. Moreover, a randomized clinical trial by Sesso et al. (2022) found that while cocoa supplementation has no effect on the primary outcome of total CV events, consumption significantly reduces CV mortality by 27%.

A recent paper by Seecheran et al. (2022) examined the effect of consumption of dark chocolate (30 g/day; 65% cocoa, for 1 week) on blood platelet function in patients with coronary artery disease (n = 20) receiving maintenance dual anti-platelet therapy: clopidogrel (75 mg/day) and aspirin (81 mg/day). Blood platelet function was determined by aspirin reaction unit (ARU) assays and the VerifyNow P2Y2 reaction unit (PRU) using blood sample. The authors indicate that consumption of dark chocolate augments the inhibitory effect of clopidogrel, but not aspirin (*in vivo* model).

The anti-platelet potential of cocoa and its various food products, as indicated in other *ex vivo* and *in vivo* models, are

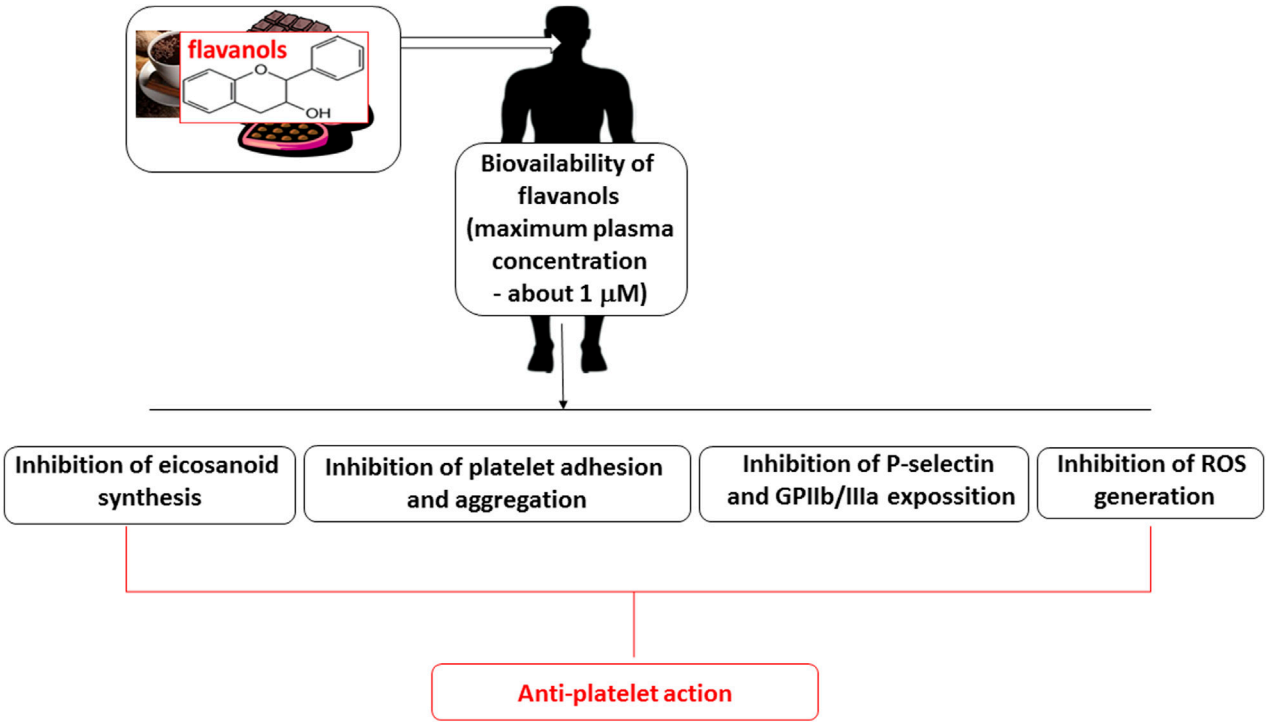

FIGURE 3
Proposed anti-platelet action of cocoa flavanols.

summarized in Table 3 and Figure 3. It is an important to note that many of the studies presented in Table 3 were based on flavanol-rich cocoa administration in both healthy subjects and patients with mild hypertension or coronary artery problems. However, their anti-platelet activity appears to be dependent on various factors, including the type of cocoa product and its chemical content, the method used for measuring blood platelet function, and the type of blood platelet agonist. Moreover, many studies fail to describe the glucose, protein, lipid and caloric content of the tested chocolate, or to demonstrate the plasma concentrations of flavanols with relatively low bioavailability; in addition, their downstream plasma metabolite concentrations may not correlate with their positive effects on CVDs. Many studies fail to consider the effects of long-term consumption. Therefore, there is a need for longer studies examining the anti-platelet action and safety of cocoa flavanols and other components.

A review by Holt et al. (2006) indicates that flavanol-rich food products, including cocoa and its products, may modulate various elements of hemostasis; for example, the authors suggest that these compounds can inhibit blood platelet adhesion and aggregation, ROS generation, eicosanoid synthesis, P-selectin and GPIIb/IIIa exposure.

The proposed mechanism of action of cocoa flavanols on blood platelets is presented in Figure 3. The mechanism by which the phenolic compounds in cocoa, especially flavanols, inhibit blood platelet function remains complex and ambiguous. It is possible that flavanols decrease ROS generation and inhibit eicosanoid biosynthesis and platelet aggregation by inhibiting GPIIb/IIIa exposure. However, without knowing at least one direct cellular target of flavanols and their metabolites, their specific mode of action remains unclear. Therefore, further studies are needed to clarify the mechanism of their anti-platelet action.

Conclusion

In recent years, cocoa and its products have been objects of various studies examining their cardioprotective action. Their findings demonstrate that cocoa consumption, especially in the form of dark chocolate, with a high flavanol concentration, has anti-platelet activity and may play a significant role in cardioprotection; they also note that cocoa consumption may be a good strategy in diminishing cardiovascular risk, including hyperactivation of blood platelets (Fernandez-Murga et al., 2011; Andjuar et al., 2012; Senturk and Gunay, 2015; Loffredo et al., 2017; Zięba et al., 2019).

Studies have described the interactions between chocolate and various drugs, such as antibiotics and statins (Antal et al., 2001; Piotrowicz et al., 2008; Scolaro et al., 2018), and these interactions are considered crucial for the efficacy of treatment in different diseases. In the case of the two antiplatelet drugs aspirin and clopidogrel, Collyer et al. (2009) suggest that chocolate acts synergistically with them by the inhibition of cyclooxygenase–I (COX-I), and this may lead to bleeding. Recently, Seecheran et al. (2022) found that the consumption of dark chocolate augments the inhibitory effect of clopidogrel in patients with coronary artery disease. However, little is known of the precise mechanisms behind the interactions of antiplatelet drugs or supplements with cocoa, its products (especially chocolate) and components (e.g., phenolic compounds and lipids), or the absorption of anti-platelet drugs and supplements after consuming cocoa and its products. Hence, the effect of cocoa on platelet activity,

and the question of whether it may be part of a sweet strategy in diminishing CVDs, remains open.

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Conflict of interest

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The cardioprotective potential of selected species of mistletoe

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Mistletoe is an evergreen woody shrub with stems measuring 30–100 cm. It has leathery, yellowish-green strap-shaped leaves, yellowish-green flowers, and bears typical berries. The most common species is *Viscum album* L., mainly present in Europe and Asia. It is commonly known as European mistletoe or simply, mistletoe. Scientific interest in mistletoe was awakened in the XX century. Mistletoe, especially *V. album* L., has historically been used in the treatment and prophylaxis of CVD, with its properties being confirmed in recent studies. This mini-review describes new aspects of the cardioprotective properties of various species of mistletoe, especially *V. album* L. The effect of oral and subcutaneous application of fresh *V. album* L. extracts on blood pressure has been studied in various models; while the data suggests that mistletoe may be a promising herbal extract with cardioprotective properties, the species has only been tested *in vitro* and *in vivo*, on animals. In addition, it is unclear whether the cardioprotective activity of mistletoe may be due to particular chemical components, as the chemical composition of mistletoe extracts can vary depending on *inter alia* the time of harvest, extraction method and plant part. Hence, this activity may instead result from synergistic interactions between various secondary metabolites. Therefore, further studies are needed to identify the mechanisms of action of mistletoe compounds on CVDs, and determine their interactions with other cardioprotective drugs, their metabolic mechanisms, pharamacokinetics and adverse effects. More research is also needed to determine the therapeutic doses of active ingredients for use in clinical trials; this would require an accurate understanding of the chemical composition of extracts from different species of mistletoe (not only *V. album* L.) and from various host trees.

KEYWORDS

cardiovascular disease, cardiovascular activity, hypertension, mistletoe, viscotoxin

Introduction

Cardiovascular diseases (CVDs) have been a leading cause of death globally over the past 20 years, according to World Health Organization (WHO). The most common forms of CVD are hypertension, stroke, myocardial infarction and atherosclerosis. CVDs may be induced by various endogenous and exogenous risk factors, including hypercholesterolemia, blood platelet hyperactivation, diabetes, obesity and oxidative stress, as well as lifestyle factors such as smoking and lack of physical activity.

Abbreviations: COX-2, cyclooxygenase-2; cGMP, guanosine-3′,5′-cyclic monophosphate; CVDs, cardiovascular diseases; GSH, glutathione; L-NAME, N-nitro-L-arginine methylester; MAPK, mitogen activated protein kinase; MDA, malondialdehyde; NO, nitric oxide; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; WHO, World Health Organization.

Fortunately, various plant components are known to possess cardioprotective properties, with one such genus being the of mistletoe. For example, a number of studies have found it to demonstrate protective potential against hypertension (Rodriguez-Cruz et al., 2003; Ofem et al., 2007; Radenkovic et al., 2009; Bachhav et al., 2011; 2012; 2015; Sekeroglu et al., 2011; Jung et al., 2013; Karagoz et al., 2016; Ko et al., 2016).

Mistletoe (*Viscum* L.) is a genus of parasitic plants from the *Viscaceae* family, which includes about 113–150 species. The range of the genus covers the tropical and temperate zone of the Old World, with the greatest diversity being in Africa and Asia (45 species), with fewer being observed in Australia (four species). Europe is home to two species, including European mistletoe (*Viscum album* L.), also known as gui, Mistel, vischio and muerdago., but *V. album* L. is the most well-known and studied mistletoe species. In addition to *Viscum album* L., *Viscum coloratum* (Kom.) Nakai (Korean mistletoe), *Viscum shimperi* Engl., *Viscum capense* L. are also well known. These species are semi-parasitic plants which grow on coniferous and deciduous trees and use suckers to take water and mineral salts from the host. They can be most often found on poplars, lindens, birches, oaks, firs and pines (Whiteman, 2023).

Mistletoe has characteristically-branching olive-green stems and thick, leathery, dark-green leaves. In late autumn and winter, spherical, white, viscous-filled berries the size of peas ripen at the ends of mistletoe shoots. These fruits are regarded as a delicacy by waxwings and thrushes. The sticky content of the fruit sticks to the feathers of the bird, allowing the mistletoe seeds to be moved from tree to tree. In folklore, mistletoe is generally known as a talisman of prosperity and good fortune (Nazaruk and Orlikowski, 2016; Szurpnicka et al., 2020; Song et al., 2021).

Mistletoe extracts, especially aqueous extracts, are applied in both traditional and modern medicine. Mistletoe itself has been known and used in traditional folk medicine for hundreds of years; indeed, Luther and Becker (1987) report that *V. album* L. has been studied for more than 2000 years, particularly with regard to its medical properties. It has long been used in the treatment of neurological diseases, including headaches, epilepsy, dizziness and other (Szurpnicka et al., 2019; 2022). Only the leaves and twigs are used for internal purposes, as the fruits contain substances harmful to humans and can only be used externally. However, in folk medicine, mistletoe fruits were used as a remedy for all sorts of skin problems; for example, fruit ointment has been used to treat many ailments including wounds, burns, frostbite, skin tumors, actinic keratosis and dermatoses. Hippocrates used mistletoe to treat diseases of the spleen, while Elder and Pliny used it to treat epilepsy (Szurpnicka et al., 2019).

Since ancient times, the anti-inflammatory, analgesic, antidiabetic, anti-arrhythmic and hypertensive properties of *V. album* L., were widely known in the traditional medicines of Asia, Africa and Europe, and remain in use in various countries. In addition, information about mistletoe is available in the European, French, German and U.S. pharmacopeias (Szurpnicka et al., 2020; Kleszken et al., 2022; Klingemann, 2024). It has been proposed that the therapeutic effect of *V. album* L. could be due to synergistic interactions between the various secondary metabolites present in its leaves (Segneanu et al., 2022). In addition, the mechanisms of mistletoe action act vary and depend on its phytochemical content and distribution (Montoya-Inzunzo et al., 2023).

Two various groups of mistletoe preparations exists: 1) which are applied at a constant dose of lektines (for example, Lektinol®, Eurixor®, and Cefalektin®), 2) which are applied homeopathically or anthroposophical produced mistletoe preparations, including Plenosol®, Helixor®, Isorel®, Iscador®, Iscucin®, and Abnobaviscum®. They are used in cancer treatment (Staupe et al., 2023).

It should also be mentioned that all parts of mistletoe are toxic. They contain viscotoxins and lectins, which are two groups of toxic proteins. For example, the consumption of mistletoe leaves or berries can result in serious stomach problems (Yousefvand et al., 2022).

This mini-review describes new aspects of the cardioprotective properties of various species of mistletoe, especially *V. album* L. The review is based on a corpus of electronic resources including GoogleScholar, PubMed, Scopus, ScienceDirect, and Web of Science (to 15the February, 2024). The following terms were used: "mistletoe," "*Viscum* L.," "*Viscum album* L.," "*V. album* L.," and "cardiovascular disease."

Chemical content of mistletoe

Mistletoe contains various bioactive substances, like alkaloids, terpenes, phenolic compounds, and proteins, associated with its potential biological activities, but its chemical content depends on the time of harvest, the manufacturing process, and species of host tree (Ko et al., 2016). Previous studies have used a range of phytochemical analysis techniques, such as high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Szurpnicka et al., 2019). Phytochemical studies based on high-performance thin layer chromatography (HPTLC) indicate that *V. album* L. contains chemical compounds from different chemical classes (Pfuller, 2000; Nicoletti and Pitera, 2020). The most characteristic group includes viscotoxins (0.05%–0.1%) and lectins, which have apoptotic and cytotoxic properties; however, other compounds were also identified, including monosaccharides, phenolic compounds, especially flavonoids, phytosterols (β-sitosterol, γ-sitosterol, stigmasterol, campesterol, and other), terpenoids, diheptanoids, amines, organic acids, amino acids and mineral salts, such as potassium, zinc and calcium compounds (Thronicke et al., 2022; Montoya-Inzunzo et al., 2023).

With regard to plant part, the leaves of *V. album* L. were found to demonstrate higher total phenolic compound contents (polyphenols and flavonoids: 57.7 mg/g dry extract and 9.5 mg/g dry extract, respectively) compared with the seeds and fruits (5.9–354 μg/g dry extract). These include various flavonoids (rhamnetin, quercetin, isoquercitrin, apigenin, luteolin, rutin, and other) and phenolic acids (chlorogenic acid, isochlorogenic acid, coumaric acid, sinapic acid, cinnamic acid, gallic acid, caffeic acid, vanillic acid, salicylic acid, ellagic acid, and other) (Pietrzak et al., 2014; 2017; Thronicke et al., 2022; Montoya-Inzunzo et al., 2023).

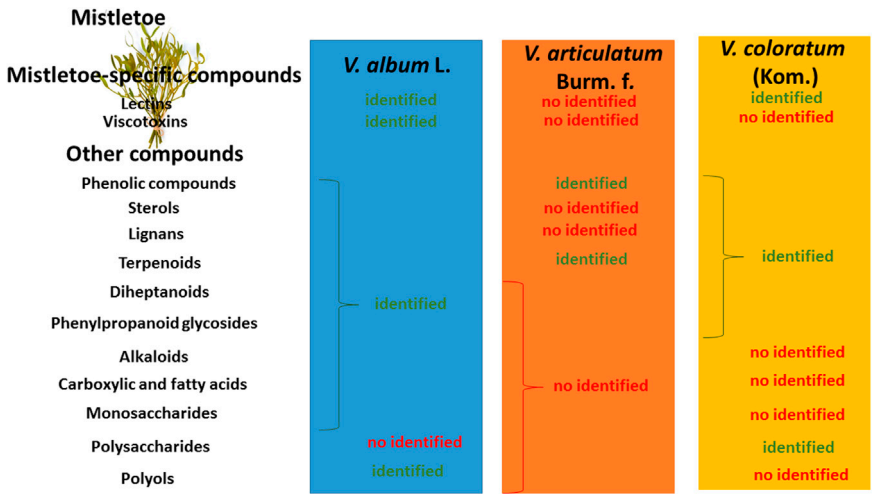

FIGURE 1
Chemical content of *V. album* L. and other species.

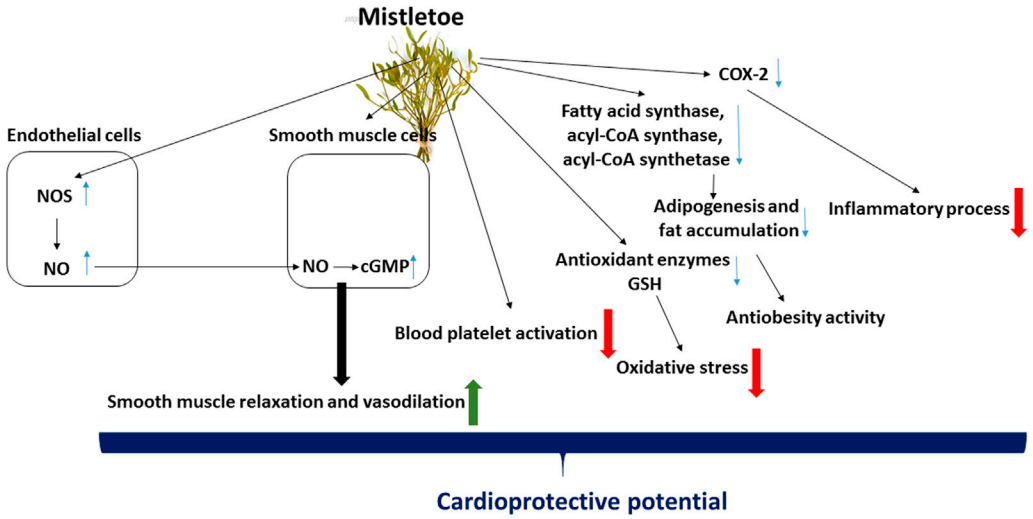

FIGURE 2
Probable mechanisms of cardioprotective potential of mistletoe. Mistletoe extract may exert their cardioprotective action via the nitric oxide (NO), including inducible and endothelial nitric oxide synthase/guanyl cyclase pathways. The anti-inflammatory properties of mistletoe extracts may be associated with inhibition of cyclooxygenase-2 (COX-2) activity. Mistletoe may also inhibit adipogenesis and fat accumulation. In addition, mistletoe has antioxidant activity and anti-platelet potential. More details are text of manuscript.

Most importantly, like in some other related species, the fruits and foliage of *V. album* L., contain low-molecular-mass thionin-type proteins, known as viscotoxins, as well as characteristic lectins known as viscolectins. Several viscotoxin isoforms have been described, viz. A1, A2, A3 and A4, with A3 being the predominant isoform (Majeed and Rehman, 2021; Yousefvand et al., 2022; Zhang et al., 2022). Moreover, three types of lectins (termed mistletoelectins I, II, and III) have been identified in *V. album* L. (Yousefvand et al., 2022; Niwa et al., 2003; Ahmad et al., 2018; Szurpnicka et al., 2019; Szurpnicka et al., 2020; de Almeida et al., 2023; Nicoletti, 2023a). The chemical content of *V. album* L. and other mistletoe species are described in Figure 1.

Cardioprotective action of mistletoe and mechamisms of action of selected chemical components

Various *in vitro* and *in vivo* studies have examined the cardioprotective activity of mistletoe extracts. For example, Suveren et al. (2017) studied the activity of *V. album* L. aqueous and methanolic extracts, prepared from dried leaf, in myocardial ischemia and reperfusion injury in rats. Both tested extracts reduced the extent of infarction compared with untreated controls. The authors propose that these extracts may exert their cardioprotective properties via the nitric oxide/guanylyl cyclase pathway.

TABLE 1 Cardioprotective potential of extracts of *V. album* L. and other species in *in vitro* and animal models.

Part of plant/host tree	Extraction solvent/ active constituents	Dose/administration	Biological activity	References
Animal model				
Fresh leaves of *V. album* L./citrus	Aqueous extract/no data	150 mg/kg/day; orally (for 6 weeks)	Antihypertensive properties	Ofem et al. (2007)
Dried leaves of *V. album* L./*Pyrus communis* L. ssp. communis	Aqueous extract/no data	250 mg/kg/day; orally (for 24 days)	Antihypertensive properties	Karagoz et al. (2016)
Dried leaves of *V. album* L./*Quercus* L.	Aqueous extract/no data	500 and 1,000 mg/kg/day	Antioxidant properties	Shahaboddin et al. (2011)
Dried leaves of *V. album* L. and quercetin/ *Pyrus communis* L. ssp. communis	Methanolic extract/no data	250 mg/kg/day; orally (for 10 days)	Reducing cyclophosphamide-induced cardiotoxicity	Sekeroglu et al. (2011)
Dried leaves of *V. album* L. and quercetin/ *Pyrus communis* L. ssp. communis	Methanolic extract/no data	250 mg/kg/day; orally (for 10 days)	Antioxidant properties	Sekeroglu et al. (2011)
Dried leaves of *V. album* L. and quercetin/ *Pyrus communis* L. ssp. communis	Methanolic extract/no data	250 mg/kg/day; orally (for 10 days)	Anti-inflammatory properties	Sekeroglu et al. (2011)
Fresh steams of *V. album* L./no data	Ethanolic, ether and ethyl acetate/ no data	3.33×10^{-5}—1×10^{-3} mg/kg; intraperitoneally	Antihypertensive properties	Radenkovic et al. (2009)
Dried herb of *V. articulatum* Burm. F/ *Cordia macleodii* (Grift) Hook and Thoms.	Methanolic extract/no data	200 and 400 mg/kg/day; orally (for 4 weeks)	Antihypertensive properties	Bachhav et al. (2012)
Cuticular wax of *V. articulatum* Burm. f/ *Cordia macleodii* (Grift) Hook and Thoms.	No data/oleanolic acid	60 mg/kg/day; intraperitoneally (for 15 days)	Antihypertensive properties	Bachhav et al. (2011)
Cuticular wax of *V. articulatum* Burm. f/ *Cordia macleodii* (Grift) Hook and Thoms.	No data/oleanolic acid	60 mg/kg/day; intraperitoneally (for 4 weeks)	Antihypertensive properties	Bachhav et al. (2015)
Mistletoe *Psittacanthus calculates* (part of plant—no data)/*Quercus candicans*	Ethanolic extract/no data	12.5–800 μg/mL	Antihypertensive properties	Rodriguez-Cruz et al. (2003)
Dried leaves of *V. coloratum* (Kom.) Nacai/ *Quercus candicans*	Aqueous extract/no data	3 g/kg/day; orally (15 weeks)	Anti-obesity properties	Jung et al. (2013)
Dried leaves of *V. coloratum* (Kom.) Nacai/ *Quercus candicans*	Aqueous and ethanolic extract/ oleanolic acid and betulin	0.2 or 0.6% extract/day; orally (8 weeks)	Anti-obesity properties	Ko et al. (2016)
In vitro model				
V. album L. (part of plant—no data)/ *Quercus* L.	Hydro-alcoholic extract/no data	10–100 μg/mL	Anti-inflammatory properties	Hegde et al. (2011)
V. album L. (part of plant—no data)/ *Quercus* L.	Hydro-alcoholic extract/no data	25 and 50 μg/mL	Anti-inflammatory properties	Saha et al. (2015)
V. album L. (part of plant—no data)/ *Armenica vulgaris* Lam.	Methanolic extract/no data	10–100 μg/mL	Antioxidant properties	Yesilada et al. (1998)
V. album L. (different parts of plant)/*Molus domestica*	Ethanolic extract/flavonoids	0.05%–0.5%	Antioxidant properties	Holandino et al. (2020)
Leaves and stems of *V. album* L./*Abies alba* Mill	Aqueous extract/flavonoids	-	Antioxidant properties	Pietrzak et al. (2014)
Dried parts of *V. album* L./no data	Ethanolic extract/flavonoids	-	Antioxidant properties	Papuc et al. (2010)

Elsewhere, a study based on isolated and perfused Guinea pig hearts found the cardioprotective potential of *V. album* L. aqueous extract to potentially derive from inducible and endothelial nitric oxide. These effects were inhibited by the presence of inhibitors and blockers. Moreover, the tested extract had inotropic effect (Tenorio-Lopez et al., 2006a; Tenorio-Lopez et al., 2006b).

Oral administration of aqueous *V. album* L. leaf extract (250 mg/kg/day) by gavage has also been found to exert a cardioprotective influence on isoproterenol-induced heart failure in rats; which was attributed to regulation of the NO pathway (n = 10) (Karagoz et al., 2016). Other studies indicate that *V. album* L. extract and quercetin supplementation attenuates cyclophosphamide-induced cardiotoxicity, genotoxicity and urotoxicity; this effect has been attributed to anti-inflammatory and antioxidant effects indicated by antioxidant enzyme activity, reduced glutathione level and malondialdehyde production (a parameter of lipid peroxidation) (Sekeroglu et al., 2011). Recently, Nicoletti (2023a) have described the antioxidant properties of *V. album* L. and other species, including various Korean mistletoe, Asian mistletoe, Argentinian mistletoe, Chilean

mistletoe, Indian mistletoe, and African mistletoe. In addition, this author suggest that not only phenolic compounds identified in *V. album* L., but also visartisides (a novel class of antioxidant) have antioxidative properties, which may play an important role in the cardioprotective action of mistletoe, although further studies concerning the mechanisms of their action are necessary. Other species of mistletoe, including Chinese mistletoes and Asian mistletoes have also antioxidant potential, which was assigned to phenolic compounds, especially flavonoids (Li et al., 2018; Ahmad et al., 2023). More details about the antioxidant activity of mistletoes are described in the review paper by Nicoletti (2023b).

Various studies have reported anti-hypertensive action of Chilean mistletoes, together with antioxidant activity (inhibition of lipid peroxidation). This activity was investigated in connection with the flavonoid-rich fraction (Dobrecky et al., 2022).

The anti-inflammatory activity of *V. album* L. extracts has been reported in other studies. They have been found to inhibit the action of cyclooxygenase-2 (COX-2), and to destabilize COX-2 mRNA (Elleru et al., 2015; Saha et al., 2015). These anti-inflammatory properties have been associated with antioxidant potential; for example, *V. album* L. contains a number of phenolic compounds, such as flavonoids, which are known to be active scavengers of ROS (Papuc et al., 2010; Vicas et al., 2011; Pietrzak et al., 2014; Saha et al., 2015; Speisky et al., 2022).

Mistletoe may also have anti-obesity potential, which may have an important role in the prophylaxis and treatment of CVDs. *V. album* L. extracts may inhibit adipogenesis and fat accumulation by decreasing the expression of fatty acid synthase, acyl-CoA synthase, acyl-CoA synthetase and other regulators of fatty acid oxidation (Kim et al., 2015).

Panossian et al. (1998) isolated four phenylproponoid glycosides from ethanol extract of *V. album* L. coniferyl alcohol-4-O-β-D-glucopyranoside (coniferin), syringenin 4-O-β-D-glucopyranoside (syringin), coniferylalcohol- and syringe 4-O-β-D-apiofuranosyl (1→2)-β-D-glucopuranosides. All substances were found to inhibit ADP-stimulated blood platelet aggregation *in vitro*. Deliorman et al. (2000) also observed that syringin, coniferin and 5,7-dimethoxy-flavanone-4′-O-β-D-apiofuranyl (1→2)-β-D-glucopuranosides isolated from *V. album* L. induce concentration-dependent contractions in rat aortic rings.

Rodriguez-Cruz et al. (2003) found that the ethanolic extract of the mistletoe *Psittacanthus calculates* (12.5–800 μg/mL), used in Mexican traditional medicine for the treatment of hypertension, has no effect on the basal tone of rat aortic rings (in the absence or presence of indomethacin (an inhibitor of the enzyme cyclooxygenase 1 and 2) or N-nitro-L-arginine methylester (L-NAME), a NO synthase inhibitor); however, low concentrations of this extract (<300 μg/mL) induced a low level of additional tension in both types of rings following precontraction by phenylephrine. In addition, at higher concentrations (>300 μg/mL), the extract relaxed the rings with an intact endothelium. This relaxation was completely reverted by the addition of L-NAME, but not by indomethacin. These results indicate that the endothelium relaxation stimulated by the plant extract was mediated by the synthesis or release of NO.

The antihypertension properties of mistletoe may be also mediated by calcium channel blockade, as indicated in a study of *V. album* L. aqueous leaf extract on rat aortic rings by Mojiminyi et al. (2008) and in a study on rabbit aortic rings by Khan et al. (2016). Ofem et al. (2007) found that *V. album* L. aqueous leaf extract to reduce blood pressure without any alternation in heart rate, and attribute this change to catecholamine-like blocking agents.

Bachhav et al. (2012) also observed that the methanolic extract of *V. articultum* Burm has anti-hypertensive activity in rats when applied at 200 and 400 mg/kg/day, for 4 weeks.

The effect of mistletoe on CVDs may be due to the presence of various phenolic compounds, such as flavonoids, as these have a number of properties that may have cardioprotective effects (Terao, 2023; Thomas et al., 2023). They are known to have anti-platelet, antioxidant and anti-inflammatory properties, and can modulate a range of related signalling pathways, such as the phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen activated protein kinase (MAPK) pathways. For example, Wen-Feng et al. (2006) noted that flavonoids from Chinese *Viscum coloratum* have antiarrhythmic properties in a rat model of arrhythmia induced by aconitine. In addition, flavonoids isolated from *V. coloratum* reduced ischemic myocardial injures in rat model of myocardial infarction by blocking the signaling pathway of platelet-activating factor (Chu et al., 2008).

Some sterols, including β-sitosterol have also demonstrated cardioprotective activity and play an important role in CVDs induced by hypercholesterolemia (Perez-Martinez et al., 2023). The antihypertensive effect of *V. articulatum* Burm. f. has also been attributed to the presence of triterpenoids, such as betulinic acid and oleanolic acid (Bachhav et al., 2012). For example, oleanolic acid isolated from this species significantly decreased systolic blood pressure and cardiac lipid peroxidation in rats (Bachhav et al., 2012). Skrypnik et al. (2022) described the concentration of oleanolic acid in different parts of *V. album* L. (stems: 4.77 ± 1.53 mg/g DW; leaves: 5.02 ± 1.47 mg/g DW, and fruits: 2.62 ± 0.89 mg/g DW).

In addition, various mistletoe extracts and isolated lectins have demonstrated radical-scavenging properties, and were found to reduce oxidative stress stimulated by reactive oxygen and nitrogen species (Sengul et al., 2009; Kim et al., 2010; 2016; Papuc et al., 2010; Kusi et al., 2015). The probable mechanisms behind the cardioprotective potential of mistletoe are presented in Figure 2.

However, little information exists regarding the cardioprotective activity of isolated chemicals in mistletoe (Panossian et al., 1998; Deliorman et al., 2000). This may suggest that the effects of mistletoe extract result from the synergic activities arising from combinations of different compounds. Mistletoe-specific compounds may also interact with other chemical groups, such as phenolic compounds or phenylproponoid glycosides. In addition, it is possible that the compounds present in mistletoe are also present in other plants, but only mistletoe has the unique combinations that can bestow cardioprotective effects.

Conclusion

Various historical references indicate the use of mistletoe, especially *V. album* L. in the treatment of CVDs, and these properties have been confirmed in recent years. Recent *in vitro* experiments and animal-based studies on cardioprotective effect of *V. album* L. and other species are presented in Table 1. However, most studies investigating this activity lack the necessary analysis of

individual chemical components, and instead relate the activity to *inter alia* total phenolic content and other chemical compounds, including triterpenoids. This is arguably due to the limited approach often taken to the study of herbal and medicinal plants, in which phytochemists and pharmacologists work independently rather than adopting a more holistic approach.

Current studies suggesting that mistletoe may be a promising herbal extract with cardioprotective properties, including antihypertensive activities, have only been based on *in vivo* models (on animals) and *in vitro* models. The compounds responsible for these properties have not been well identified due to the considerable variation present in the chemical composition of mistletoe extracts, which can depend on *inter alia* time of harvest, extraction technique and plant part. The cardioprotective action may result from synergistic interactions of different secondary metabolites rather than individual compounds (Rodriguez-Cruz et al., 2003; Ofem et al., 2007; Rodenkovic et al., 2009; Bachhav et al., 2011; 2012; Karagoz et al., 2016).

Further studies of the effect of mistletoe compounds on CVDs should address their interactions with other drugs with cardioprotective properties, metabolic mechanisms, pharamacokinetics and adverse effects. More research is needed to determine the exact chemical composition of extracts from different species of mistletoe and host trees. These extracts may be used to determine the therapeutic doses of active ingredients for use in clinical trials.

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BO: Conceptualization, Writing–original draft, Writing–review and editing.

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Alchemilla vulgaris modulates isoproterenol-induced cardiotoxicity: interplay of oxidative stress, inflammation, autophagy, and apoptosis

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Introduction: Isoproterenol (ISO) is regarded as an adrenergic non-selective β agonist. It regulates myocardial contractility and may cause damage to cardiac tissues. Alchemilla vulgaris (AV) is an herbal plant that has garnered considerable attention due to its anti-inflammatory and antioxidant bioactive components. The present investigation assessed the cardioprotective potential of AV towards ISO-induced myocardial damage.

Methods: Four groups of mice were utilized: control that received saline, an ISO group (85 mg/kg, S.C.), ISO + AV100, and ISO + AV200 groups (mice received 100 or 200 mg/kg AV orally along with ISO).

Abbreviations: AV, *Alchemilla vulgaris*; Bcl-2, B-cell lymphoma protein 2; Bax, Bcl-2-associated X; CAT, catalase; CK-MB, creatine kinase-myoglobin binding; GSH, reduced-glutathione; HMGB1, high mobility group box 1 protein; ISO, isoproterenol; IL-1β, interleukin-1β; LDH, lactate dehydrogenase; LPO, lipid peroxidation; MDA, malondialdehyde; LC3, microtubule-associated protein light chain 3; NF-κB/p65, nuclear factor Kappa-B/p65; PCA, principal component analysis; RAGE, receptor of advanced glycation end products; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α.

Results and discussion: ISO induced notable cardiac damage demonstrated by clear histopathological disruption and alterations in biochemical parameters. Intriguingly, AV treatment mitigates ISO provoked oxidative stress elucidated by a substantial enhancement in superoxide dismutase (SOD) and catalase (CAT) activities and reduced glutathione (GSH) content, as well as a considerable reduction in malondialdehyde (MDA) concentrations. In addition, notable downregulation of inflammatory biomarkers (IL-1β, TNF-α, and RAGE) and the NF-κB/p65 pathway was observed in ISO-exposed animals following AV treatment. Furthermore, the pro-apoptotic marker Bax was downregulated together with autophagy markers Beclin1 and LC3 with in ISO-exposed animals when treated with AV. Pre-treatment with AV significantly alleviated ISO-induced cardiac damage in a dose related manner, possibly due to their antioxidant and anti-inflammatory properties. Interestingly, when AV was given at higher doses, a remarkable restoration of ISO-induced cardiac injury was revealed.

KEYWORDS

***Alchemilla vulgaris*, isoproterenol, myocardial injury, oxidative stress, inflammatory cytokines, HMBG1/RAGE pathway**

1 Introduction

Isoproterenol (isoprenaline hydrochloride, ISO; $C_{11}H_{17}NO_3$) is a synthetic non-selective β-adrenergic receptor agonist, which is the iso-propylamine analog of adrenaline (Song et al., 2020; Pandi et al., 2022). It stimulates heart rate and myocardial contractility and is commonly used to treat bradycardia, heart block, cardiac arrest, and occasionally asthma (Timercan et al., 2019; Asiwe et al., 2023). Cardiotoxicity is one of the most common adverse effects of ISO administration (Pandi et al., 2022). The underlying mechanism of ISO-triggered cardiac damage is complicated and multifactorial, with oxygen-derived free radicals and oxidative stress presumed to be critical mechanisms implicated in ISO cardiotoxicity (Obeidat et al., 2022; Asiwe et al., 2023). ISO generates quinone, which is converted to superoxide anions (O_2^-) and hydrogen peroxides (H_2O_2) by molecular oxygen (Othman et al., 2017). Overproduction of reactive oxygen species (ROS) causes cellular dysfunction, protein and lipid degradation, and DNA damage, resulting in irreparable cell damage and is associated with the pathogenesis of most cardiovascular conditions (Tsutsui et al., 2011). Although treatment using modern medicine is effective, it is associated with more adverse effects. Therefore, exploring natural products as an innovative alternative for the management of cardiac ailments has recently gained great attention (Abdel-hady et al., 2021; Li et al., 2022). Plant-based drugs are affordable and have fewer side effects (Song et al., 2020). Previous research have demonstrated that herbal remedies or their active compounds can mitigate ISO-induced cardiotoxicity such as *Sanguisorba minor* (Hosseini et al., 2023), *Nerium oleander* Linn (Gayathri et al., 2011), *Fumaria indica* (Sajid et al., 2022), and *Esculetin* (Pullaiah et al., 2021).

Alchemilla vulgaris (AV), or "lady's mantle," or "lion's foot," or "bear's foot" as known, is an herbaceous perennial plant that corresponds to the Rosaceae family (Vlaisavljević et al., 2019). This herbal plant is quite effective against many gynecological diseases and reproductive problems (Jakimiuk and Tomczyk, 2024). It also is used to treat skin and digestive disorders (Boroja et al., 2018). According to the European Pharmacopoeia 6.0, AV has been designated a medicinal plant with diverse pharmacodynamic activities (Beck, 2008). AV contains a wide range of phytochemical compounds, including tannins (catechin, gallic acid, and ellagic acid), flavonoids (quercetin, luteolin, and proanthocyanidins), phenolic acids, and terpenes, as well as fatty acids and their esters including antibacterial, antioxidant, anti-inflammatory and antifungal properties (Vlaisavljević et al., 2019; Kovač et al., 2022). Several reports have documented the potential of AV against oxidative damage associated with different toxicants, such as cisplatin-induced hepatorenal and testicular injury (Jurić et al., 2020), zinc sulphate-induced reproductive injury (Mohammed and Ali, 2019), and carbon tetrachloride-induced hepatorenal damage (El-Hadidy et al., 2018).

Notwithstanding the considerable research that has been carried out to explain the beneficial effects of AV, there are no published reports investigating the efficacy of AV against myocardial injury. Consequently, this study evaluated the cardioprotective impacts of AV extract on cardiotoxicity induced by ISO injection in albino mice. Cardiac enzyme biomarkers, oxidative state, inflammation-related gene expression levels, apoptotic signaling pathways, and myocardial histoarchitecture were evaluated.

2 Materials and methods

2.1 Molecular docking assessment

2.1.1 Ligand preparation

Using SDF format, the three-dimensional (3D) structures of ISO were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Moreover, the 3D structures of AV's bioactive compounds were retrieved from the LOTUS (https://lotus.naturalproducts.net/) database.

2.1.2 Protein preparation

The 3D structures of mouse glutathione synthetase, CAT, SOD1, SOD2, SOD3, Bcl-2, TNF-α, IL-1β, NF-κB1, Bax, RAGE, and HMBG1 were retrieved from UniProt database (https://www.uniprot.org/). Target protein energy minimization was prepared for docking using UCSF Chimera software.

2.1.3 Visualization and docking interactions

Protein-ligand interactions and docking were carried out using InstaDock (Mohammad et al., 2021), while their visualization was done using BIOVIA discovery visualization 2024 Client software.

2.2 Preparation of *Alchemilla vulgaris* extract

The plant material of AV (aerial parts and roots) was procured from a local market in Mansoura, Egypt. Taxonomic identification was provided by the Department of Botany, Faculty of Science, Mansoura University, Egypt. The dried plant material was ground into powder. Each 10 g of dried plant material was soaked and mixed with 100 mL of 60°C distilled water before ultrasound treatment (Ultrasonic Cleaner USC-500 TH, VWR, Darmstadt, Germany) for 1 h at room temperature. Before being used, the plant extracts were filtered. Finally, the AV extract was phytochemically analyzed using HPLC as described in the Supplementary Material and Supplementary Table S1.

2.3 Animal study and protocol endorsement

This study was approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Mansoura University [Approval no. MU-ACUC (VM.R.23.06.69)]. Also, the experimental protocol is in agreement with ARRIVE 2.0 guidelines for animal experiments.

Male albino mice weighing 15–20 g and aged 5–6 weeks old, were procured from the animal unit at the Faculty of Pharmacy, Mansoura University, Egypt. Prior to the experiment, mice were kept in standard housing for 2 weeks under appropriate environmental conditions (21°C ± 2°C, 55% ± 5% humidity, and 12/12 h light/dark cycle). All mice received a standard diet along with unlimited access to water.

After 2 weeks of acclimation, mice were evenly divided into four groups of five mice each. Control group (CTL) mice were given normal saline; ISO group mice were injected subcutaneously (S.C) with ISO (Sigma-Aldrich, MO, United States; 85 mg/kg bw) (Meeran et al., 2021); ISO + AV100 group and ISO + AV200 group mice received the same dose of ISO and 100 or 200 mg/kg of AV extract orally (Özbilgin et al., 2019). Mice were pretreated with AV for seven consecutive days and ISO was given on the 8th and 9th days.

On the 10th day, the experiment was terminated. All mice were euthanized with an intraperitoneal pentobarbital injection (45 mg/kg). Then, blood was withdrawn from the orbital sinus, centrifuged for 10 min at 3,000 ×g and sera collected and stored at −20°C for further biochemical assessment. The heart was rapidly removed and washed with cold physiological saline to remove any blood clots and cut into three pieces. One portion was preserved in buffered formalin (10%) for histopathology. The second fresh piece of tissue from each heart was used to isolate RNA for gene expression analysis and preserved at−80°C. The remaining fresh tissue piece was kept at −20°C for subsequent oxidative cascade marker investigation.

2.4 Assay of cardiac serum markers

Creatine kinase-myoglobin binding (CK-MB) and lactate dehydrogenase (LDH) activities were estimated utilizing colorimetric kits acquired from Human (Wiesbaden, Germany).

2.5 Oxidative biomarkers assessment

Oxidative biomarkers, including malondialdehyde (MDA; a lipid peroxidation marker), and reduced glutathione (GSH), were assessed at 532 nm, and at 412 nm, respectively. In addition, antioxidant enzymes as superoxide dismutase (SOD) and catalase (CAT) were measured at 560 nm and 240 nm in cardiac homogenates according to the manufacturer's instructions (Laboratory Biodiagnostics, Cairo, Egypt).

2.6 Assessment of cardiac inflammatory biomarkers

The assessment of inflammation in cardiac tissue homogenates was completed using commercially available ELISA kits for interleukin-1β (IL-1β; Catalog No.; MBS763059, MyBioSource, CA, United States) and tumor necrosis factor-alpha (TNF-α; Catalog No.; LS-F5192, LifeSpan Biosciences Inc., MA, United States) in accordance with the manufacturer's instructions.

2.7 RNA isolation and qRT-PCR

Total RNA was extracted from cardiac tissue samples using TRIzol reagent. cDNA was synthesized utilizing Revert Aid™ H Minus Reverse Transcriptase (Fermentas, Thermo Fisher Scientific Inc., Canada) following the manufacturer's instructions. PCR was performed with a Quanti Fast SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). All reactions were carried out in duplicate using the ViiA™ 7 System (Thermo Fisher Scientific, CA, United States). The PCR cycling procedures were completed based on the protocols published by Habotta et al. (2023). The ΔΔCt approach was employed to ascertain the relative gene expression levels among the various groups. β-actin was used as the housekeeping gene. The primer sequences (Jena Bioscience, Jena, Germany) used to determine the gene expression levels of B-cell lymphoma protein 2 (Bcl-2), Bcl-2-associated X (Bax), Beclin1, a receptor for advanced glycation end products (RAGE), and NF-κB/p65 are provided in Supplementary Table S2.

2.8 Histoarchitectural assessment

The formalin-fixed cardiac tissue samples were dehydrated in a graded alcohol series. Then, the tissues were cleared with xylene prior to paraffin embedding. The tissue blocks were cut into 5 μm thick sections mounted on glass microscope slides, deparaffinized, stained with H&E for histological evaluation, and scanned using a digital camera-integrated imaging system (DM300, Leica, Germany) (Banerjee et al., 2002). Microscopic lesions were graded as "0", no pathological lesions detected; "1" mild, lesions affecting <10% of heart tissue; "2"

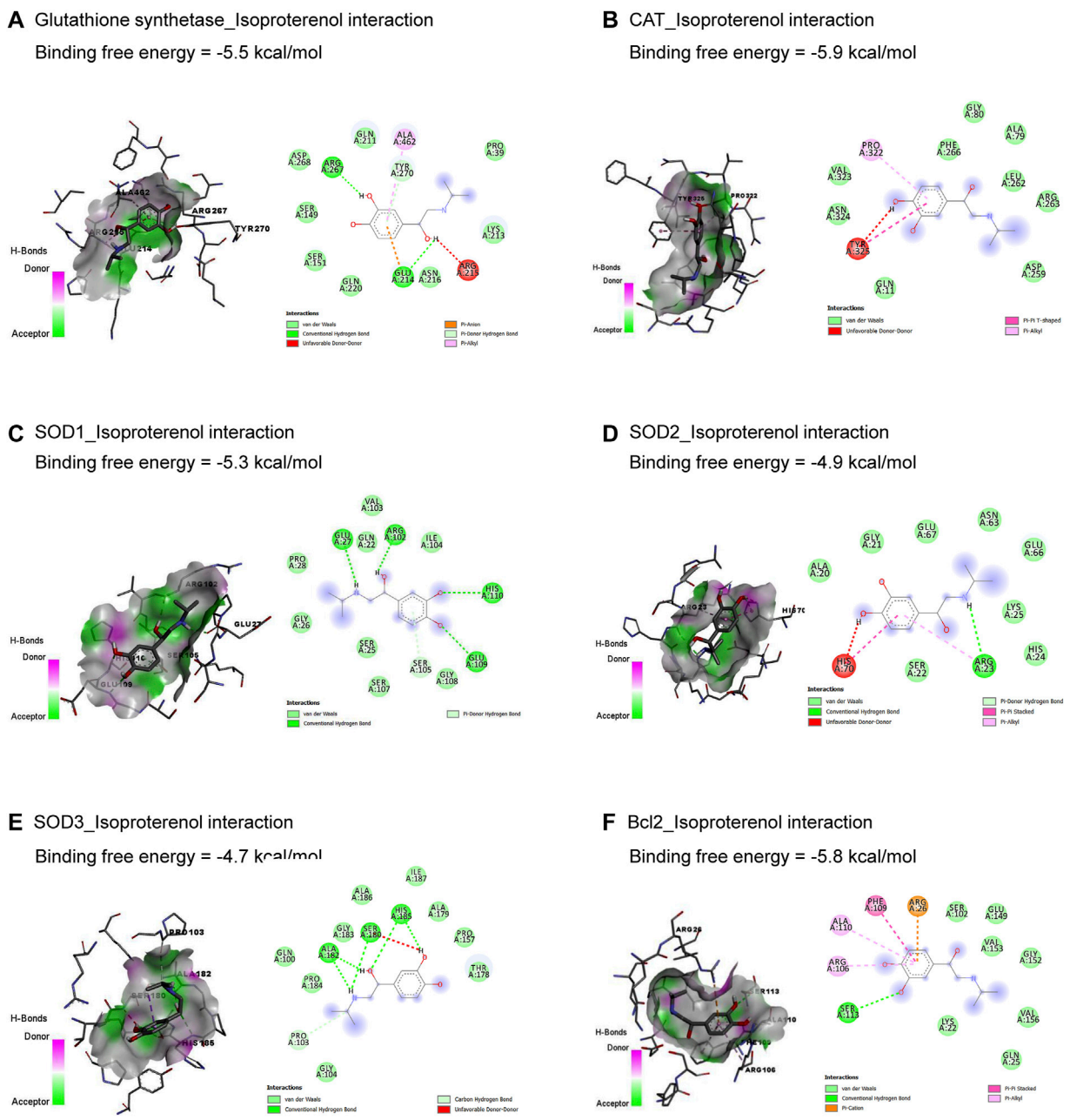

FIGURE 1
Molecular docking interactions of isoproterenol against glutathione synthetase **(A)**, CAT **(B)**, SOD1 **(C)**, SOD2 **(D)**, SOD3 **(E)**, and Bcl-2 **(F)**. CAT, catalase; Bcl-2, B-cell lymphoma protein 2; SOD, superoxide dismutase.

moderate, lesions affecting 10%–50% of heart tissues; and "3" severe, lesions affecting >50% of heart tissues (Rizzardi et al., 2012).

2.9 Immunohistochemistry analysis

Immunostaining was carried out utilizing the avidin-biotin-peroxidase complex procedure. Briefly, paraffin slices were sequentially deparaffinized and dehydrated using a graded ethanol series. Antigens were retrieved using heat-induced epitope retrieval for 30 min cooled to room temperature for 10 min. Subsequently, cardiac endogenous peroxidases were inhibited for 5 min using a 3% H_2O_2 solution. This was followed by three 5-minute washings in phosphate-buffered saline (PBS). The sections were incubated at 4°C overnight with rabbit polyclonal antibodies for high mobility group box 1 (HMGB1; Wuhan Servicebio Technology, Wuhan, Hubei, China, 1:1,000 dilution) and microtubule-associated protein light chain 3 (LC3 I, 1:100 dilution). Then the sections were incubated for 24 h at 4°C with rabbit polyclonal secondary antibodies. The sections were rinsed with PBS, biotinylated goat anti-rabbit IgG was added, and the sections were

TABLE 1 Molecular docking interaction of *Alchemilla* vulgaris's bioactive compounds against TNF-α, IL-1β, NF-κB1, Bax, RAGE, and HMBG1.

Ligands	Binding free energy (kcal/mol)					
	TNF-α	IL-1β	NF-κB1	Bax	RAGE	HMBG1
Corosolic acid	−7.0	−8.0	−6.6	−7.0	−7.6	−7.1
Euscaphic acid	−7.1	−7.8	−7.1	−7.0	−7.5	−6.8
Oleanolic acid	−7.3	−8.0	−6.8	−7.2	−8.0	−7.1
Quercetin	−6.4	−6.8	−6.0	−6.2	−6.5	−6.3
Tormentic acid	−6.7	−7.8	−7.0	−7.0	−7.4	−6.9
Ursolic acid	−7.2	−8.3	−6.7	−7.0	−7.6	−7.1

incubated for 45 min at 30°C with streptavidin-peroxidase complexes. The peroxidase activity was measured using 3,3′-diaminobenzidine. Images were captured with a Nikon Eclipse E200-LED at an original magnification of ×400. Positive-stained regions for HMGB1 or LC3 were evaluated using ImageJ software (version 1.53 t; Wayne Rasband and contributors, NIH, United States). A quantitative scoring system for the immunostained proteins (H-Score) that utilized "Area Percentage" was carried out. Percent Area for the HMGB1 and LC3 stained sections was determined using the Nikon Eclipse E200-LED image analyzer computer system (United States). The image analyzer included a Nikon research microscope, color monitor, color video camera, and a computer hard disc linked to the microscope. The system was controlled using ImageJ software (version 1.53 t; Wayne Rasband and contributors, National Institute of Health, United States). H-Score analysis software was used to record and identify the staining intensity and positive rate (%) of each intensity category for the target cells using the following scale: negative (score; 0), weak, mild (score; 1), moderate (score; 2) and strong (score; 3).

2.10 Statistical analyses

Means ± SEM were used to represent the results. A one-way ANOVA was employed to assess the results. Duncan's test was used as a *post hoc* test to compare the significance among the groups, and *P*-values less than 0.05 were deemed statistically significant. R version 4.0.2 of RStudio was utilized to analyze and visualize the data.

3 Results

3.1 Molecular docking

The molecular interactions of ISO with glutathione synthetase, CAT, SOD1, SOD2, SOD3, and Bcl-2 are represented in Figure 1. ISO interacted with the binding sites of glutathione synthetase (Figure 1A), CAT (Figure 1B), SOD1 (Figure 1C), SOD2 (Figure 1D), SOD3 (Figure 1E), and Bcl-2 (Figure 1F) by binding free energy of −5.5, −5.9, −5.3, −4.9, −4.7, and −5.8 kcal/mol, respectively.

Results in Table 1 and Figure 2 explored the molecular docking interaction of AV's bioactive compounds with TNF-α, IL-1β, NF-κB1, Bax, RAGE, and HMBG1. Oleanolic acid interacted with the binding sites of TNF-α (Figure 2A), Bax (Figure 2D), RAGE (Figure 2E), and HMBG1 (Figure 2F) by binding free energy of −7.3, −7.2, −8.0, and −7.1 kcal/mol, respectively. Another compound, ursolic acid, was bound to the binding site of IL-1β by binding free energy of −8.3 kcal/mol (Figure 2B). In addition, euscaphic acid interacted with NF-κB1's binding site with −7.1 kcal/mol binding free energy (Figure 2C).

3.2 Cardiac function parameters

ISO-induced cardiac injury was characterized by a notable increase in CK-MB and LDH compared to the normal control group. On the contrary, treatment with AV extract at either a high (AV200) or low (AV100) dose resulted in a considerable decline in cardiac serum levels of CK-MB, and LDH. Noticeably, the higher dose of AV dramatically altered the CK-MB level compared to the low dose AV supplementation (Figure 3).

3.3 Cardiac lipid peroxidation and antioxidant markers assay

ISO triggered substantial oxidative injury and lipid peroxidation (LPO), as depicted in Figure 4. A remarkable increase in the cardiac MDA levels accompanied by a discernible decrease in the enzyme activities of SOD and CAT and the GSH level was observed in mice treated with ISO in contrast to the other groups. Notably, the ISO-prompted oxidative stress was considerably reduced by AV supplementation, as indicated by a substantial decrease in the MDA level and drastic increase in GSH, SOD, and CAT indices in a dose-dependent manner.

3.4 Inflammatory pathway

As depicted in Figure 5, the ISO-exposed mice exhibited cardiac inflammation. This was elucidated by a dramatic increase in the levels of pro-inflammatory cytokines (TNF-α, and IL-1β) along with upregulation of NF-κB/P65 mRNA levels and RAGE inflammation-

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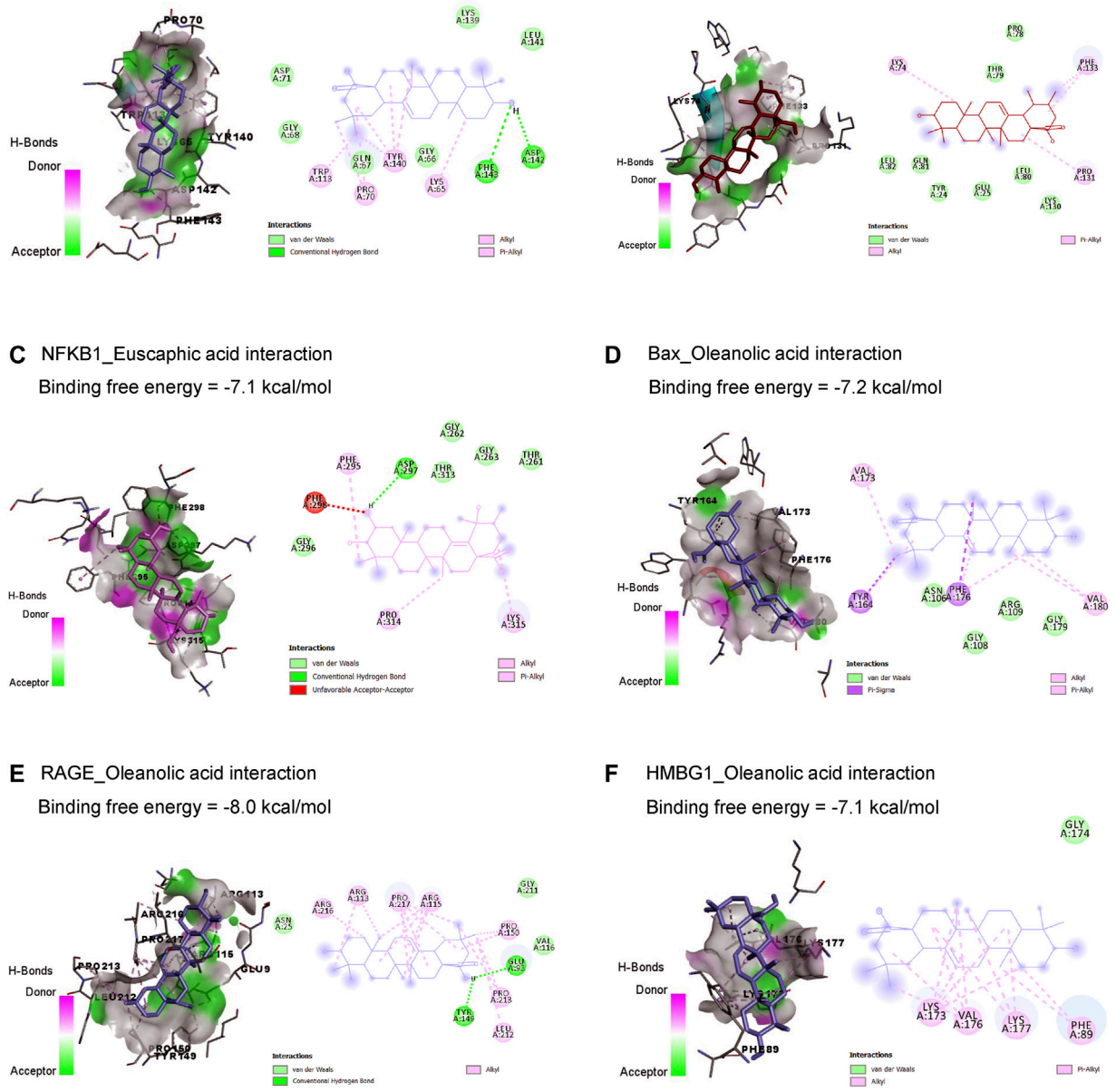

FIGURE 2
Molecular docking interactions of *Alchemilla* vulgaris's bioactive compounds with TNF-α **(A)**, IL-1β **(B)**, NF-κB1 **(C)**, Bax **(D)**, RAGE **(E)**, and HMBG1 **(F)**. Bax, Bcl-2-associated X; HMGB1, high mobility group box 1 protein; IL-1β, interleukin-1β; NF-κB, nuclear factor Kappa-B; TNF-α, tumor necrosis factor-α.

related genes in the ISO- intoxicated group in comparison to the other groups. Remarkably, pretreatment with AV100 and AV200 showed considerable decreases in ISO-induced cardiac inflammation in a dose-dependent pattern.

3.5 Autophagy and apoptotic pathways

ISO exposure induced cardiac apoptosis as indicated by alterations in mRNA expression of apoptotic and anti-apoptotic biomarkers (Figure 6). Compared to the control mice, ISO exposure substantially increased the expression of Bax and induced considerable downregulation of Bcl-2 (anti-apoptotic) gene expression levels, indicating stimulation of apoptotic cell death. On the other hand, we observed well-regulated expression of these genes in cardiac tissue when ISO-exposed mice were pretreated with AV (low or high doses) in a dose-dependent pattern. Together, these results indicated the promotion of the apoptotic pathway with ISO exposure. Furthermore, ISO exposure induced cardiac autophagy as exhibited by upregulation of Beclin1 mRNA expression compared to control mice. In addition, AV treatment significantly modulated Beclin1 expression in a dose-response manner.

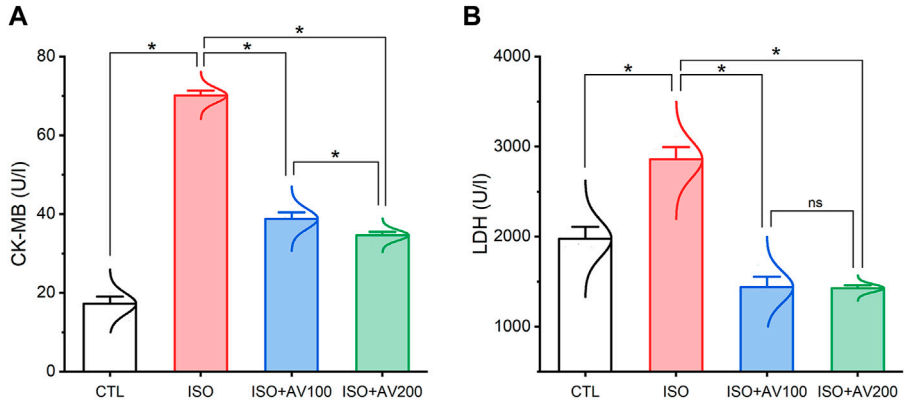

FIGURE 3
Bar plots of cardiac function parameters of ISO-exposed mice with AV supplementation. **(A)** CK-MB, creatine kinase-myoglobin binding; **(B)** LDH, lactate dehydrogenase. Values are expressed as means ± SEM (*$p < 0.05$). Colored spikes indicate the data distribution. AV, *Alchemilla vulgaris*; CTL, control; and ISO, isoproterenol.

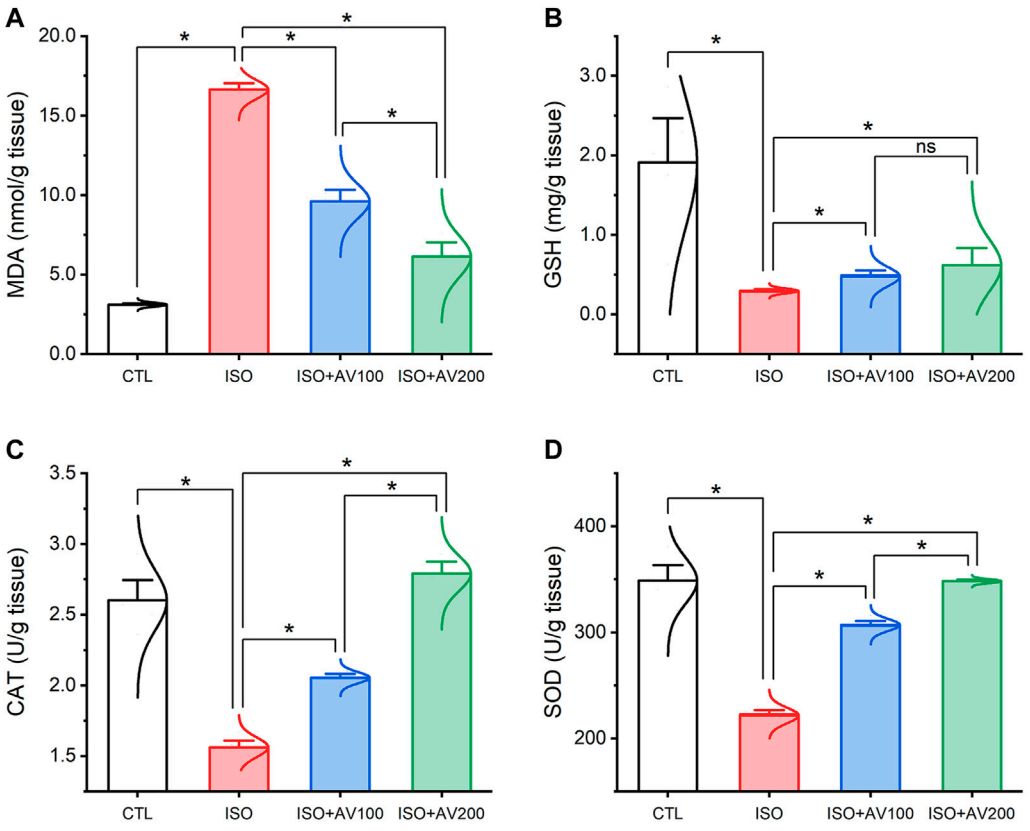

FIGURE 4
Bar plots of oxidative stress markers in cardiac tissue of ISO-exposed mice with AV supplementation. **(A)** MDA, malondialdehyde; **(B)** GSH, reduced glutathione; **(C)** CAT, catalase; **(D)** SOD, superoxide dismutase. Values are expressed as means ± SEM (*$p < 0.05$). Colored spikes indicate the data distribution. AV, *Alchemilla vulgaris*; CTL, control; and ISO, isoproterenol.

3.6 HMGB1 and LC3 expression in the cardiac tissue

HMBG1 and LC3 expression in cardiac tissue sections of ISO-exposed mice with AV supplementation are shown in Figure 7. ISO exposure resulted in considerable upregulation of HMBG1 and LC3 expression in cardiac tissue (prominent positive brown staining of cardiomyocytes). In contrast, we observed moderate to minimal expression of both proteins when mice were pre-treated with AV100 and AV200, respectively. The

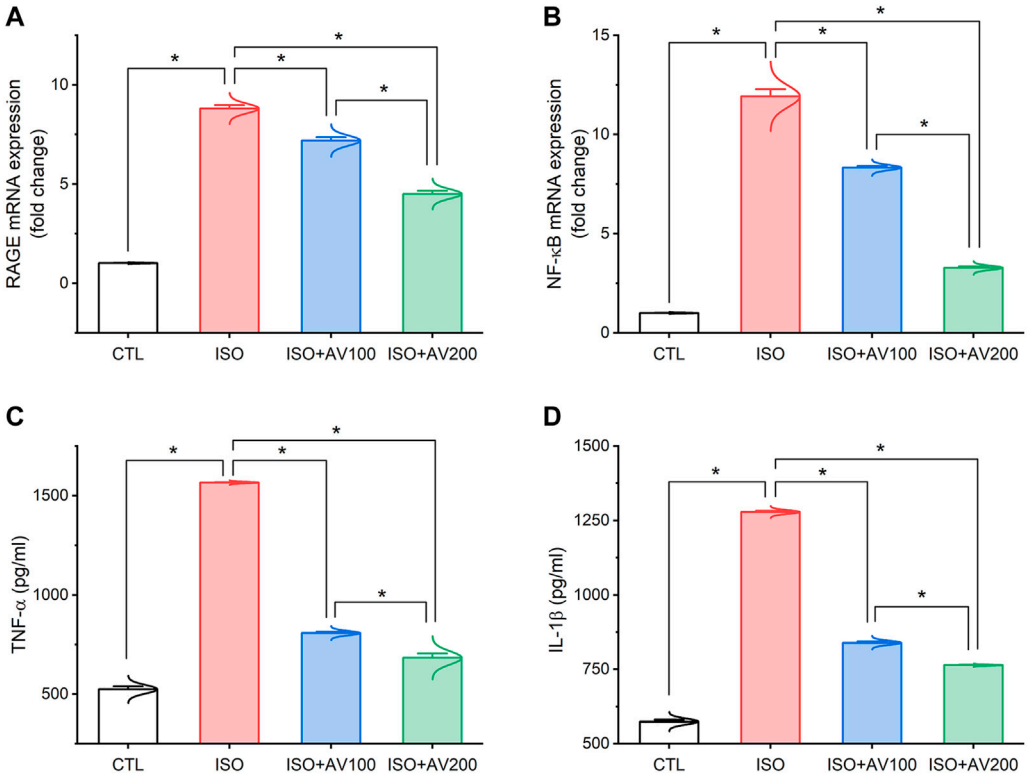

FIGURE 5
Bar plots of inflammation-related genes and inflammatory cytokines in cardiac tissue of ISO-exposed mice with AV supplementation. **(A)** mRNA expression of RAGE, receptor for advanced glycation end products; **(B)** mRNA expression of NF-κB/p65, nuclear factor kappa-B transcription factor/p65; **(C)** TNF-α, tumor necrosis factor alpha; **(D)** IL-1β, interleukin-1β. Values are expressed as means ± SEM (*$p < 0.05$). Colored spikes indicate the data distribution. AV, *Alchemilla vulgaris*; CTL, control; and ISO, isoproterenol.

semiquantitative analysis of positive-stained sections in the different treatment groups corroborated these results. Therefore, the results revealed improved in ISO-upregulated HMBG1 and LC3 expression following AV pretreatment. Intriguingly, the high dose of AV (200 mg/kg) reduced the ISO-mediated cardiac injury substantially and in a dose-dependent manner.

3.7 Cardiac histoarchitecture

A histological assessment was done to confirm the previously reported findings that evaluated alterations in cardiac tissue sections in ISO-exposed mice that received AV supplementation. The control mice showed normal cardiac tissue architecture, including normal arrangement of muscle fibers, normal elongated branching pattern, and oval central striated nuclei, as shown in Figure 8A. On the other hand, mice exposed to ISO (Figures 8B–D) exhibited overt cardiac damage, as indicated by various degenerative and necrotic changes in myocardial fibers and cardiomyocytes. Degranulation, vacuolation of the cytoplasm, and leukocytic cellular infiltration also were observed. Nevertheless, pre-treatment of ISO by AV100 demonstrated moderate improvement in the histological findings, as indicated by mild degenerative changes in nearly all the cardiac tissue sections, with little vacuolation (Figure 8E). Interestingly, myocardial histological architecture was nearly completely recovered following the high AV dose (absence of myocardial degenerative changes) (Figure 8F). The histopathological findings corroborated the biochemical data, indicating that pre-exposure to AV exhibited a profound protective impact against ISO-inflicted cardiac damage in a dose-dependent manner (Figure 8G).

3.8 Multivariate analyses

Multivariate analyses were performed to ascertain the correlation between the measured parameters and treatment groups, as depicted in Figure 9. Principal component analysis (PCA) was used to investigate the association between different treatments and covariates (Figure 9A). The PCA distinguished three main dimensional components for all variables, which collectively represented 96.6% of the variation. Component 1 discriminated most of the examined variables and therefore expressed the larger proportion of variation (83.7%), whereas components 2 and 3 accounted for smaller proportions of the variance. The PCA revealed that the ISO-exposed group was clearly different from the control group and the ISO + AV200 group. These findings elucidated a significant distinction between the AV pretreated animals and those exposed to ISO.

In addition, correlation networks were constructed among variables in the control and exposed groups (Figure 9B). Beclin1 and NF-κB/p65 correlated positively with each other and with RAGE, Bax, Bax-Bcl2

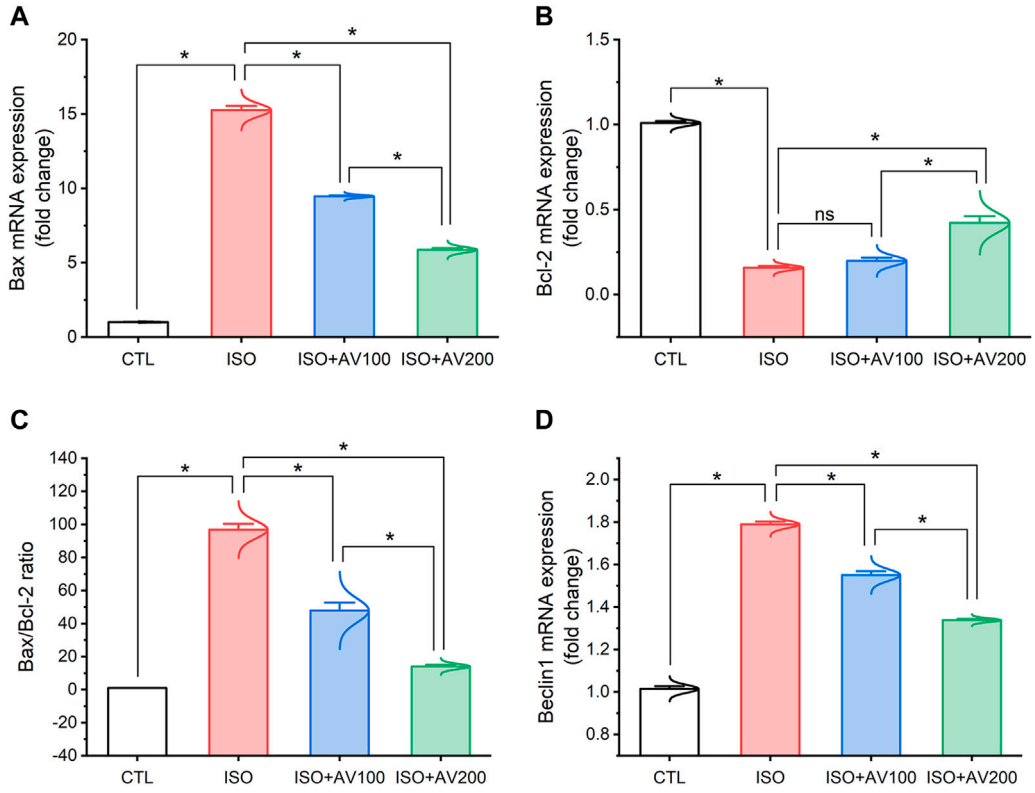

FIGURE 6
Bar plots of apoptosis and autophagy-related genes in cardiac tissue of ISO-exposed mice with AV supplementation. **(A)** mRNA expression of Bax, **(B)** mRNA expression of Bcl-2, **(C)** Bax/Bcl-2 ratio, **(D)** mRNA expression of Beclin1. Values are expressed as means ± SEM (*$p < 0.05$). Colored spikes indicate the data distribution. AV, *Alchemilla vulgaris*; CTL, control; and ISO, isoproterenol.

ratio, and MDA. Beclin1 and NF-κB/p65 were negatively correlated with Bcl-2. In addition, CK-MB was positively correlated with RAGE, Beclin1, and IL-1β. Furthermore, the clustering heatmap provided an intuitive visualization of all data sets (Figure 9C). The clustering heatmap revealed a noticeable distinction between the concentration values of all evaluated variables following ISO exposure in contrast to the other treatment groups. These data demonstrated that the ISO-exposed mice displayed greater injury than mice in the other groups. On the other hand, the intensity of the cell color with AV treatment revealed an intermediate color density at all measured variables, indicating the mitigating effect of AV against ISO exposure.

Finally, we performed a correlation analysis to investigate the relationship of CK-MB levels with other measured parameters. Figure 9D depicts the different parameters positively or negatively correlated with the CK-MB level in cardiac tissue. All measured variables had a strong positive correlation with CK-MB level, while only GSH, SOD, CAT, and Bcl-2 exhibited negative correlation.

4 Discussion

The current *in silico* study explored whether ISO had a binding affinity to glutathione synthetase, CAT, SOD1, SOD2, SOD3, and Bcl-2, which revealed the association of oxidative and apoptotic pathways to ISO-induced cardiac injury. In addition, AV's bioactive compounds exhibited binding affinity to TNF-α, IL-1β, NF-κB1, Bax, RAGE, and HMBG1, which attests to potential antioxidant, anti-inflammatory, and antiapoptotic activities.

ISO is categorized as a synthetic, non-selective, β-adrenergic agonist (Pandi et al., 2022). It produces significant cardiotoxicity, particularly infarct-like damage in the myocardium. Several mechanisms have been proposed in the pathogenesis of ISO-induced myocardial damage, but the production of ROS during autoxidation of catecholamines is one of the most critical causal reasons (Song et al., 2020). Quinone is produced when ISO is oxidized, producing a range of free radicals that cause LPO and exhaustion of the cellular antioxidant system (Othman et al., 2017; Song et al., 2020). This study provided compelling evidence that ROS contributed to the toxic consequences of ISO treatment, which is exhibited by noteworthy reductions in GSH levels and enzymatic activities of SOD and CAT in cardiac tissues. SOD is the first line of enzymatic defense in mitochondria and responsible for the dismutation of the generated $O_2^{\bullet-}$ to O_2 and H_2O_2 (Abdeen et al., 2023;Shanab et al., 2024) Furthermore, CAT accelerates the dissolution of H_2O_2 into water and O_2, quenching oxidative damage (Hassan et al., 2023). In the case of CAT exhaustion, substantial quantities of $OH^{\bullet}$, the most potent reactive radical, are generated from H_2O_2 by Fenton's reaction. The $OH^{\bullet}$ molecules drastically destroy lipid membranes, producing LPO and boosting the production of another harmful substance, MDA (Abdelnaby et al., 2022). MDA itself can dramatically disrupt the membrane

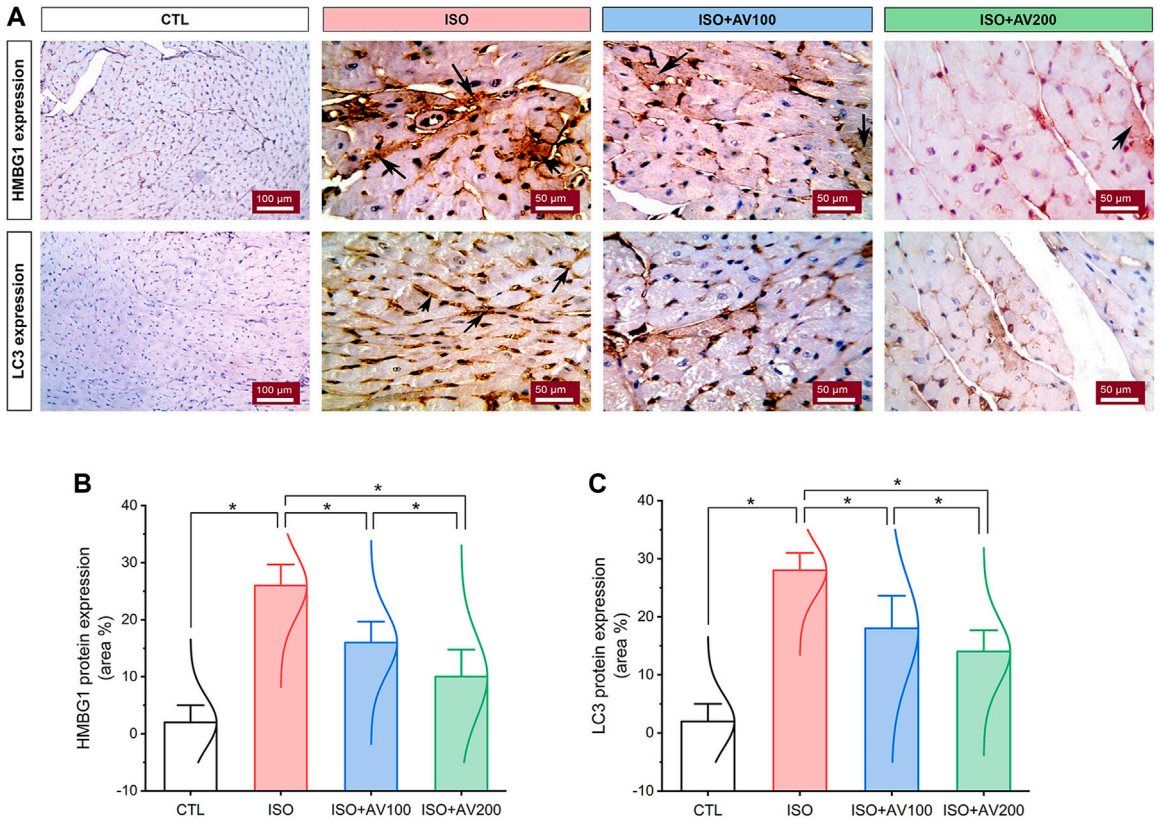

FIGURE 7
Changes in HMBG1 and LC3 expression in cardiac sections of ISO-exposed mice with AV supplementation. **(A)** CTL group exhibits passive expression of both proteins. Prominent positive brown staining of cardiomyocytes in the ISO group. ISO + AV100 and ISO + AV200 groups exhibited moderate and minimal expression of both proteins, respectively. The positive staining is indicated by the arrows and the brown color. **(B)** Quantitative analysis of protein expression of HMBG1. **(C)** Quantitative analysis of protein expression of LC3. Values are expressed as means ± SEM (*$p < 0.05$). Colored spikes indicate the data distribution. AV, *Alchemilla vulgaris*; CTL, control; and ISO, isoproterenol.

potential of mitochondria and alter cellular proteins and DNA integrity, leading to substantial cellular damage (Shanab et al., 2023). Our investigation documented substantial increase in MDA levels, corroborating the possibility of membrane damage due to ISO exposure.

As anticipated, the enhanced LPO disrupted the membrane integrity and permeability of the cardiomyocytes, liberating cardiac enzymes (CK-MB and LDH) into the blood, elevating their levels (Liu et al., 2018). This result was confirmed by our correlation analysis, which revealed a robust positive correlation between CK-MB levels and MDA accumulation in cardiac muscle. Our oxidative stress markers were consistent with earlier research that reported a reduction in cellular antioxidants upon exposure to ISO (Abdel-hady et al., 2021). Furthermore, the result mentioned above correlated with those of Sammeturi et al. (2019), who corroborated LPO and enhanced MDA levels following ISO treatment. The enhanced LPO corresponded with our histology findings, which revealed degenerative alterations within the cardiomyocytes and vividly mirrored the biochemical findings.

Mounting evidence revealed a robust correlation between inflammation and oxidative stress. Therefore, we suggest that the inflammatory pathway is an additional mechanism underlying ISO-induced cardiotoxicity (Garg and Khanna, 2014; Jain et al., 2018). Increased ROS production triggers a cellular signaling cascade that enhances the expression of proinflammatory genes and releases inflammatory mediators, culminating in enhanced inflammation (Shanab et al., 2023). In accordance with the outcomes of this study, ISO considerably increases cardiac levels of proinflammatory cytokines (IL-1β, and TNF-α) along with enhanced HMGB1 immunostaining. Moreover, the expression of NF-κB/p65 and RAGE m-RNA genes were upregulated. The involvement of the HMGB1–RAGE axis in the pathogenesis of inflammatory cardiac disease has received considerable attention recently (Bangert et al., 2016). HMGB1 binds to RAGE and Toll-like receptors in response to cellular injury, causing the release of chemokines and inflammatory cytokines (Bangert et al., 2016; Habotta et al., 2023). RAGE is a cell membrane receptor expressed in cardiomyocytes (Zhang et al., 2021; Dong et al., 2022), during cell death, it can be passively released and actively secreted into the extracellular space (Yang et al., 2023).

RAGE signaling also can provoke an inflammatory reaction via amplifying various cellular cascades that enhance inflammatory cytokine release and promote activation of the NF-κB/p65 pathway, culminating in an enormous inflammatory reaction (Jangdea et al., 2020; Habotta et al., 2023). NF-κB/p65 is a redox-regulated transcription factor that is inactive under normal conditions, but when triggered, enters the nucleus (Habotta et al., 2023; Hassan et al., 2023) and promotes

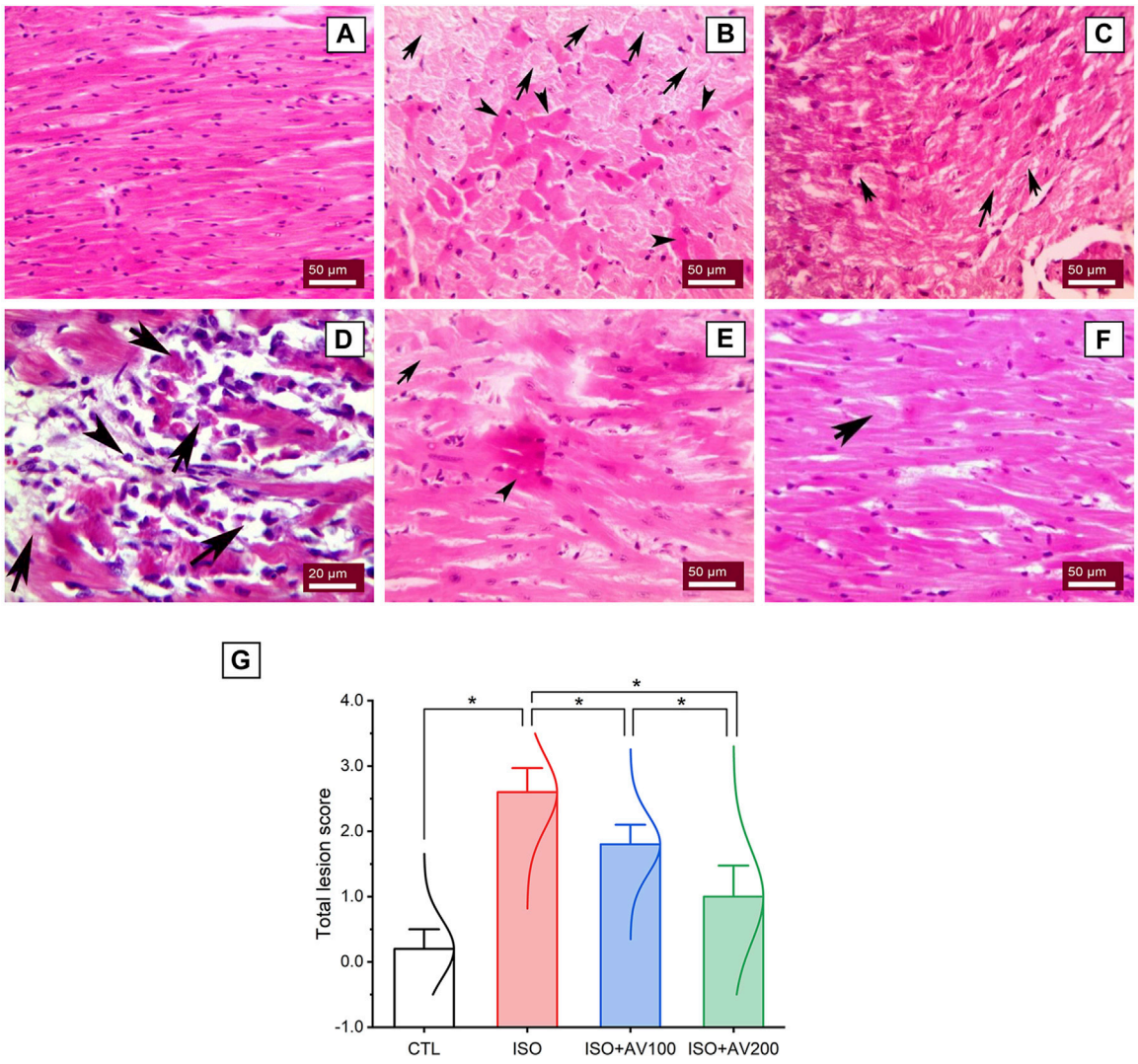

FIGURE 8
Histoarchitectural changes in cardiac tissue of ISO-exposed mice with AV supplementation. **(A)** Control group exhibits normal cardiac structure. **(B, C)**, ISO-treated group shows Zenker's degeneration (arrowheads) and necrotic changes (arrows) in the myocardial fibers and degranulation in the cardiomyocytes. **(D)** leukocytic infiltrations (arrowheads) replaced the necrotic myocardial muscle fibers (arrows). **(E)** ISO + AV100 group presents mild degenerative changes in almost all cardiac tissue sections, with little vacuolation. **(F)** ISO + AV200 group depicts a remarkable recovery of cardiac histology. **(G)** Lesion scores of histopathological alterations of cardiac cells among the different treatment groups. Values are expressed as means ± SEM (*$p < 0.05$). Colored spikes indicate the data distribution. AV, *Alchemilla vulgaris*; CTL, control; and ISO, isoproterenol.

proinflammatory mediators, including TNF-α that enhance the activation of IL-6 and adhesion molecules, encouraging leukocytes to congregate at the zone of inflammation (Shanab et al., 2024). Our correlation network further confirmed the robust relationship between oxidative stress and inflammation with ISO-induced cardiac damage. Furthermore, our histological evaluation of heart tissue verified these effects, as indicated by notable infiltration of inflammatory cells. These findings are consistent with previous studies that reported enhanced expression of these inflammation-associated markers in the cardiac tissue (Liu et al., 2018; Asiwe et al., 2023). Besides, Bangert and his gorup observed considerable increases in the expression of HMGB1 and RAGE, in myocardial biopsies of patients suffering from acute myocarditis (Bangert et al., 2016).

It has been documented that mitochondrial oxidative stress triggers numerous cell signaling pathways, including apoptosis (Meeran et al., 2021). Apoptosis is cell death brought on by the initiation of specific cellular programs controlled by complex regulatory mechanisms (Xiao et al., 2019; Sun et al., 2024). However, autophagy, a lysosomal breakdown pathway, is responsible for the degradation of dysfunctional and superfluous organelles and proteins which plays a crucial role in cell survival and homeostasis (El-Ashmawy et al., 2022; Sun et al., 2024). The relationship between apoptosis and autophagy is complicated, as the balance between autophagy and apoptosis is essential for cell survival (Wang et al., 2018). Apoptosis is regulated by two cytoplasmic proteins, pro-apoptotic Bax and anti-apoptotic Bcl-2. In apoptosis, Bax protein triggers the cascade of mitochondrial intrinsic pathways, eventually leading to cell death (Kulsoom

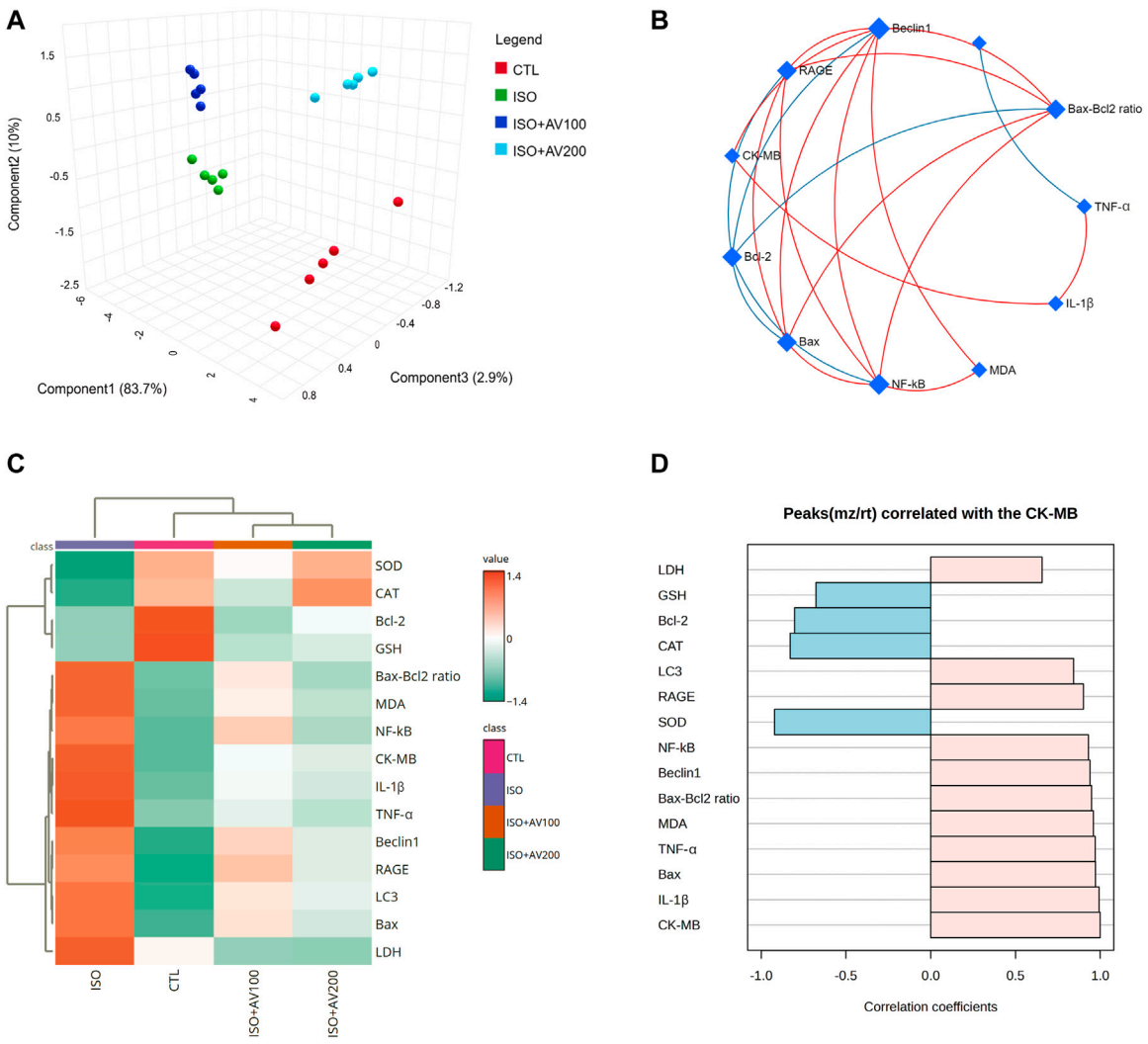

FIGURE 9
Clustering analysis of whole datasets in cardiac tissue of ISO-exposed mice with AV supplementation using Pearson's rank correlation coefficient. **(A)** 3D plot of principal component analysis (PCA) identifying the four experimental groups (CTL, ISO, ISO + AV100, and ISO + AV200). **(B)** Correlation network. The measured variables are represented by the nodes in the network, while the correlation measures are represented by the edges. Line color is proportional to the strength of the correlation. The blue lines display a negative correlation, while the red lines display a positive correlation. The node size is proportional to the mean abundance of measured variables. **(C)** Clustering heatmap. Each colored cell in the map represents a concentration value, and the rows and columns are made of different averages and treatment sets, respectively. Dark orange has the highest value on the gradation scale, while green has the lowest. **(D)** A pattern hunter plot correlation coefficient between CK-MB level in cardiac muscle and other measured parameters. Pink bars represent the positive correlation (Pearson r > +0.5) while blue bars show the negative correlation (Pearson r > −0.5).

et al., 2018). On the other hand, Bcl-2 prevents apoptosis by suppressing the Bax proteins, restricting downstream activation of apoptotic machinery pathways (Kulsoom et al., 2018; Sun et al., 2024). In line with this evidence, our investigation discovered enhanced apoptosis as shown by marked increased mRNA expression levels of Bax alongside downregulation of Bcl-2 in myocardial tissue of the ISO-treated group in contrast to the control group.

On the other hand, Beclin1 is the critical gene that regulates cardiac autophagy and autophagosome formation (Yang et al., 2023). Bcl-2 binds to Beclin1 forming a Beclin1/Bcl-2 complex. Consequently, Beclin1 is sequestered, and autophagy is suppressed (Dong et al., 2019). HMGB1, a critical modulator of autophagy, is translocated from the nucleus to the cytosol via a ROS-dependent pathway (Tang et al., 2010). Then, it binds to Beclin1 and encourages its dissociation from Bcl-2, thereby enhancing the autophagic flux (Dong et al., 2019; Yang et al., 2023). In addition, HMGB1 is passively secreted into the extracellular space following cell death and modulates cellular autophagic activity by binding with RAGE (Yang et al., 2023). Moreover, LC3 is a well-known biomarker of autophagy activity that strongly correlates with autophagosome biogenesis (Xiao et al., 2019). Our investigation documented autophagy dysfunction, as indicated by upregulation of Beclin1 m-RNA expression alongside exaggerated immunostaining for LC3 and HMGB1 proteins in myocytes following ISO exposure. In accordance with our data, El-Ashmawy et al. (2022) and Sun et al. (2024) reported myocardial autophagy following ISO treatment that was expounded by overexpression of the initiation marker LC3.

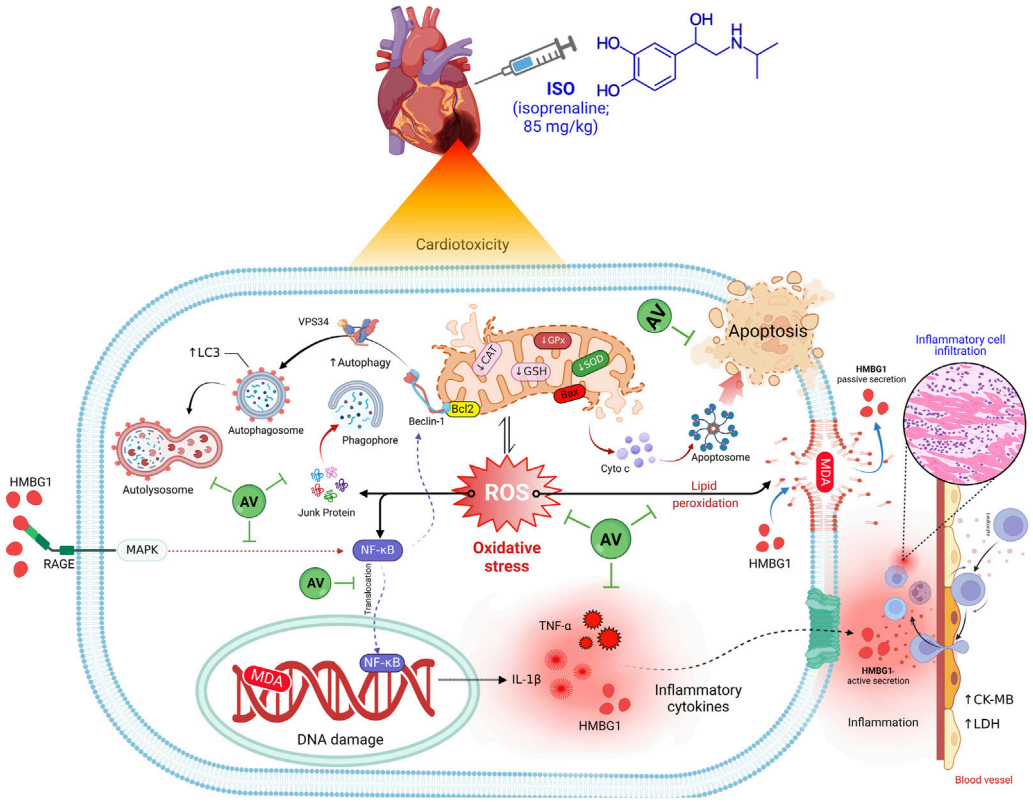

FIGURE 10
The molecular pathways behind AV's mitigating function following ISO exposure.

AV is extensively used in folk remedies throughout the world due to its well-known anti-inflammatory and immunomodulatory effects (Boroja et al., 2018; Kanak et al., 2022), as well as its antioxidant capabilities (Jain et al., 2021; Jelača et al., 2022). AV's antioxidant activity is the primary plausible mechanism underlying its protective action against heart injury (Yücel and Yücel, 2020; Jakimiuk and Tomczyk, 2024). This study also revealed that AV treatment could have a robust antioxidant impact on the oxidative stress induced by ISO in a dose-dependent manner. The protective capability of AV to scavenge free radicals is attributed to its richness in total polyphenolic and flavonoid content, especially the very high concentrations of soluble tannins and saponins (El-Hadidy et al., 2018; Jurić et al., 2020; Jakimiuk and Tomczyk, 2024). The hydroxyl groups found in the chemical structure of phenolic compounds might offer critical reasons for their ability to function as radical scavengers (Jain et al., 2021). In addition, quercetin, one of the phytochemicals in AV, can decrease the risk of arteriosclerosis by inhibiting the oxidation of low-density lipoprotein, which reduces cardiac damage (Yücel and Yücel, 2020). Many compelling publications have endorsed the idea that AV supplementation boosts the effectiveness of antioxidant enzymes and mitigates the action of LPO (Jurić et al., 2020; Tadić et al., 2020).

Notably, ongoing research indicates that pre-treatment with AV could alleviate the cardiac damage triggered by ISO, as revealed by the considerable improvement in cardiac serum biomarkers in a dose-dependent manner. The abundance of heart-strengthening compounds in AV such as pedunculin, agrimoniin, sanguin, castalagin, vescalagin, gallic, and chlorogenic acids may have critical roles in these actions (Yücel and Yücel, 2020). Furthermore, these flavonoids have been reported to have cardioprotective effects (Said et al., 2010).

The current work also revealed a dose-dependent, immune-modulating action of AV in ISO-exposed mice, which was elucidated by alterations in the expression levels of proinflammatory cytokines and suppression of inflammatory cell infiltration into cardiac tissues. Intriguingly, AV is a rich source of ellagitannins that have a crucial function in alleviating the inflammatory response via the NF-κB/p65 pathway, thus, inhibiting the release of proinflammatory cytokines (Schink et al., 2018). Our findings align with other previous reports (Katare et al., 2017; Jelača et al., 2022). Also, AV supplementation exerted an anti-apoptotic effect against ISO-prompted enhanced mRNA expression of Bax and downregulated Bcl-2 mRNA expression in cardiomyocytes. Additionally, both doses of AV downregulated the expression of Beclin1 and LC3 proteins and affected cardiac autophagy, which is a potential mitigating mechanism against ISO-triggered myocardial damage. Figure 10 highlights the molecular pathways underlying AV's mitigating function following ISO exposure.

5 Conclusion

Our data elucidated the potential mitigating effect of AV on ISO-inflicted cardiac injury. This effect was likely due to AV's

enriched antioxidant, ROS-scavenging, and anti-inflammatory attributes. Mice pretreated with AV maintained the integrity of cardiac tissue architecture and function. AV exerted notable improvements in the heart's antioxidant defenses by reducing the levels of MDA and increasing GSH levels and SOD and CAT activities. Notable decreases in the gene expression of pro-inflammatory cytokines and NF-κB/p65 were observed in the AV-treated mice. Targeting the HMGB1/RAGE signaling pathway also contributed to the cardioprotective action of AV. Furthermore, AV alleviated the ISO-induced apoptotic death and autophagy. Thus, our data suggest that AV supplementation is a promising treatment for ISO-induced cardiotoxicity that acts in a dose-dependent manner.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by The Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Mansoura University (Approval no. MU-ACUC (VM.R.23.06.69). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NA: Validation, Formal Analysis, Data curation, Writing–review and editing, Visualization, Conceptualization, Software, Writing–original draft. AhA: Writing–original draft, Investigation, Writing–review and editing, Conceptualization, Methodology. ET: Writing–review and editing, Conceptualization, Methodology, Writing–original draft, Investigation. AfA: Investigation, Writing–original draft, Writing–review and editing, Methodology, Conceptualization. HAA: Writing–original draft, Writing–review and editing, Investigation, Validation, Methodology, Conceptualization. MG: Writing–original draft, Conceptualization, Investigation, Writing–review and editing, Methodology. NEA: Investigation, Writing–review and editing, Methodology, Conceptualization, Writing–original draft. RA-S: Validation, Formal Analysis, Visualization, Data curation, Writing–original draft, Writing–review and editing, Software. SS: Investigation, Conceptualization, Methodology, Writing–original draft, Writing–review and editing. HMA: Conceptualization, Investigation, Methodology, Writing–review and editing, Writing–original draft. EE: Writing–original draft, Writing–review and editing, Software, Data curation, Validation, Formal Analysis, Visualization. ME-S: Writing–review and editing, Software, Data curation, Validation, Visualization, Writing–original draft, Formal Analysis. FI: Formal Analysis, Writing–original draft, Data curation, Visualization, Software, Writing–review and editing, Validation. II: Validation, Visualization, Writing–original draft, Data curation, Software, Formal Analysis, Writing–review and editing. MM: Validation, Data curation, Methodology, Visualization, Software, Writing–review and editing. OH: Investigation, Methodology, Writing–original draft, Conceptualization, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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Suppression of NLRP3 inflammasome orchestrates the protective efficacy of tiron against isoprenaline-induced myocardial injury

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The major contribution of myocardial damage to global mortalities raises debate regarding the exploration of new therapeutic strategies for its treatment. Therefore, our study investigated the counteracting effect of tiron against isoprenaline (ISO)-mediated cardiac infarction in mice. Tiron was administered to mice for 7 days prior to two consecutive injections of ISO on days 8 and 9 of the treatment protocol. Tiron significantly reduced the levels of CK-MB, LDH, and AST in serum samples of ISO-challenged mice. A considerable increase in the cardiac antioxidant response was observed in tiron-treated mice, as indicated by depletion of MDA and enhancement of antioxidant activities. Furthermore, tiron induced a marked decrease in NLRP3, ASC, and caspase-1 levels accompanied by weak immune reactions of IL-1β, NF-κB, TLR4, and iNOS in the infarct cardiac tissues. Histopathological screening validated these variations observed in the

cardiac specimens. Thus, tiron clearly mitigated the oxidative and inflammatory stress by repressing the NLRP3 inflammasome and the TLR4/NF-κB/iNOS signaling cascade.

KEYWORDS

inflammatory cytokines, isoproterenol, myocardial infarction, NOD-like receptor protein 3 inflammasome, oxidative stress, tiron

1 Introduction

Isoproterenol (ISO) is a synthetic non-selective β-adrenergic receptor agonist and an isopropylamine analog of adrenaline (Song et al., 2020; Pandi et al., 2022). Since ISO controls myocardial contractility and metabolism, it is frequently used to treat heart block, cardiac arrest, bradycardia, and occasionally asthma (Timercan et al., 2019; Asiwe et al., 2023). However, cardiotoxicity is a common side effect of ISO (Pandi et al., 2022). Oxidative stress and the production of oxygen-derived free radicals are considered to be the primary causes of the many and varied processes underlying ISO-induced myocardial damage (Obeidat et al., 2022; Asiwe et al., 2023). Myocardial infarction (MI) is one of the leading causes of death in cardiac disorders (Meeran et al., 2020). Accumulating evidence suggests that oxidative and inflammatory stresses play substantial roles in the pathogenesis of MI (Cinar et al., 2021). Highly reactive radicals could evoke lipid peroxidation and trigger cell death *via* multiple pathways, such as apoptosis and autophagy (Zou et al., 2022). ISO has been implicated in these types of destructive mechanisms in damaged heart tissue (Hamed et al., 2020).

Notably, the NOD-like receptor protein 3 (NLRP3) inflammasome has been implicated in the inflammatory response in numerous diseases such as renal ischemic/reperfusion injury, diabetes, tumors, and atherosclerosis (Ding et al., 2019; Lei et al., 2022). Upon activation, the NLRP3 inflammasome activates caspase-1 and apoptosis-associated speck-like protein, ASC (Schulz et al., 1995; Yu et al., 2018). Subsequently, caspase-1 and ASC stimulate the generation of related inflammatory cytokines, including interleukin-1β (IL-1β), which are critically involved in ischemic injury (Shao et al., 2022). Previous reports have established that cardiac ischemic injury is mediated through stimulation of the NLRP3 inflammasome in various animal models (Xiao et al., 2020; Chen et al., 2022; Lei et al., 2022). Toll-like receptor 4 (TLR4) exacerbates ischemic tissue damage by triggering nuclear factor kappa B (NF-κB) signaling, which increases the expression of inflammation-related genes (Othman et al., 2022; Kassab et al., 2022; Wang et al., 2022; Habotta O. A. et al., 2023). Additionally, inhibition of NF-κB transcription has been reported to repress activation of the NLRP3 inflammasome and attenuate proinflammatory mediators (Zhao et al., 2020; Hua et al., 2022; Habotta O. et al., 2023). Therefore, targeting the NF-κB/NLRP3/caspase-1 signaling pathway is a promising therapeutic approach for managing MI-related cardiac damage.

Numerous therapeutic agents have been developed for heart diseases, but these agents are associated with undesirable adverse effects and expensive costs (Khan et al., 2022). Tiron is a non-toxic, water-soluble alpha-tocopherol analog. It is an effective chelating agent as it forms a water-soluble complex with metal ions (Mohamed et al., 2021). Previous reports have documented its effectiveness in mitigating various metal toxicities, including magnesium and titanium (Morgan et al., 2017; Abdel-Magied et al., 2019). Tiron possesses potent ROS scavenging action by removing ROS inside mitochondria where tiron is located (Abdel-Magied et al., 2019). Morgan et al. (2017) found that tiron protected rat kidneys from exposure to titanium oxide nanoparticles by increasing antioxidants and decreasing lipid peroxidation. Furthermore, tiron mitigated oxidant and inflammatory responses in the pulmonary tissue of asthmatic mice challenged with ovalbumin (El-Sherbeeny et al., 2016). In an acute pancreatitis model using rats, tiron had significant antioxidant effects in the pancreas, liver, lung, and kidney (Ateyya et al., 2016).

Based on these considerations, our study expanded our understanding of the ameliorative effects of tiron on cardiac injury induced by ISO. ISO induces a pathological condition that mimics heart injuries, including MI in humans. This study evaluated cardiac, oxidant, and inflammatory biomarkers, as well as histopathological changes that occurred in cardiac tissues.

2 Materials and methods

2.1 Chemicals and experimental animals

Male Swiss albino mice weighing between 15 and 20 g were used in the current experiment. All mice were purchased from the Animal Unit of Mansoura University, Egypt. They were kept in a conventional laboratory setting with a 12-h light and dark cycle, a temperature range of 23°C–25°C, and a relative humidity facility. They were given free access to water and a balanced diet. ISO was obtained from Sigma-Aldrich, MO, United States and tiron was purchased from Acros Organics, Geel, Belgium, and then it was dissolved in normal saline.

2.2 Experimental protocol and sampling

Four groups of mice (n = 5 mice each) were randomly assigned as follows: a CTL group, where mice received only normal saline and served as negative controls; an ISO group, where mice were injected with ISO hydrochloride (85 mg/kg body weight, subcutaneously); an ISO + T1 group, where mice received ISO (85 mg/kg body weight, subcutaneously) and tiron (140 mg/kg body weight, intraperitoneally); and an ISO + T2 group, where mice were given ISO (85 mg/kg body weight, subcutaneously) and tiron (280 mg/kg body weight, intraperitoneally) (Mohamed et al.,

2021; Chu et al., 2021). Mice were administered tiron for 7 consecutive days, while ISO was given on days 8 and 9.

Mice were sedated with secobarbital (Sigma-Aldrich, MI, United States) at a dose rate of 50 mg/kg, i. p., and blood samples were collected from the radio-orbital plexus. The heart tissues were collected from all treated groups 24 h after the last ISO management. Subsequently, each mouse was decapitated, and the heart was dissected and divided into two parts. The first part was used to prepare a 10% (wt/vol) homogenate by directly mixing the tissue with ice-cold 10 mM phosphate buffer (pH 7.4) and then centrifuged at 4 C for 10 min. The supernatant was used for biochemical tests. The second part was preserved in buffered formalin for histopathological assessment.

2.3 Determination of cardiac enzymes

Analysis of aspartate transaminase (AST), creatine kinase–myoglobin binding (CK-MB), and lactate dehydrogenase (LDH) activities was determined using colorimetric kits that were acquired from Human (Wiesbaden, Germany).

2.4 Measurement of cardiac oxidant/antioxidant parameters

Malondialdehyde (MDA) content and the enzymatic activities of superoxide dismutase (SOD) and catalase (CAT) were estimated based on the manufacturer's protocols.

2.5 Assessment of cardiac inflammatory biomarkers

The assessment of inflammation in the cardiac homogenates was achieved using ELISA kits for caspase-1 (MY-Bio-Source Co., United States), NLRP3 (Aviva Systems Biolog, United States), and ASC (LifeSpan Biosciences Co., United States), according to the manufacturer's protocols.

2.6 Histopathological examination

The myocardial tissues were dissected from the mice and immediately fixed in 10% neutral buffered formalin for 24 h. The fixed tissue was processed and embedded in paraffin wax. Embedded samples were cut into 5 μm thickness. The sliced heart sections were stained using hematoxylin and eosin. The left ventricle focusing on the deep muscle layer was examined under a light microscope (Olympus CX 31 microscope, Tokyo, Japan). The necroinflammatory lesions were quantitatively scored in the different experimental groups using ImageJ software.

2.7 Immunohistochemistry

IL-1β, inducible nitric oxide synthase (iNOS), NF-κB, and TLR4 expression were assessed in the fixed mouse heart tissues using standard protocols (Buchwalow and Böcker, 2010). Here, 5 μm tissue sections of paraffin-embedded specimens were deparaffinized in an oven (70°C–75°C) for 20 min. The deparaffinized sections underwent antigen retrieval for 30 min in a hot water bath, followed by 10 min of cooling at room temperature. After 30-min incubation in hydrogen peroxide (0.3%), the sections were treated with a preblocking solution for 10 min and then washed with phosphate-buffered saline (PBS) to prevent non-specific antibody binding. The sections were treated with a 1:100 dilution of rabbit anti-caspase 3 polyclonal antibody for 1 hour in a humid environment. After washing, the sections were incubated in a biotinylated secondary antibody for 10 min with additional incubation with streptavidin labeled with horseradish peroxidase (HRP). The sections were washed with PBS and incubated for 5 minutes at ambient temperature in a dark, humid environment using a DAB substrate chromogen system. Subsequently, they were rinsed with tap water. After cleaning the sections with xylol, they were counterstained for 10–20 min with hematoxylin, rinsed in distilled water, rinsed in acid ammonia water, dehydrated in a graded alcohol series, and cover-slipped. The sections were examined, and images were captured using an Olympus CX31 microscope (Tokyo, Japan). The positive stained area was semi-quantitatively assessed in the different experimental groups using ImageJ software.

2.8 Molecular docking

The three-dimensional structures of mice's β1AR, β2AR, β3AR, caspase-1, NLRP3, and ASC were retrieved from the UniProt database (https://www.uniprot.org/). The solvent was removed, and polar hydrogens were added by MOE docking software. Moreover, the three-dimensional structure of ISO was retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Molecular docking interactions and visualization were performed using MOE software.

2.9 Statistical analyses

Means ± SE were used to represent the results. One-way ANOVA was used to carry out statistical comparisons of the various groups. Duncan's test was used as a *post hoc* test to compare between groups, with a p-value less than 0.05 set as the limit for significance. RStudio were used to analyze and visualize the data.

3 Results

3.1 Impact of tiron on the cardiac enzyme activities in ISO-induced mice

We examined the activity of myocardial injury markers in serum samples to determine whether tiron could lessen ISO-induced myocardial injury. As depicted in Figure 1, compared to healthy controls, ISO injection induced cardiac dysfunction, as

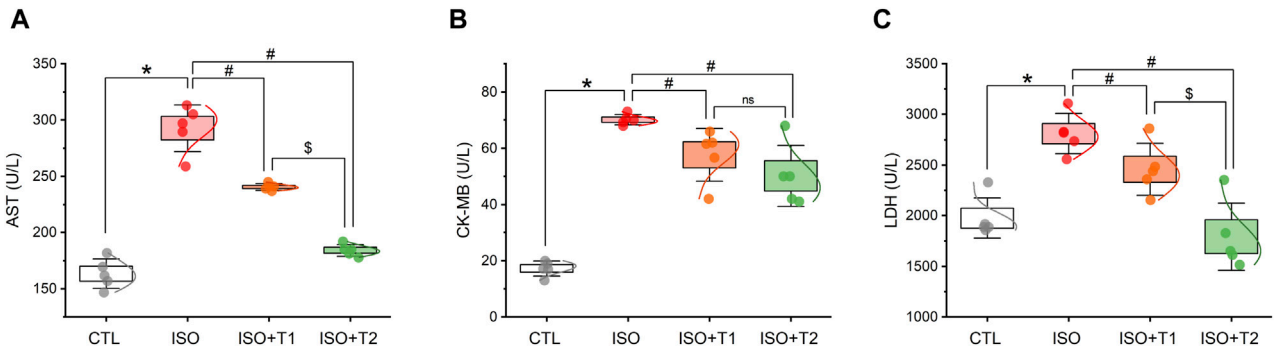

FIGURE 1
Effect of tiron on ISO-induced myocardial injury markers. **(A)** Serum AST, **(B)** serum CK-MB, and **(C)** serum LDH. AST, aspartate transaminase enzyme; CK-MB, creatine kinase-MB; LDH, lactate dehydrogenase enzyme. Data are presented as means ± SE (n = 5). *p < 0.05 vs. CTL group; $^{\#}p$ < 0.05 vs. ISO group; and $^{\$}p$ < 0.05 vs. ISO + T1.

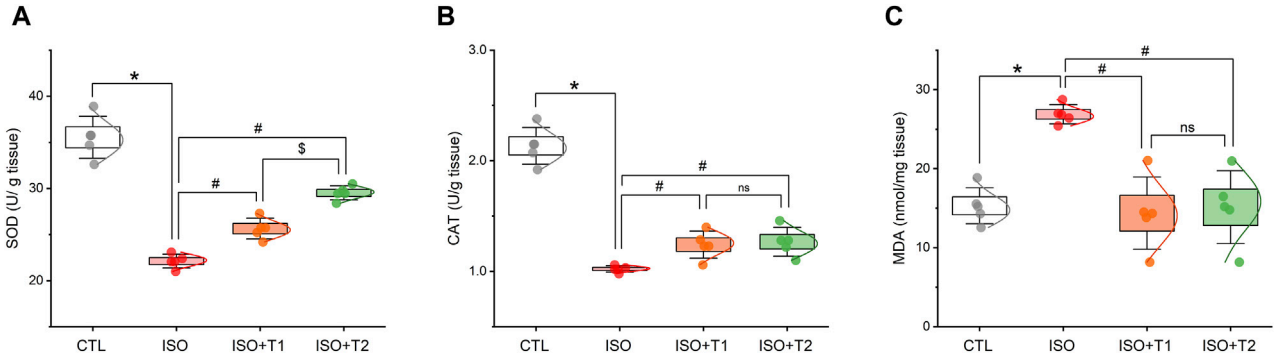

FIGURE 2
Effects of tiron on ISO-induced myocardial oxidant/antioxidant markers. **(A)** SOD activity, **(B)** CAT activity, and **(C)** MDA concentration. SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde. Data are presented as means ± SE (n = 5). *p < 0.05 vs. CTL group; $^{\#}p$ < 0.05 vs. ISO group; and $^{\$}p$ < 0.05 vs. ISO + T1.

indicated by significant increases in CK-MB, LDH, and AST activity; tiron administration reduced their activity in this animal model. The LDH activity in the ISO + T1 and ISO groups showed no appreciable difference. In addition, no discernible difference in the CK-MB levels was observed when comparing the ISO + T1 and ISO + T2 groups. Noticeably, the tiron administration decreased the AST activity in a dose-dependent manner.

3.2 Effect of tiron on cardiac oxidative status and antioxidant biomarkers

As seen in Figure 2, a considerable increase in MDA, a marker of lipid peroxidation, was observed in hearts from the ISO group compared to the control mice. Furthermore, the mice that received ISO exhibited lower antioxidant activity levels of SOD and CAT. Tiron, however, markedly decreased the cardiac MDA content and elevated SOD and CAT activities compared to ISO-treated mice. On the other hand, no discernible differences were observed in MDA and CAT between the ISO + T1 and ISO + T2 groups. These findings indicate that ISO treatment caused oxidative stress in the hearts of mice, which was suppressed by tiron treatment.

3.3 Effect of tiron on NLRP3 inflammasome activation

The protein levels of NLRP3, ASC, and caspase-1 were assessed in the heart specimens to clarify the effect of tiron on the NLRP3 inflammasome in MI. The activation of NLRP3 caused by the injection of ISO was accompanied by marked increases in cardiac caspase-1, NLRP3, and ASC expression levels. Furthermore, tiron treatment markedly decreased the upregulated proteins compared to the ISO group (Figure 3).

3.4 Multivariate analyses

As depicted in Figure 4A, principal component analysis (PCA) was used to analyze the overall data and evaluate the relationship between the different interventions and covariates. The 3D PCA revealed three dimensions for all variables, which accounted for

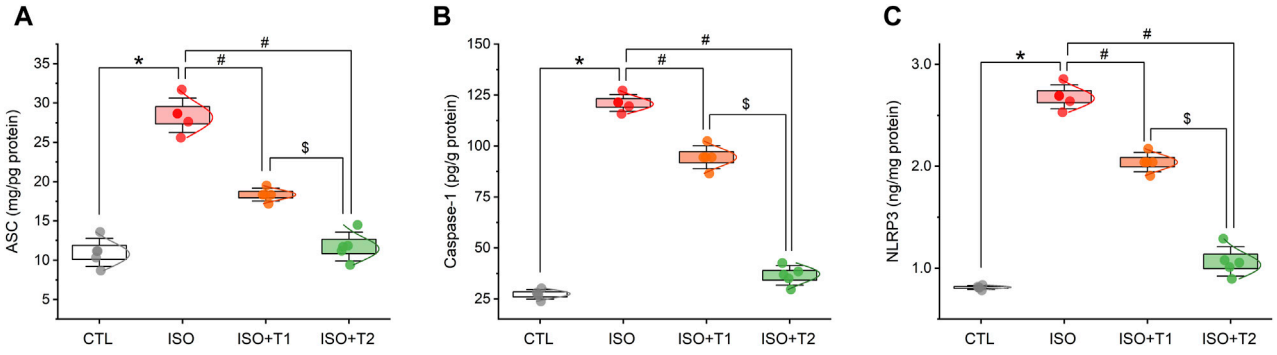

FIGURE 3
Effects of tiron on ISO-induced myocardial inflammatory markers. **(A)** ASC level, **(B)** caspase-1 level, and **(C)** NLRP3 level. ASC, apoptosis-associated speck-like protein containing a CARD; NLRP3, NOD-like receptor protein 3. Data are presented as means ± SE (n = 5). $^{*}p < 0.05$ vs. CTL group; $^{\#}p < 0.05$ vs. ISO group; and $^{\$}p < 0.05$ vs. ISO + T1.

96.3% of the variation. Although dimensions 2 (10%) and 3 (5.6%) revealed the lowest variance proportions, dimension 1 provided most of the variation (80.7%). PCA indicated that the ISO-injured mice were distinctively separated from the other treated mice, including CTL, ISO + T1, and ISO + T2 on the opposite side. Therefore, mice pretreated with tiron exhibited a remarkable difference compared to mice given ISO.

The clustering heatmap (Figure 4B) illustrates an intuitive visualization of the entire data set, highlighting and summarizing the concentration values of all assessed markers following different interventions. ISO-injured heart tissue was more likely to show damage, according to the given parameters than in the other groups (CTL, ISO + T1, and ISO + T2). The tiron and ISO co-administered groups displayed intermediate color intensities for all measured parameters, indicating that animals treated with ISO responded positively to the protective action of the tiron pretreatment in a dose-dependent manner. Since CK-MB is a commonly approved marker for cardiac injury, a hunter heatmap (Figure 4C) was generated to explore the correlation between the degree of heart injury and other concentration values of measured variables. These data indicated that NLRP3 and SOD exhibited the strongest positive and negative correlations, respectively, with the degree of damage. In addition, as seen in Figure 4D, the variable importance in projection (VIP) scores indicated that CAT, CK-MB, SOD, MDA, LDH, ASC, AST, and NLRP3 had a considerable effect in the existing study, with scores up to 2.0.

3.5 Histopathological findings

The H&E-stained heart section exhibited a normal myocardial architecture in the control group (Figures 5A, B, I). However, high necroinflammation was observed in the ISO group compared to other groups. In addition, marked extensive vascular congestion with edema-separated necrotic muscle fibers, loss of striation, multifocal-to-coalescing inflammatory aggregates were detected in the ISO group (Figures 5C, D, J). On the other hand, mice treated with ISO and low doses of tiron (ISO + T1) presented a remarkable reduction of ISO deleterious effect with moderate myonecrosis and inflammation (Figures 5E, F, K). Interestingly, same findings but with better improvements were documented in the ISO + T2 group, which was attested by the scoring data indicated in Figure 5M.

3.6 Assessment of inflammation

The inflammatory markers, IL-1β, iNOS, NF-κB, and TLR4 exhibited significant reductions in the ISO + T1 and ISO + T2 groups compared to the ISO group (Figures 6, 7). In contrary, the control animals showed weak cytoplasmic IL-1β, iNOS, and TLR4 expressions in the sarcoplasm of cardiac muscle fibers with cytoplasmic and nuclear expression of NF-κB. However, strong expression of these inflammatory markers in the inflammatory aggregates that replaced the necrotic myocytes with faint expression in the surrounding myocytes was recorded. The semi-quantitative data obtained from immunostaining of those proteins indicated significant differences between the ISO group and other treated groups, and a dose-dependent response was seen between the ISO + T1 and ISO + T2 groups (Figures 6, 7).

3.7 Molecular docking

ISO interacted with the binding site of β1AR with an energy of −6.0 kcal/mol (Figure 8A). In addition, ISO bound with ASN293 (H-donor) residue in the binding site of β2AR with an energy of −5.66 kcal/mol (Figure 8B). Similarly, with an energy of −5.88 kcal/mol, ISO interacted with ASP180 (H-donor) and ARG312 (H-acceptor) residues in the binding site of β3AR (Figure 8C).

Furthermore, tiron interacted with the binding site of caspase-1 by H-donor (GLN239, GLU240, and ASP258 residues), H-acceptor (ARG285, LYS256, and ASP258 residues), and pi-H (LEU293 residue) with a binding energy of −6.10 kcal/mol (Figure 9A). Furthermore, tiron bound with SER370 (H-acceptor) and ARG374 (H-acceptor) residues in the binding site of NLRP3 with an energy of −6.11 kcal/mol (Figure 9B). With −4.33 kcal/mol of energy, tiron interacted with the GLN31 (H-donor) in the binding site of ASC, as represented in Figure 9C.

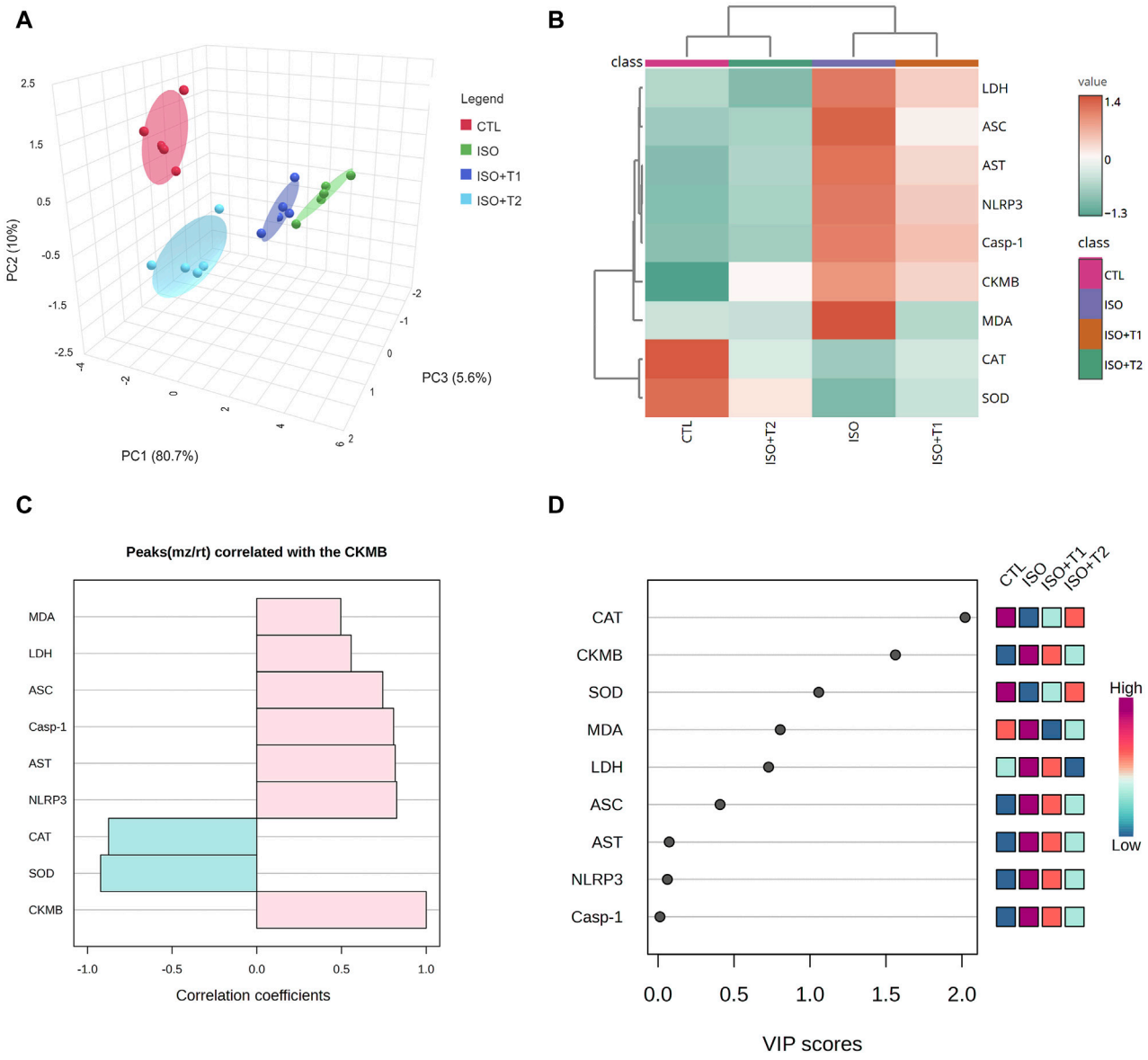

FIGURE 4
Multivariate analyses of all data sets after ISO and tiron intervention. **(A)** Three-dimensional scoring plot of principal component analysis (PCA). **(B)** Clustering heatmap for the variable averages and various groups; the concentration levels are illustrated by each colored cell on the map. **(C)** Hunter heatmap. **(D)** Variable important project scores.

4 Discussion

Myocardial damage is recognized as a leading cause of morbidity and mortality worldwide (Ardjmand et al., 2019). Even though several medical and interventional treatments have recently seen substantial advancements, many patients still have poor prognoses, lowering their quality of life and increasing the risk mortality (Hamed et al., 2020). Isoprenaline (ISO) is a catecholamine that stimulates β1 adrenergic receptors, disrupting myocardial contractions under stressful situations that can lead to heart failure (Shahzad et al., 2019). ISO was reported to induce cardiac abnormalities in experimental animals similar to the pathogenic pathways observed in humans. Thus, it offers an appropriate experimental model for investigating the beneficial effects of various medications (Hamed et al., 2020). In this study, the possible protective effects of tiron against ISO-mediated myocardial injury were investigated by focusing on the NLRP3 inflammasome and TLR4/NF-κB/iNOS signaling. We observed that ISO exposure caused severe myocardial damage that was verified by alterations in heart function and structure. Several studies have suggested that ISO produces myocardial necrosis through inducing ischemia (Malik et al., 2011; Ojha et al., 2013). This concurs with our study, where marked myonecrosis, infiltration of inflammatory cells, edema, and hemorrhage were observed. However, mice treated with tiron (140 mg/kg) exhibited a marked reduction in myocardial damage, as demonstrated by the presence of minimal, occasional necrosis, inflammation, and decreased edema. Mice treated with

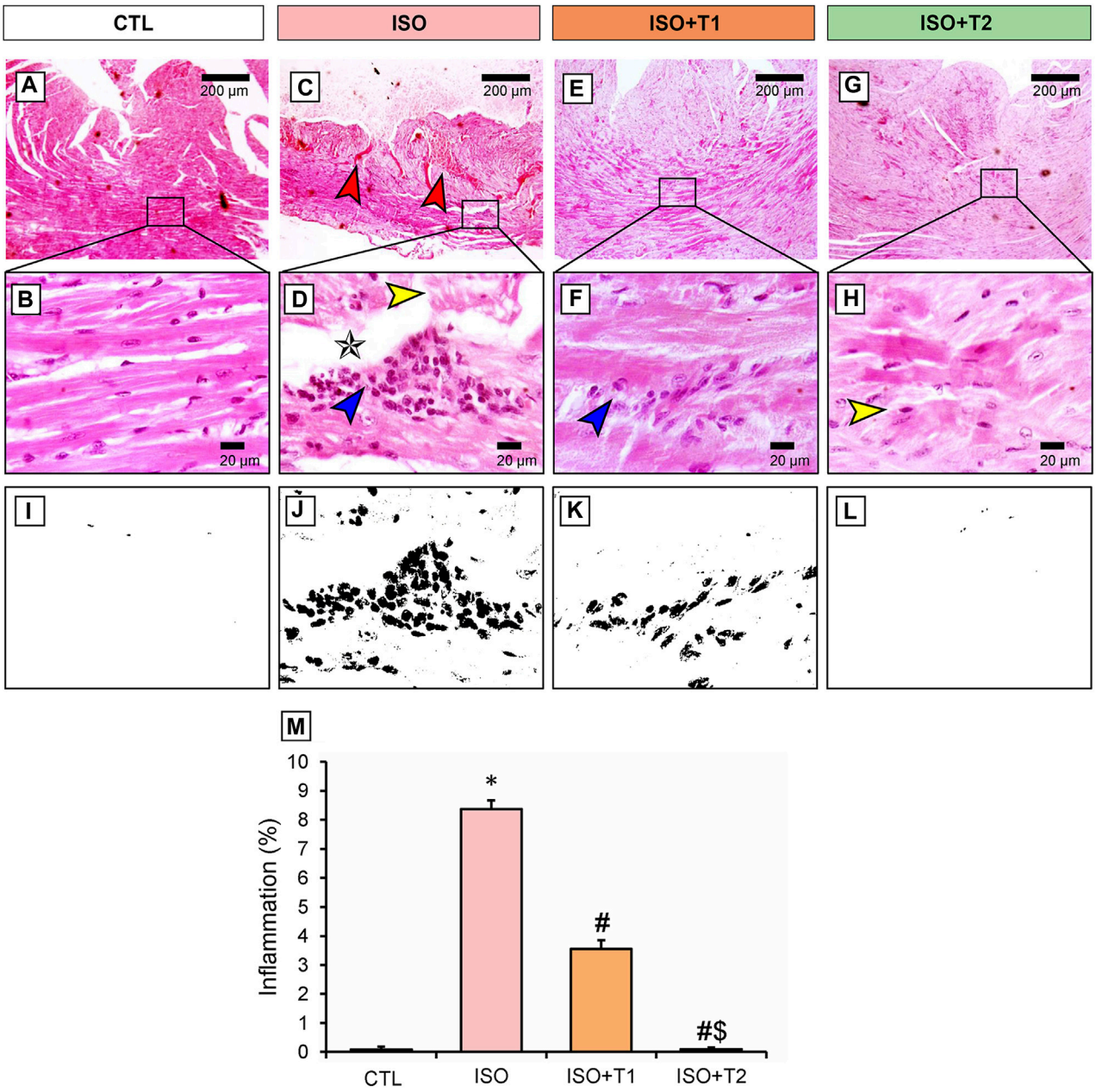

FIGURE 5
Representative photomicrograph of the heart section from different treatment groups. **(A)** Control cardiac muscle presents normal histological appearance of muscle fibers with centrally located nucleus. **(B)** Higher magnification of A (Scale bar = 20 µm). **(C, D)** ISO group shows extensive vascular congestion (red arrow) with edema (star) separated a necrotic muscle fiber (yellow arrow) with loss of striation and multifocal-to-coalescing inflammatory aggregates (blue arrow). **(D)** Higher magnification of C (Scale bar = 20 µm). **(E, F)** ISO + T1 exhibits few inflammatory aggregates replaced an occasional necrotic myocyte (blue arrow). **(E)** Higher magnification of F (Scale bar = 20 µm). **(G, H)** ISO + T2 indicates partial restoration of cardiac architecture with few necrotic myocytes (yellow arrow) and without any inflammation. **(H)** Higher magnification of G (Scale bar = 20 µm). **(I–M)** Corresponding qualitative image and semi-quantitative analysis of inflammation. Data are presented as means ± SE. $^{*}p < 0.05$ vs. CTL group; $^{\#}p < 0.05$ vs. ISO group; and $^{\$}p < 0.05$ vs. ISO + T1.

tiron (280 mg/kg) also showed minimal-to-mild myocardial necrosis, inflammation, and edema.

Regarding heart function, ISO injection substantially increased the enzymatic activities of heart injury markers (CK-MB, AST, and LDH). It has been reported that the myocardial cells contain LDH and CK under normal conditions (Song et al., 2020). However, when these cells are destroyed under pathological conditions such as disturbed metabolism and inadequate oxygen delivery, these enzymes leak into the bloodstream (Song et al., 2020; Al-Brakati et al., 2021). Administration of tiron at both doses markedly decreased the serum activity of CK-MB compared to the ISO group. Concerning LDH activity, only tiron at the highest dose succeeded in restoring normal LDH activity. These observations suggest that tiron has a membrane-stabilizing effect in the myocardium of treated mice.

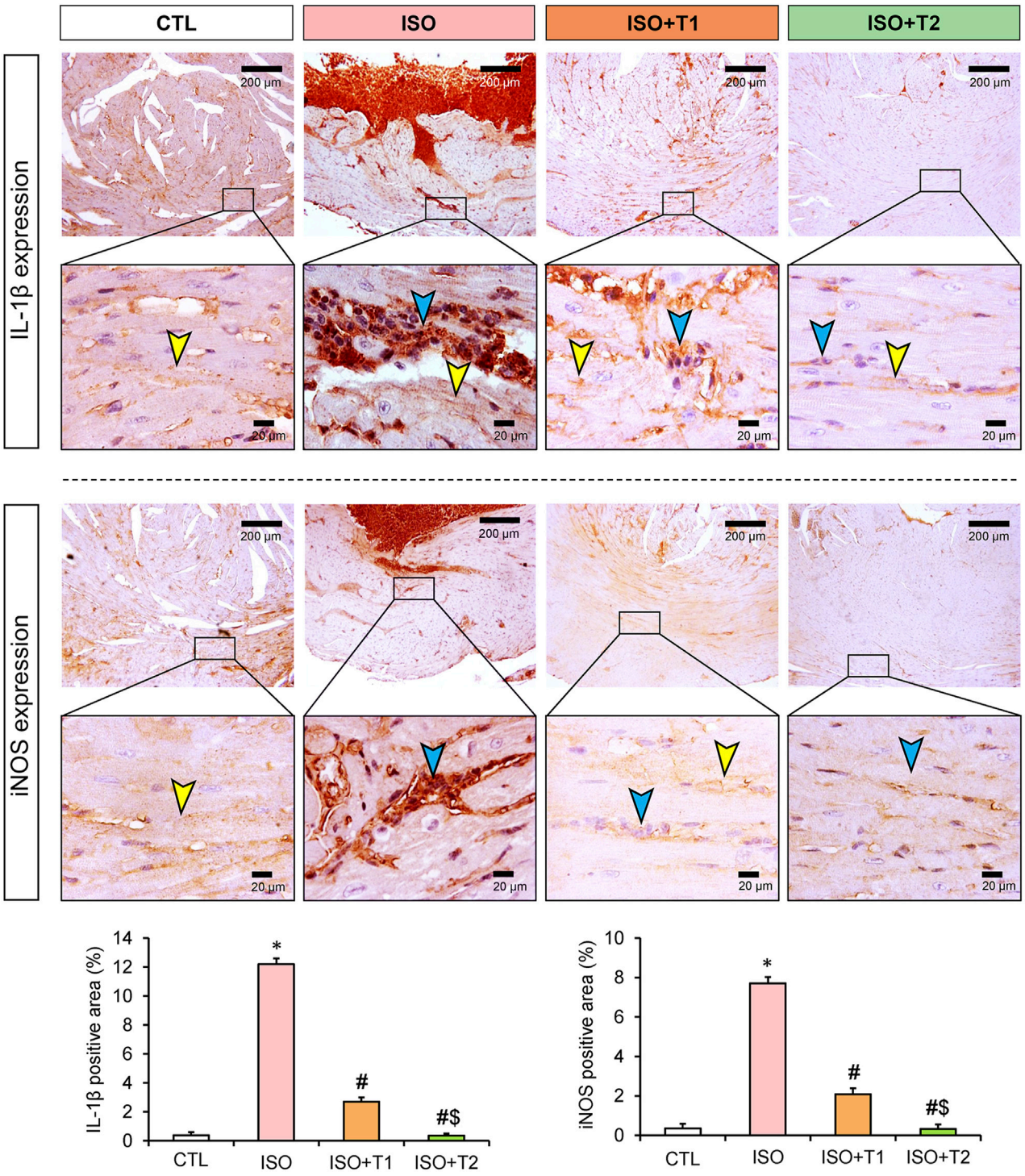

FIGURE 6
Representative immunohistochemical staining of IL-1β and iNOS expressions in heart tissue at low and high magnification scales (scale bars = 200 and 20 μm, respectively). The blue arrow indicates the positive immunostained inflammatory cells, and the yellow arrow points the positive immunostained myocytes. Bar plots show the semi-quantitative expression levels of IL-1β and iNOS presented in the upper images. Data are presented as means ± SE. *$p < 0.05$ vs. CTL group; $^{\#}p < 0.05$ vs. ISO group; and $^{\$}p < 0.05$ vs. ISO + T1.

It is well-known that ISO produces deleterious oxidative insults in myocardial tissue, which evokes infarct-like damage in the heart. Numerous processes have been proposed to explain how ISO might cause myocardial injury, and one of the most important causes is the formation of ROS during catecholamine autoxidation (Mert et al., 2018). Similarly, our results revealed that ISO caused disturbance in the oxidant/antioxidant balance, as demonstrated by a significant elevation in MDA content and reduction in the activities of SOD and

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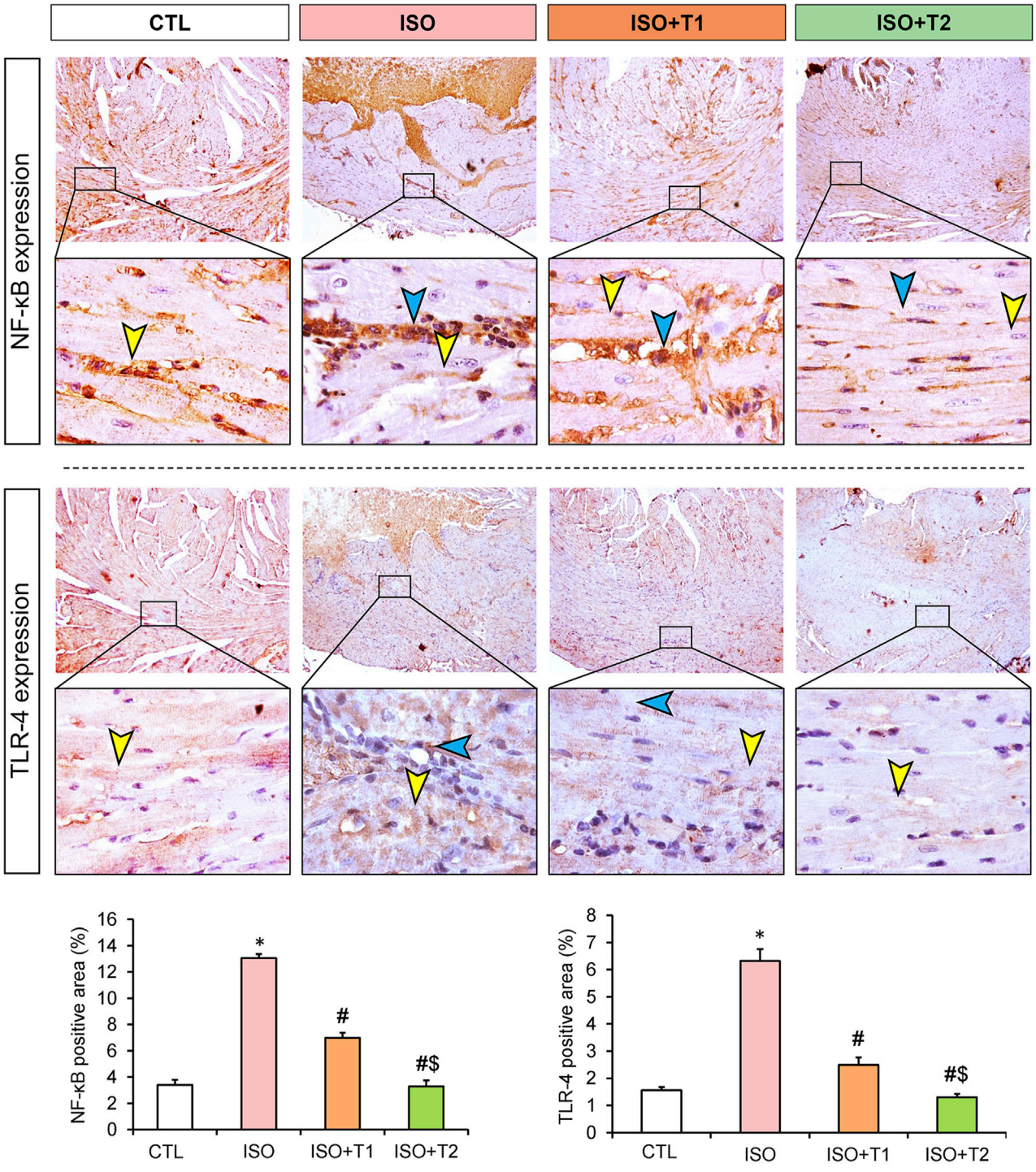

FIGURE 7
Representative immunohistochemical staining of NF-κB and TLR4 expressions in heart tissue at low and high magnification scales (scale bars = 200 and 20 μm, respectively). The blue arrow indicates the positive immunostained inflammatory cells, and the yellow arrow points the positive immunostained myocytes. Bar plots show the semi-quantitative expression levels of NF-κB and TLR4 presented in the upper images. Data are presented as means ± SE. *$p < 0.05$ vs. CTL group; $^{\#}p < 0.05$ vs. ISO group; and $^{\$}p < 0.05$ vs. ISO + T1.

CAT compared to the controls. Notably, pretreatment with tiron significantly decreased the MDA content and increased the activity of both enzymes compared to ISO-treated mice. This indicates that tiron might protect the heart against lipid peroxidation by eliminating surplus damaging free radicals induced by ISO.

Earlier studies have clarified the crucial function that oxidative stress plays in the NLRP3 inflammasome (Abais et al., 2015; El-Kashef and Serrya, 2019). Thus, suppression of oxidative stress could prevent NLPR3 inflammasome activation. In this work, ISO induced significant increases in cardiac levels of NLRP3, ASC, caspase-1, and protein

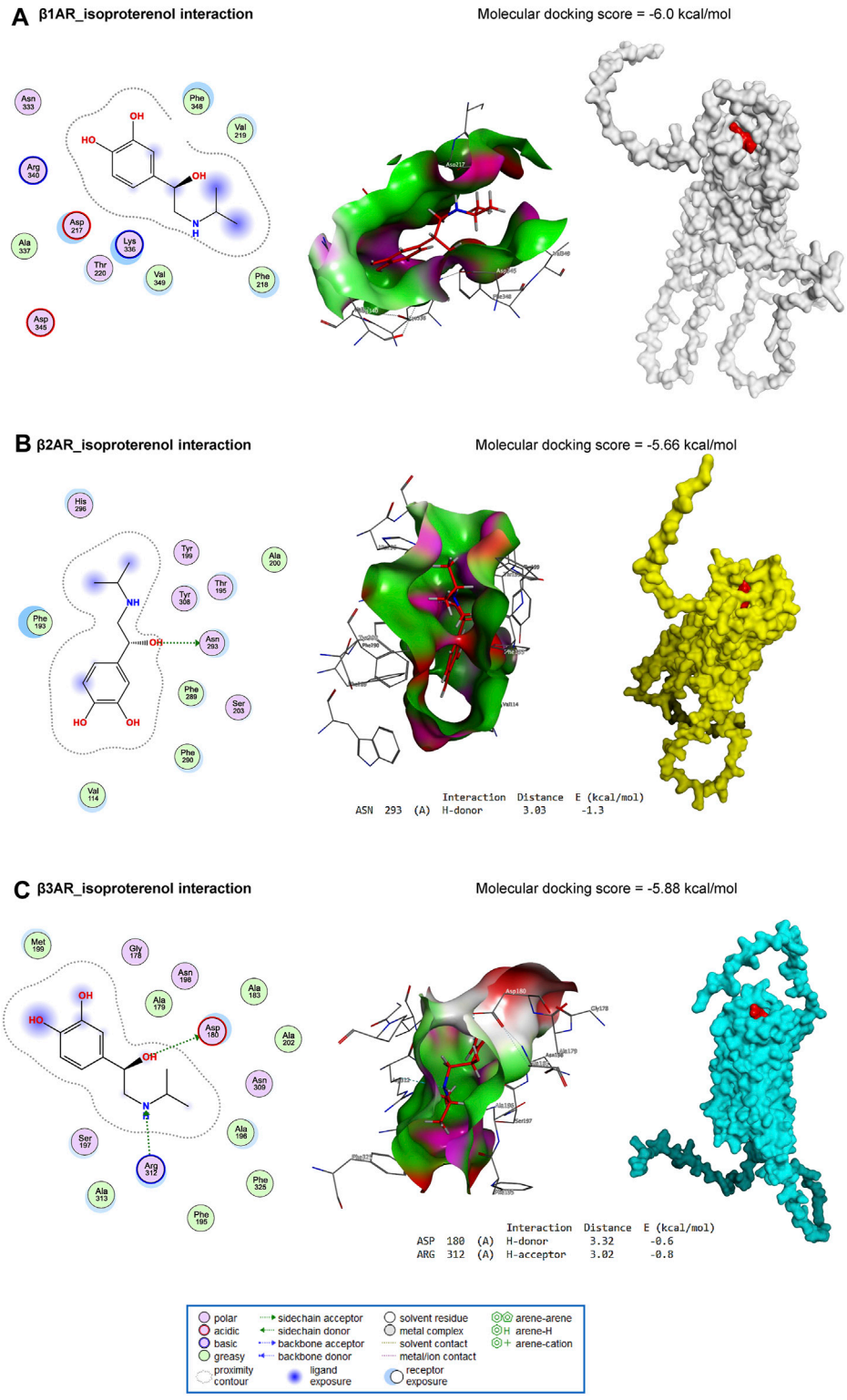

FIGURE 8
Molecular docking interaction of ISO against mouse β1AR **(A)**, β2AR **(B)**, and β3AR **(C)**.

expression of IL-1β compared to the controls. However, pretreatment with tiron resulted in a profound reduction in their protein expression levels compared to the ISO group. These findings suggest that tiron might exert anti-inflammatory effects by blocking the NLRP3 pathway.

The inflammatory response during myocardial infarction is regulated by several inflammatory markers, including IL-1β, iNOS, NF-κB, and TLR4 (Viswanadha et al., 2020). This investigation detected notable increases in the immune

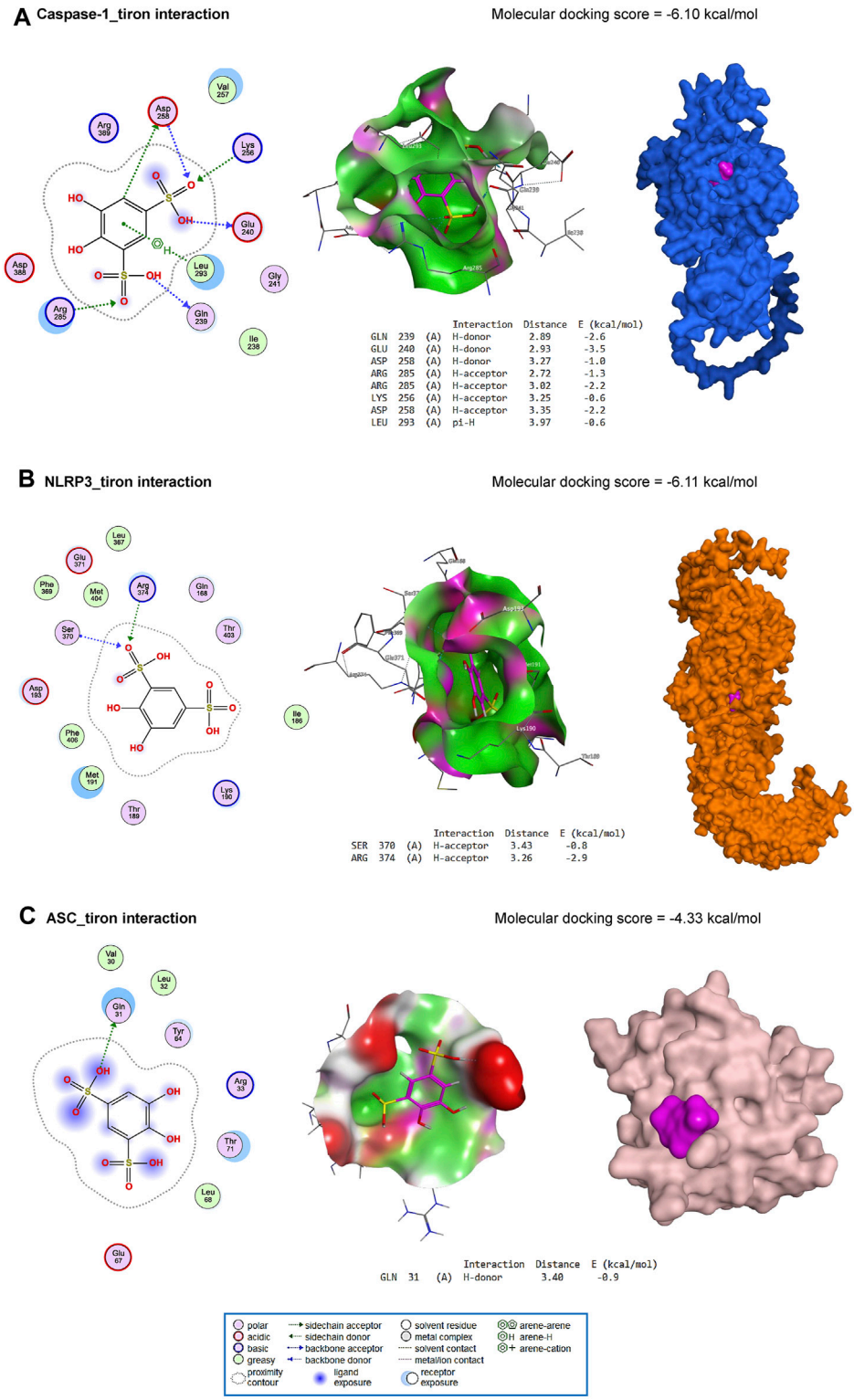

FIGURE 9
Molecular docking interaction of tiron against mouse caspase-1 **(A)**, NLRP3 **(B)**, and ASC **(C)**.

reactions of IL-1β, iNOS, NF-κB, and TLR4 in cardiac tissue, following the ISO insult compared to the controls. On the other hand, tiron-treated groups showed mild-to-moderate expression of these inflammatory biomarkers when compared to the ISO group. Accordingly, the myocardial damage in the ISO-treated group could be attributed to the responsive mechanism of TLR4 to damage-associated molecular patterns (DAMPs), which promote upregulation of proinflammatory cytokines

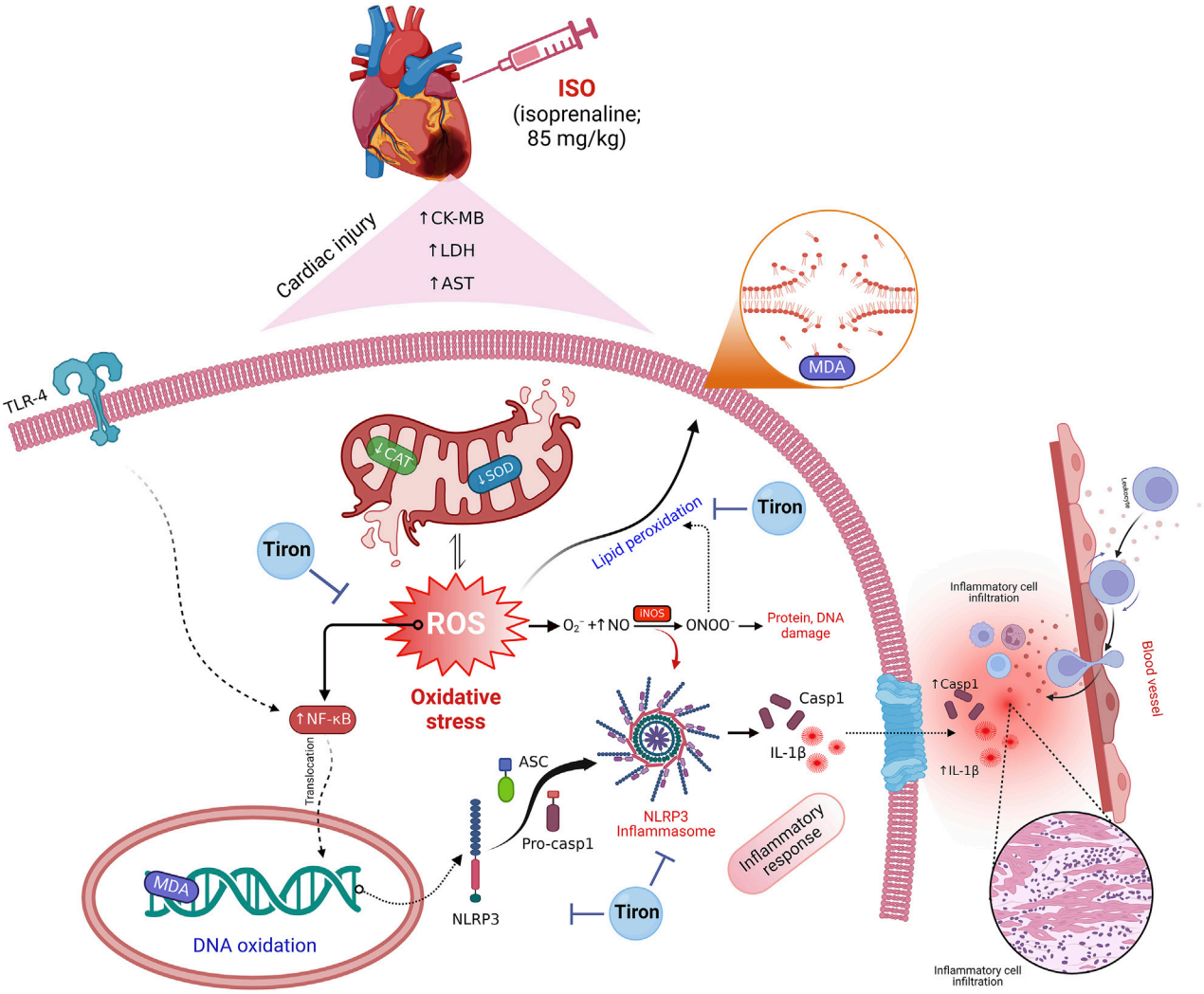

FIGURE 10
Proposed mechanistic insights involved in the protective activity of tiron against ISO cardiotoxicity.

such as IL-1β (Liu et al., 2015). The elevation of proinflammatory regulators might hasten the progression of heart damage. Elevated IL-1β expression might enhance specific intracellular pathways, including NF-κB and ROS production in cardiomyocytes (Machida et al., 2003; Ha et al., 2011; Lokman et al., 2022). Furthermore, IL-1β activates iNOS in cardiac critical for myocardial contractility (Schulz et al., 1995).

The results obtained from the molecular docking experiment revealed comparable free energies of binding of ISO with β1AR, β2AR, and β3AR in mice, suggesting the potential impact of ISO on the myocardial function attested in the present study. The *in silico* study also revealed how tiron could directly interact with caspase-1, NLRP3, and ASC proteins. This interaction pattern was in harmony with the data obtained from the current *in vivo* experiment.

The multivariate analyses provide an intuitive visualization of the entire data set, which summarize all measurements in one output and highlight the influence of all variables in the current study after different interventions. Herein, PCA, a clustering heatmap, a hunter heatmap, and VIP scores were created to assess the effects of the different interventions on cardiac tissue. The data obtained from PCA revealed that the toxic group (ISO) could influentially discriminate other treatments, where the ISO-injured mice were distinctly set apart from other mice by clustering independently on the far right along the PC1 axis. In the same data frame, the clustering heatmap illustrated the changes in the investigated variables due to ISO exposure along all studied parameters in all groups. It also emphasized the extreme alterations in the ISO-treated mice in comparison to the control mice. However, the ISO + T1 and ISO + T2 groups were located in the middle range among those of ISO and CTL groups with more pronounced effects in the ISO + T2-pretreated animals. Moreover, the strong positive or negative correlations between CK-MB and other oxidant/antioxidants, inflammation, and apoptotic markers exhibited by the hunter heatmap confirm the potential involvement of those selected measurements in the proposed mechanisms underlay the ISO-induced injury and the mitigating effects of tiron in the heart tissue. Alongside the VIP

scores, those parameters are sorted in order according to their impact in this study; therefore, CAT, CK-MB, SOD, MDA, and LDH were the top influencing ones. Collectively, these data robustly corroborated the cardioprotective potential of tiron for ISO-induced heart injury. Figure 10 summarizes the proposed mechanistic insights involved in the protective activity of tiron against ISO-induced cardiotoxicity.

5 Conclusion

Collectively, the myocardial preservation in the tiron-treated groups could be attributed to increased antioxidant capacity inflammation inhibition. These actions suggest that the enhancement of the cardiac antioxidant defense was mediated by increased levels of SOD and CAT concurrent with decreased MDA levels. Moreover, suppression of TLR4/NF-κB/iNOS and the NLRP3/caspase-1/IL-1β signaling pathways mediated the cardioprotective effect of tiron. Therefore, tiron could potentially be a valid, safe, and therapeutic substance for myocardial infarction. Further clinical studies are necessary to verify its clinical use in cases of myocardial infarction.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by the Institutional Animal Ethics Committee for animal care and use at the Faculty of Veterinary Medicine, Mansoura University (Approval Number: R/136). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DA: conceptualization, investigation, methodology, writing–original draft, and writing–review and editing. OH: conceptualization, investigation, methodology, writing–original draft, and writing–review and editing. ET: conceptualization, investigation, methodology, writing–original draft, and writing–review and editing. EE: investigation, methodology, writing–original draft, writing–review and editing, and conceptualization. II: conceptualization, investigation, methodology, writing–original draft, and writing–review and editing. AA: conceptualization, data curation, formal analysis, investigation, methodology, software, visualization, writing–original draft, and writing–review and editing. AI: data curation, formal analysis, software, validation, visualization, writing–original draft, and writing–review and editing. RI: conceptualization, investigation, methodology, writing–original draft, and writing–review and editing. HA: conceptualization, investigation, methodology, writing–original draft, and writing–review and editing. OM: writing–review and editing, data curation, formal analysis, software, validation, visualization, and writing–original draft. RO: data curation, formal analysis, software, validation, visualization, writing–original draft, and writing–review and editing. KA: data curation, formal analysis, software, validation, visualization, writing–original draft, and writing–review and editing. RA-S: data curation, software, validation, visualization, writing–review and editing. ME-S: data curation, software, validation, visualization, writing–review and editing. SS: data curation, methodology, software, validation, visualization, writing–review and editing. DE-K: conceptualization, investigation, methodology, writing–original draft, and writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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