

Edaravone and Acetovanillone Upregulate Nrf2 and PI3K/Akt/mTOR Signaling and Prevent Cyclophosphamide Cardiotoxicity in Rats

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Emad HM Hassanein¹
Omnia AM Abd El-Ghafar²
Marwa A Ahmed³
Ahmed M Sayed⁴
Wail M Gad-Elrab⁵
Jamaan S Ajarem⁶
Ahmed A Allam⁷
Ayman M Mahmoud^{7,8}

¹Department of Pharmacology and Toxicology Faculty of Pharmacy, Al-Azhar University, Assiut, Egypt; ²Department of Pharmacology and Toxicology, Faculty of Pharmacy, Nahda University, Beni-Suef, Egypt; ³Department of Pharmacology, Faculty of Medicine, Assiut University, Assiut, Egypt; ⁴Biochemistry Laboratory, Chemistry Department, Faculty of Science, Assiut University, Assiut, Egypt; ⁵Human Anatomy & Embryology Department Faculty of Medicine, Al-Azhar University, Assiut, Egypt; ⁶Zoology Department, College of Science, King Saud University, Riyadh, Saudi Arabia; ⁷Zoology Department Faculty of Science, Beni-Suef University, Beni-Suef, Egypt; ⁸Biotechnology Department, Research Institute of Medicinal and Aromatic Plants, Beni-Suef University, Beni-Suef, Egypt

Correspondence: Ayman M Mahmoud
Physiology Division, Zoology Department, Faculty of Science, Beni-Suef University, Salah Salim Street, Beni-Suef 62514, Egypt
Tel +20 1278355478
Email ayman.mahmoud@science.bsu.edu.eg

Jamaan S Ajarem
Zoology Department, College of Science, King Saud University, Riyadh 11451, Saudi Arabia
Email jajarem@ksu.edu.sa

Introduction: Cyclophosphamide (CP) causes redox imbalance and its use is associated with marked cardiotoxicity that limits its clinical applications. The present study investigated the protective effects of acetovanillone (AV) and edaravone (ED) against CP-induced oxidative stress and cardiac damage, emphasizing the role of PI3K/Akt/mTOR and Nrf2 signaling.

Materials and Methods: Rats received either AV (100 mg/kg) or ED (20 mg/kg) orally for 10 days and CP (200 mg/kg) on day 7. At day 11, the rats were sacrificed, and samples were collected for analysis.

Results: AV and ED ameliorated serum troponin I, CK-MB, LDH, AST and ALP, and prevented cardiac histological alterations in CP-intoxicated rats. Both treatments decreased cardiac lipid peroxidation and enhanced GSH, SOD and cytoglobin in CP-induced rats. AV and ED downregulated Keap1, whereas increased the expression of PI3K, Akt, mTOR and Nrf2 in the heart of rats received CP. Additionally, the binding modes of AV and ED to Keap1 were pinpointed in silico using molecular docking simulations.

Conclusion: AV and ED prevent CP cardiotoxicity by attenuating oxidative stress and tissue injury, and modulating cytoglobin, and PI3K/Akt/mTOR and Keap1/Nrf2 signaling. Therefore, AV and ED may represent promising agents that can prevent cardiac injury in patients receiving CP.

Keywords: chemotherapy, acetovanillone, edaravone, oxidative stress, cardiotoxicity, Nrf2, mTOR

Introduction

Cyclophosphamide (CP) is a cytotoxic alkylating phosphoramidate ester extensively used as an anti-neoplastic and immunosuppressive agent in organ transplantation and systemic lupus erythematosus. CP has the ability to modulate DNA synthesis and prevent cellular proliferation.^{1,2} Despite the therapeutic effects of CP, pulmonary injury, hepatotoxicity, nephrotoxicity, bone marrow suppression, cardiotoxicity, genotoxicity and other adverse effects are associated with its use. The incidence of CP cardiotoxic effects remains high and was found to be dose-related.³ Exposure to high doses of CP leads to acute cardiotoxic effects, including extravasation of toxic metabolites, myocyte damage and diastolic contractile dysfunction.⁴ Tachyarrhythmias, myocarditis, hypotension, pericardial disease and heart failure represent the common manifestations of CP cardiotoxicity. These manifestations typically present within the first 48 h after the administration of CP.^{5,6} Acute heart

failure occurs in 7–33% of patients receiving more than 150 mg/kg CP.⁷ Numerous studies have shown a surplus increase in reactive oxygen species (ROS) following CP administration.^{8–11} Excess ROS can damage several cellular biomolecules, including proteins, lipids and DNA, resulting in cell death. Additionally, acrolein, the toxic metabolite of CP, can induce toxicity through altering the antioxidant defense system manifested by the depletion of cellular reduced glutathione (GSH) and superoxide dismutase (SOD).¹² Subsequently, a massive increase in lipid peroxidation (LPO), nitric oxide (NO) and protein carbonyls occurs as a result of CP administration, culminating in tissue injury.^{8–11} Such effects can interfere with the heart regulatory mechanisms and increase the risk of cardiovascular diseases. Thus, agents with radical-scavenging activity could be effective against CP-induced oxidative injury and cardiotoxicity.

The nuclear factor erythroid 2-related factor 2 (Nrf2) is an antioxidant intracellular defense system widely distributed in various tissues. Under physiological conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) which facilitates the ubiquitination and subsequent proteolysis of Nrf2. Upon exposure to ROS or electrophilic chemicals, Nrf2 is released from Keap1, translocates into the nucleus and binds to the antioxidant response element (ARE), resulting in coordinated antioxidant and anti-inflammatory response by stimulating the expression of many cytoprotective genes.¹³ Studies have demonstrated a decline in Nrf2 signaling in CP-intoxicated rodents.^{8–11} Therefore, activation of Nrf2 signaling is an effective strategy to counteract CP-induced oxidative stress and cardiotoxicity and other adverse effects. In this context, Nrf2 activation enhanced antioxidant defenses and attenuated oxidative injury, inflammation, and cell death in the liver and kidney of CP-intoxicated rats,^{8–11} making it a valuable therapeutic target to prevent cardiac injury associated with the use of CP. Moreover, the PI3K/Akt/mTOR is another signaling pathway that has been reported to impede cell survival process upon its downregulation.^{14,15} Dysregulation of this signaling has also been implicated in cardiomyopathy.¹⁶

Acetovanillone (AV) is a small molecule extracted from *Picrorhiza kurroa* and has been reported to exert antioxidant, anti-inflammatory and anti-apoptosis activities.¹⁷ It is a potent NADPH oxidase (NOX) inhibitor that attenuates excessive ROS generation in different disease models.^{18–21} The beneficial effects of AV have been attributed to its ability to suppress ROS generation via inhibiting NOX activity and enhancement of the cellular

antioxidant defenses.^{21,22} Edaravone (ED; 3-methyl-1-phenyl-2-pyrazolin-5-one) is a low molecular weight agent with potent radical-scavenging properties that protect the cells from death.²³ ED has a neuroprotective activity mediated via its ability to suppress oxidative stress,^{24,25} and protected against bleomycin-induced lung injury²⁶ and amitriptyline-induced cardiotoxicity²⁷ in murine models. Given the potent antioxidant properties of AV and ED, this study explored their protective effect against CP cardiotoxicity, pointing to the possible involvement of PI3K/Akt/mTOR and Nrf2 signaling.

Materials and Methods

Drugs and Chemicals

AV, ED and CP were obtained from Sigma-Aldrich (USA). Assay kits of cardiac troponin I (CTnI) was purchased from Lifespan BioSciences (USA). Creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) kits and TRIzol were supplied by Thermo Fisher Scientific (USA). Aspartate aminotransferase (AST) and alkaline phosphatase (ALP) kits were purchased from BioDiagnostics (Cairo, Egypt). Primary antibodies for Nrf2, cytoglobin, PI3K, Akt and mTOR were obtained from Santa Cruz (USA). Primers were purchased from Vivantis Technologies (Malaysia) and SYBR green master mix was obtained from Fermentas (USA).

Experimental Animals and Treatments

Forty-eight male Wistar rats weighing 180–210 g were purchased from the central animal house, Faculty of Medicine, Assiut University (Egypt). The Animals were housed in controlled temperature, humidity and 12 h light-dark cycles, and supplied a standard laboratory diet of known composition and water ad libitum. All experiments were conducted in line with the guidelines of the National Institutes of Health (NIH publication No. 85–23, revised 2011) and approved by the Animal Care and Use Committee of Assiut University (IRB No. 17,300,462).

The animals were allocated into six groups (n = 8) as follows:

Group I (Control): received 0.5% carboxymethyl cellulose (CMC) via oral gavage for 10 days and a single intraperitoneal (i.p.) injection of saline at day 7.

Group II (AV): received 100 mg/kg AV¹⁹ for 10 days.

Group III (ED): Rats received 20 mg/kg ED²⁸ for 10 days.

Group IV (CP): received 0.5% CMC for 10 days and a single dose of CP (200 mg/kg) at day 7.²⁹

Group V (AV + CP): received 100 mg/kg AV for 10 days and a single dose of CP (200 mg/kg) at day 7.

Group VI (ED + CP): received 20 mg/kg ED for 10 days and a single dose of CP (200 mg/kg) at day 7.

AV and ED were dissolved in 0.5% CMC and administered orally, and CP was injected i.p. At day 11, all rats were sacrificed under pentobarbital anesthesia and blood was collected via cardiac puncture. Serum was prepared by centrifugation at 4000 rpm for 10 min. The animals were immediately dissected, and the heart was removed, washed in cold phosphate-buffered saline (PBS) and samples were fixed in neutral buffered formalin. Other samples were kept frozen at -80°C in RNAlater whereas others were homogenized in cold PBS (10% w/v), centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was collected and kept frozen at -80°C .

Measurement of Cardiac Function Biomarkers

Serum CTnI was estimated by a specific ELISA kit and the activities of LDH, CK-MB, AST and ALP were determined following the manufacturers' instructions.

Histology and Immunohistochemistry

Heart specimens, fixed in 10% neutral buffered formalin for 48 h, were dehydrated, cleared, embedded in paraffin, sectioned (4–5 μm) and stained with hematoxylin and eosin (H&E) stain according to the previously described method.³⁰ The sections were examined blindly under light microscope (Leica Q 500 MCO, Germany).

Other sections were used for the immunohistochemical staining of PI3K, Akt, mTOR, Nrf2 and cytoglobin. Briefly, the sections were deparaffinized and treated with 3% hydrogen peroxide (H_2O_2) for 10 min to inactivate endogenous peroxidases, heated in 10 mM citrate buffer at 121°C for 30 min for antigen retrieval and then blocked by 5% bovine serum albumin (BSA) in Tris-buffered saline. The slides were incubated with the primary antibodies overnight at 4°C and then washed and incubated with horseradish peroxidase (HRP)-conjugated

secondary antibodies for 30 min at room temperature. The sections were subjected to diaminobenzidine staining and hematoxylin counterstaining. The percentage of area occupied by brown color was measured in randomly selected six fields in each slide using ImageJ (NIH, USA).

Determination of LPO, GSH and SOD

Malondialdehyde (MDA), a marker of LPO, was assayed in the heart homogenate as previously described.³¹ The antioxidants GSH and SOD were assayed according to the methods of Ellman³² and Marklund and Marklund,³³ respectively.

Gene Expression Analysis

The effect of AV and ED on mRNA abundance of Keap1 and Nrf2 genes was determined by qRT-PCR using the ABI 7500 real-time PCR system (Applied Biosystems, USA). Briefly, total RNA was isolated using TRIzol reagent. The isolated RNA was quantified using a nanodrop and reverse transcribed into cDNA. The obtained cDNA was amplified using SYBR green master mix and the primers listed in Table 1 in a total reaction volume of 20 μL . The amplification data were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method³⁴ and normalized to GAPDH as a housekeeping gene.

Molecular Docking

The binding mode of AV and ED with Keap1 was determined by molecular docking using Autodock vina 1.5.6.³⁵ The crystal structure of Keap1 and its (1S,2R)-2-[(1S)-1-[(1,3-dioxo-2,3-dihydro-1H-isoindol-2-yl)methyl]-1,2,3,4-tetrahydroisoquinolin-2-carbonyl]cyclohexane-1-carboxylic acid (compound (S, R, S)) was obtained from protein data bank with PDB ID: 4I7b.³⁶ The water molecules and ligand were removed and the binding pocket of Keap1 was used for docking against AV and ED. PyMOL 1.7.6 software was used to visualize the docking models of AV and ED with Keap1.

Statistical Analysis

The results were expressed as mean \pm SEM and all the statistical comparisons were performed using one-way ANOVA followed by Tukey's post-hoc on GraphPad

Table 1 Primer Used for qRT-PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>Nrf2</i>	ATTGCTGTCCATCTCTGTCAG	GCTATTTTCCATTCCTGAGTTAC
<i>Keap1</i>	TCAGCTAGAGGCGTACTGGA	TTCGGTTACCATCCTGCGAG
<i>GAPDH</i>	TGCTGGTGTCTGAGTATGTCG	TTGAGAGCAATGCCAGCC

Prism 7.0 (GraphPad software, USA). Statistical significance was considered for a P value less than 0.05.

Results

AV and ED Ameliorate Heart Injury in CP-Intoxicated Rats

The ameliorative effect of AV and ED on CP cardiotoxicity was assessed through determination of CTnI, CK-MB, LDH, AST and ALP (Figure 1). CP induced a significant elevation of serum CTnI (Figure 1A), CK-MB (Figure 1B), LDH (Figure 1C), AST (Figure 1D) and ALP (Figure 1E) when compared with the control rats ($P<0.001$). Oral administration of either AV or ED decreased serum CTnI, CK-MB, LDH, AST and ALP in CP-intoxicated rats. Treatment with ED decreased serum CK-MB (Figure 1B) and LDH (Figure 1C) significantly ($P<0.001$) in CP-administered rats when compared with AV. Normal rats received either AV or ED exhibited non-significant changes in all assayed heart function markers (Figure 1).

AV and ED Prevent CP-Induced Heart Injury in Rats

The cardioprotective effect of AV and ED on CP-induced cardiac injury was further confirmed by the histological

findings. Examination of H&E-stained sections revealed well-organized and intact cardiomyocytes with normal nuclei and cytoplasmic striation in the control (Figure 2A), and AV- (Figure 2B) and ED-supplemented rats (Figure 2C). Administration of CP resulted in histological alterations, including degenerative changes, fragmented myofibrils, nuclear pyknosis, increased intercellular spaces between adjacent cardiomyocytes and many congested blood vessels (Figure 2D–F). CP-intoxicated rats treated with AV (Figure 2G) or ED (Figure 2H) exhibited an improvement in the histological architecture of the heart with fewer degenerative changes and congestions (Table 2).

AV and ED Attenuate Cardiac Oxidative Stress in CP-Induced Rats

Given the role of oxidative stress in mediating the deleterious effects of CP, we evaluated the effects of AV and ED on cardiac MDA and the antioxidants GSH and SOD in the heart of rats (Figure 3). MDA showed a significant increase in heart of CP-treated rats when compared with the control group (Figure 3A; $P<0.001$). In contrast, GSH and SOD were significantly declined in the heart of CP-intoxicated rats as represented in Figure 3B and C, respectively. Oral

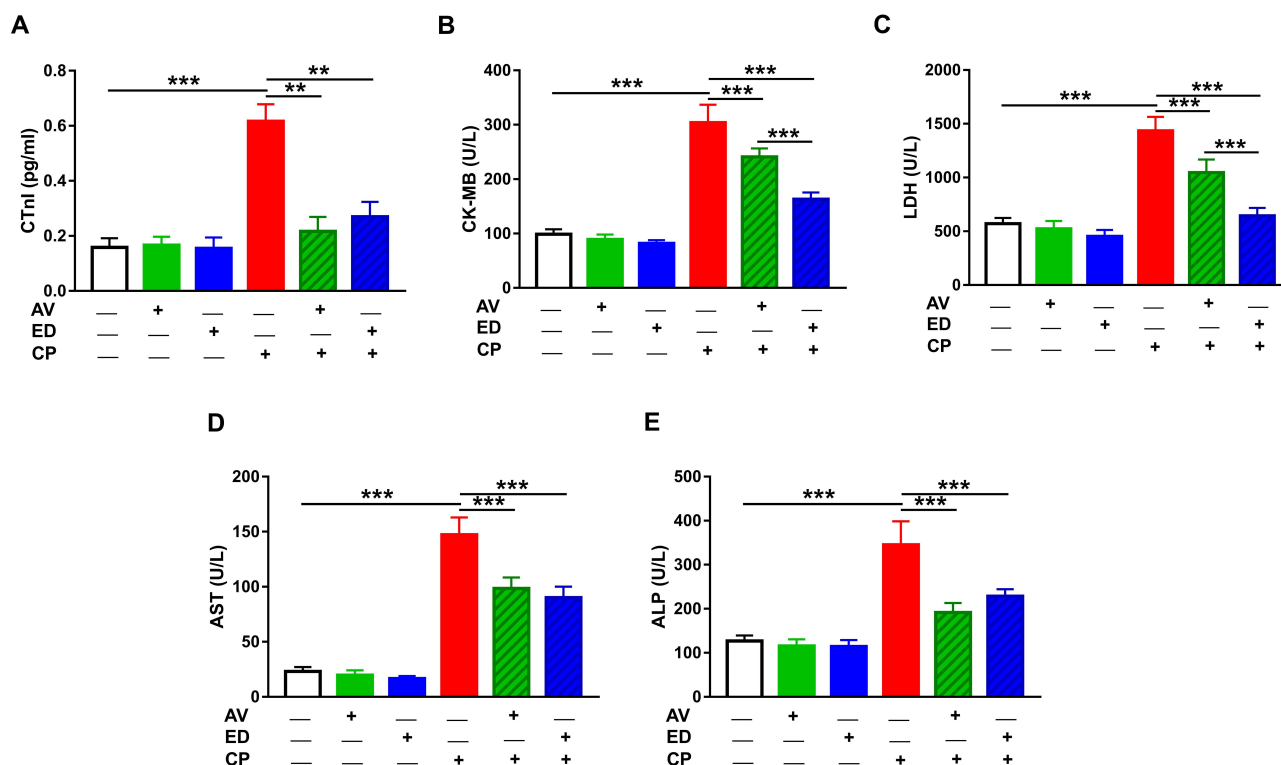


Figure 1 AV and ED ameliorate heart function in CP-intoxicated rats. Treatment with AV and ED decreased serum (A) CTnI, (B) CK-MB, (C) LDH, (D) AST and (E) ALP in CP-intoxicated rats. Data are mean ± SEM, (n = 8). ** $P<0.01$ and *** $P<0.001$.

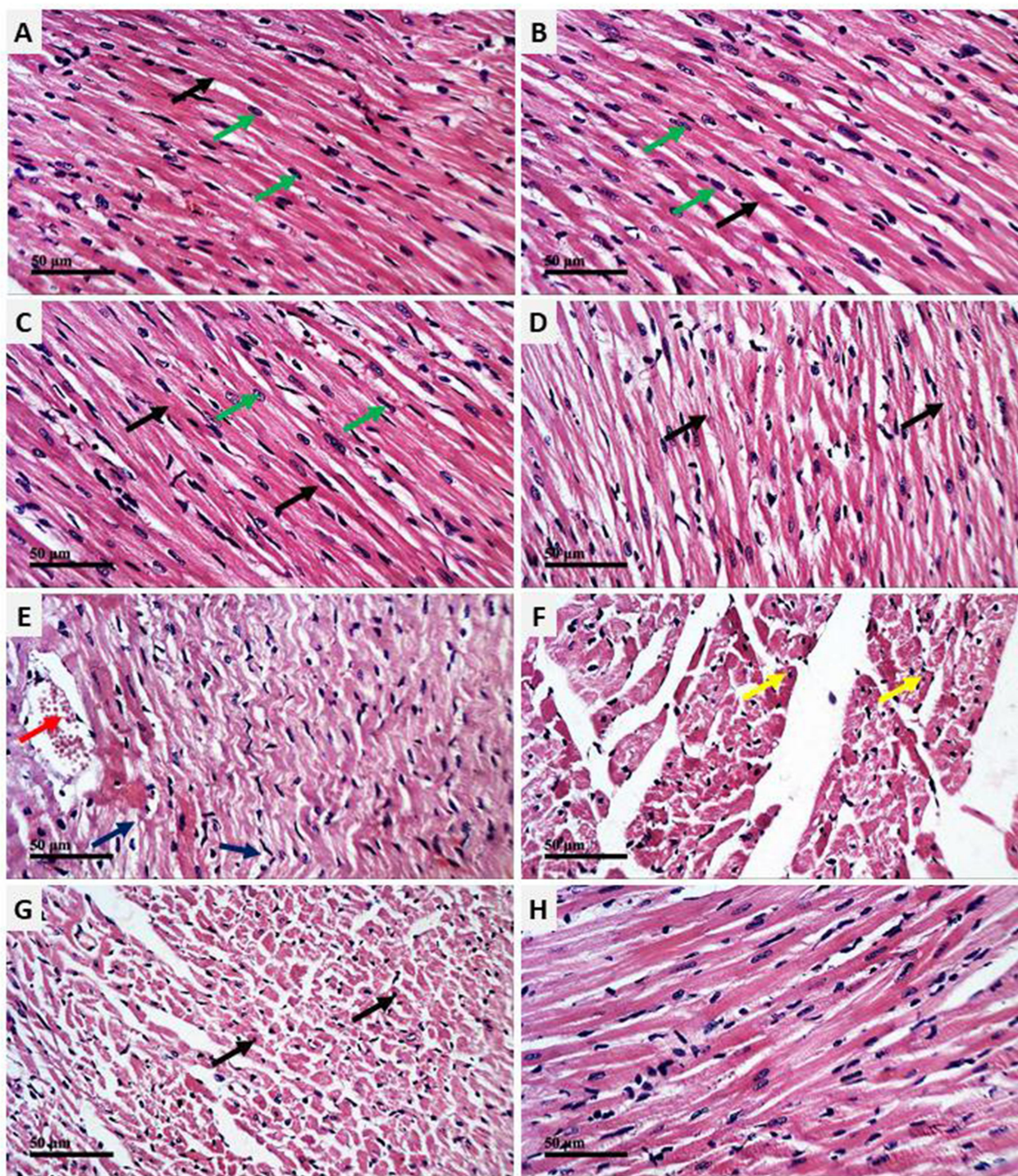


Figure 2 AV and ED prevent CP-induced cardiac injury in rats. Photomicrographs of the heart tissue sections of control (A) and rats treated with AV (B) and ED (C) showing normal structure of the cardiomyocytes with normal nuclei (green arrow) and cytoplasmic striation (black arrow), CP-intoxicated rats (D–F) showing degenerative changes (blue arrow), fragmented myofibrils (black arrow), nuclear pyknosis (yellow arrow), increased intercellular spaces between adjacent cardiomyocytes and many congested intermuscular blood vessels (red arrow), and CP-intoxicated rats treated with AV (G) and ED (H) showing improved histological architecture of the heart with fewer degenerative changes and congestions (arrow). (H&E, Scale bar = 50 µm).

Table 2 Heart Histopathological Score Lesions

	Control	AV	ED	CP	AV +CP	ED +CP
Degenerative changes	–	–	–	++	++	+
Intercellular spaces	–	–	–	++	+	+
Blood vessels congestion	–	–	–	++	+	+

Abbreviations: AV, acetovanillone; ED, edaravone; CP, cyclophosphamide.

supplementation of either AV or ED decreased cardiac MDA and increased GSH and SOD in CP-induced rats. Of note, both treatment agents did not alter cardiac redox balance in normal rats.

AV and ED Upregulate Keap1/Nrf2 Signaling Pathway in the Heart of CP-Induced Rats

The effect of AV and ED on the Nrf2 signaling was assessed by evaluating the expression levels of Keap1 and Nrf2 in the heart of normal and CP-intoxicated rats (Figure 4). In

addition, molecular docking simulations were performed to explore the binding mode of AV and ED with Keap1 (Figure 5). The results showed a significant increase in the mRNA abundance of cardiac Keap1 (Figure 4A) in CP-intoxicated rats when compared with the control group ($P<0.001$). While AV and ED did not affect the expression levels of AV and ED in normal rats, both treatments downregulated Keap1 mRNA in rats that received CP. Nrf2 gene and protein expression levels exhibited a different pattern as shown in Figure 5B–D. Nrf2 mRNA and protein were decreased significantly in the heart of rats administered with CP. Treatment with AV and ED resulted in a significant increase in Nrf2, both mRNA and protein, in the heart of both normal and CP-intoxicated rats.

Molecular docking analysis showed that AV docks into the determined active site of Keap1 and forms four hydrogen bonds with the binding site (R336, S363 and N382) of Keap1 at the dimeric interface (Figure 5). The benzene ring of AV exhibits hydrophobic interaction with the side chains of P384 and Y334. The binding affinity was -5.96 ± 0.27 kcal mol⁻¹. ED forms two hydrogen bonds with V463 and A510 of Keap1. The benzene ring of ED showed a tight fit in the hydrophobic pocket of Keap1 (V418, V463, V465

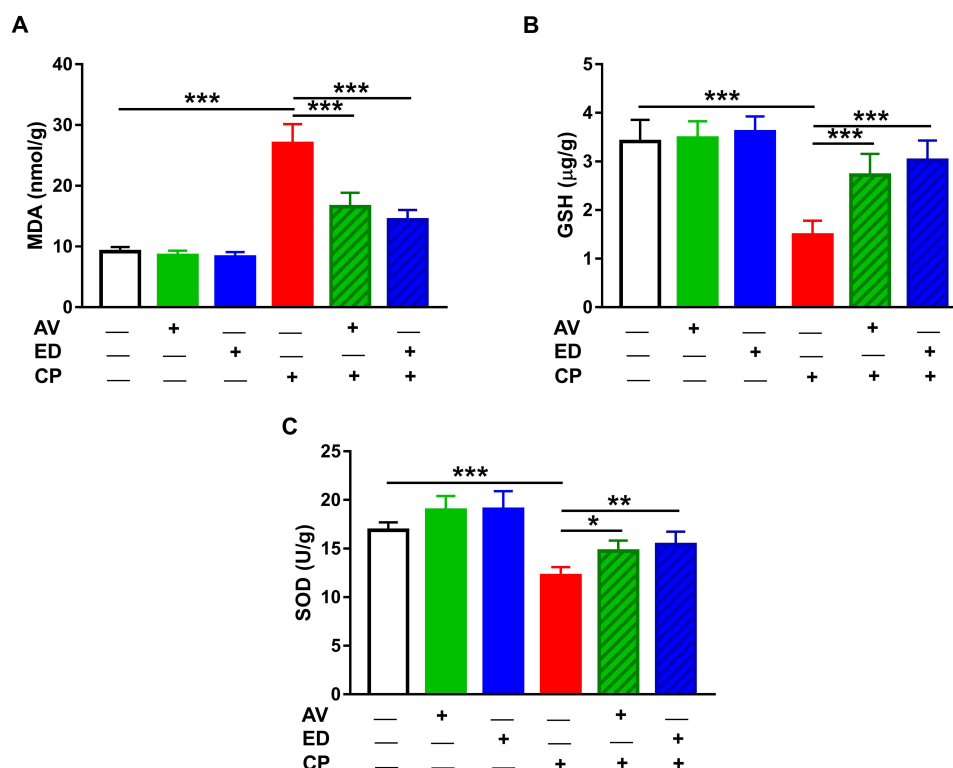


Figure 3 AV and ED attenuate cardiac oxidative stress in CP-induced rats. AV and ED decreased cardiac MDA (A) and increased GSH (B) and SOD (C) in CP-intoxicated rats. Data are mean \pm SEM, (n = 8). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

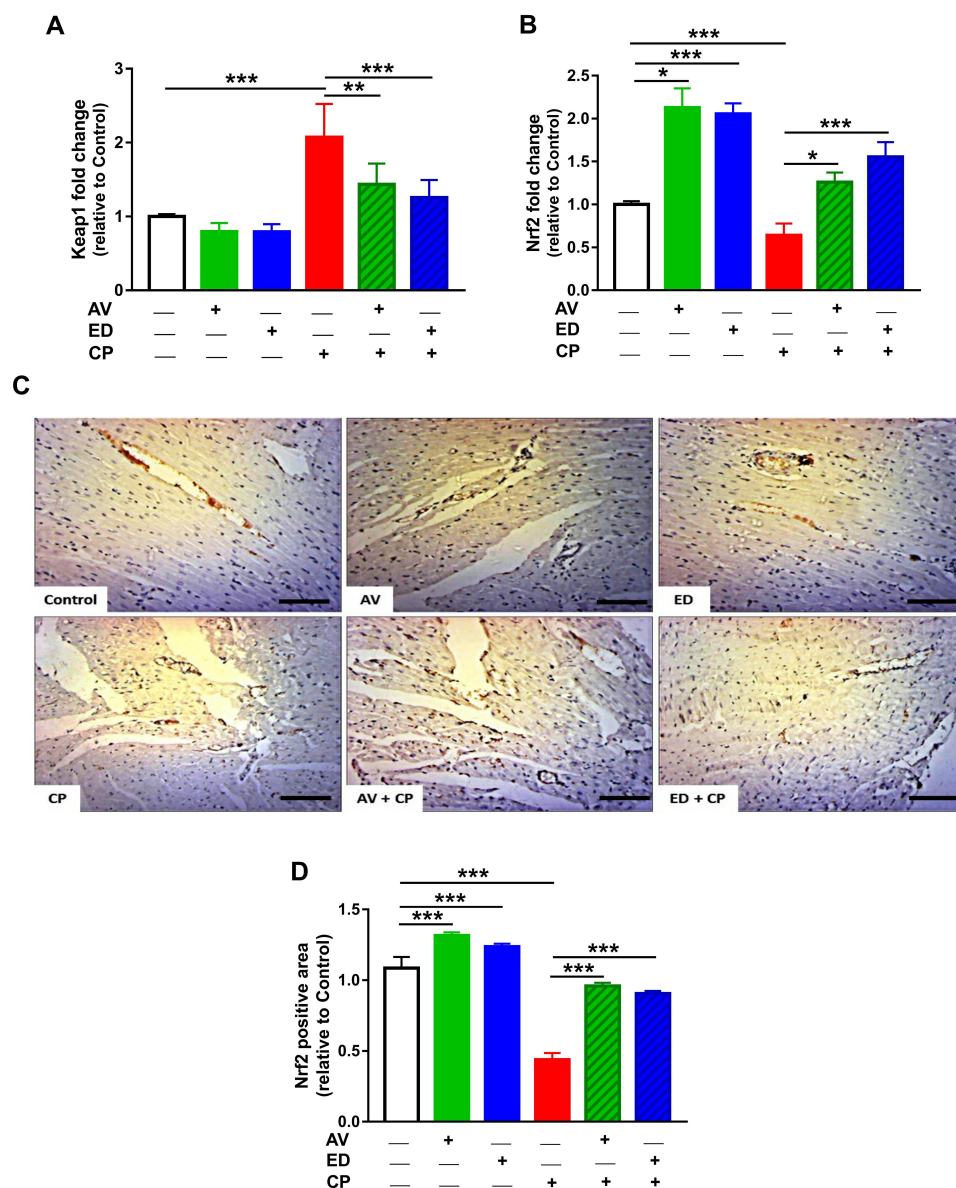


Figure 4 AV and ED upregulate Keap1/Nrf2 signaling pathway in the heart of CP-induced rats. AV and ED downregulated Keap1 mRNA (**A**) and increased Nrf2 gene (**B**) and protein expression (**C**, **D**) in rats [Scale bar = 100µm]. Data are mean ± SEM, (n = 8). *P<0.05, **P<0.01 and ***P<0.001.

and V512). The binding affinity was -6.23 ± 0.10 kcal mol⁻¹ (Figure 5).

AV and ED Upregulate PI3K/Akt/mTOR Signaling in the Heart of CP-Induced Rats

Immunohistochemical staining was used to evaluate the effect of AV and ED on the expression levels of PI3K, Akt and mTOR in the heart of both normal and CP-intoxicated rats. Oral administration of AV and ED upregulated cardiac PI3K (Figure 6A and B), Akt (Figure 6C and D) and mTOR (Figure 6E and F) in normal rats. CP administration significantly decreased the expression levels of PI3K,

Akt and mTOR in the heart tissue, an effect that was significantly reversed in rats treated with either AV or ED. The effect of ED on PI3K and TOR levels in CP-intoxicated rats was significant when compared with AV (P<0.001).

AV and ED Increase Cardiac Cytochrome Expression in CP-Induced Rats

Treatment with AV significantly increased the expression of cardiac cytochrome in the heart of normal rats when compared with the control (Figure 7; P<0.001). CP administration resulted in a significant downregulation of cardiac

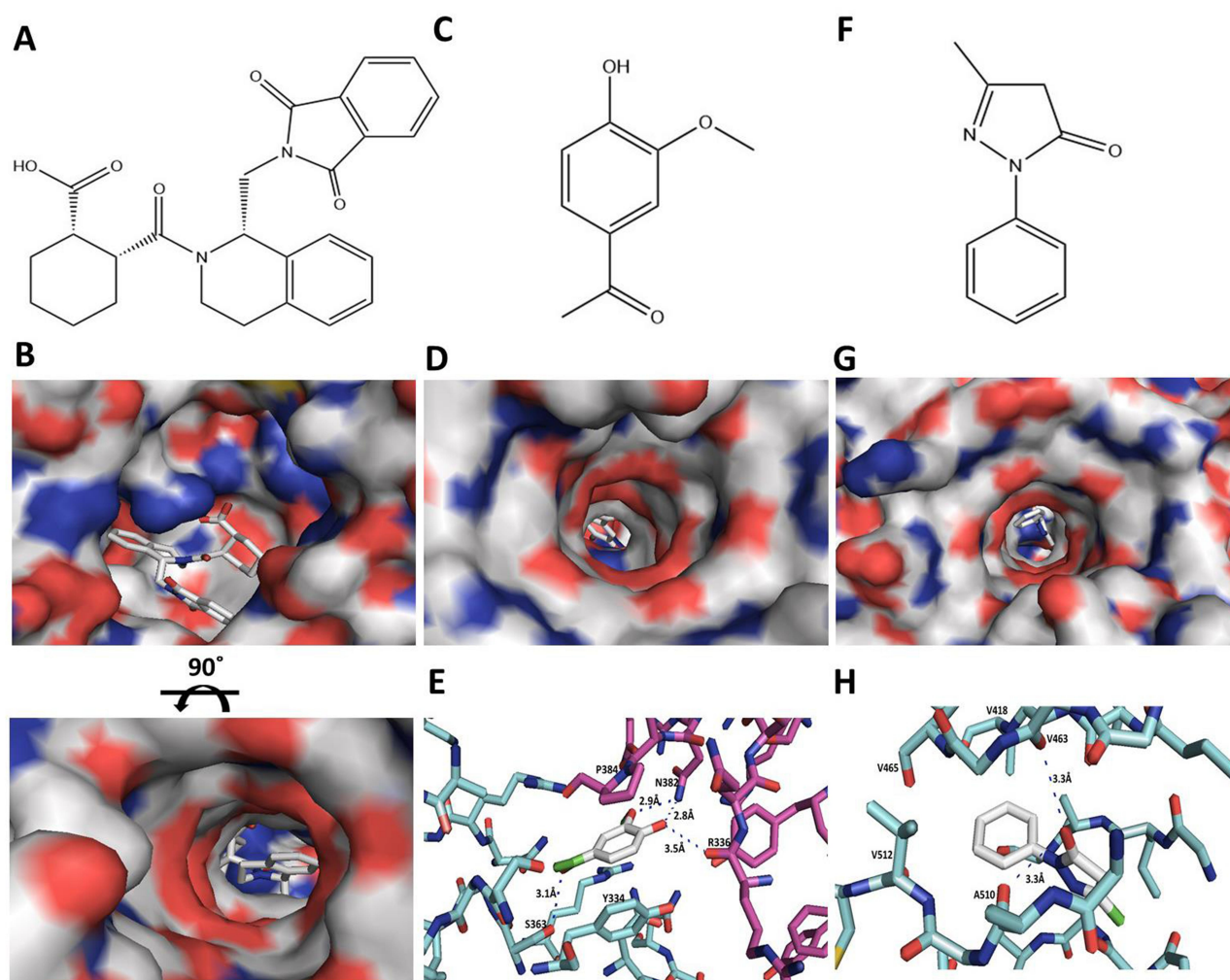


Figure 5 Molecular interactions of AV and ED with the Keap1. (A, B) Chemical structure of SRS (A) and surface map of the SRS complex with Keap1 (B). (C–E) Chemical structure of AV (C) surface map of the docking model of AV with the binding site of Keap1 (D) and the amino acid residues on Keap1 within a 5 Å contact radius of AV (E). (F–H) Chemical structure of ED (F) surface map of the docking model of ED with the binding site of Keap1 (G) and the amino acid residues on Keap1 within a 5 Å contact radius of ED (H).

cytoglobin expression. On the other hand, CP-intoxicated rats treated with AV or ED exhibited a significant increase in cardiac cytoglobin. When compared to ED, AV increased cardiac cytoglobin significantly ($P < 0.01$) in CP-intoxicated rats.

Discussion

Cardiotoxicity is a life-threatening complication of chemotherapy and CP is well known to cause cardiac adverse effects.^{3,37} Given the role of oxidative stress in mediating its adverse effects, we investigated the protective effects of AV and ED on CP cardiotoxicity in rats with an emphasis on PI3K/Akt/mTOR and Nrf2 signaling.

CP administration caused cardiac injury manifested by the increased serum CTnI, CK-MB, LDH, AST and ALP

along with the histological alterations, including degenerative changes, fragmented myofibrils, nuclear pyknosis and congested blood vessels. Similar findings have been reported in previous studies where CTnI, CK-MB and LDH were increased in rodents following the administration of CP.^{38,39} Treatment of the CP-intoxicated rats with AV or ED ameliorated the circulating levels of all assayed cardiac function markers and effectively prevented the histological alterations in the heart tissue. The cardioprotective effects of AV and ED have been demonstrated in different studies. For instance, AV showed protective effects against cisplatin cardiotoxicity,⁴⁰ isoproterenol-induced cardiac hypertrophy⁴¹ and diabetic cardiomyopathy.²⁰ ED has also shown protective effects in animal models of methamphetamine-,⁴² valproic acid-⁴³

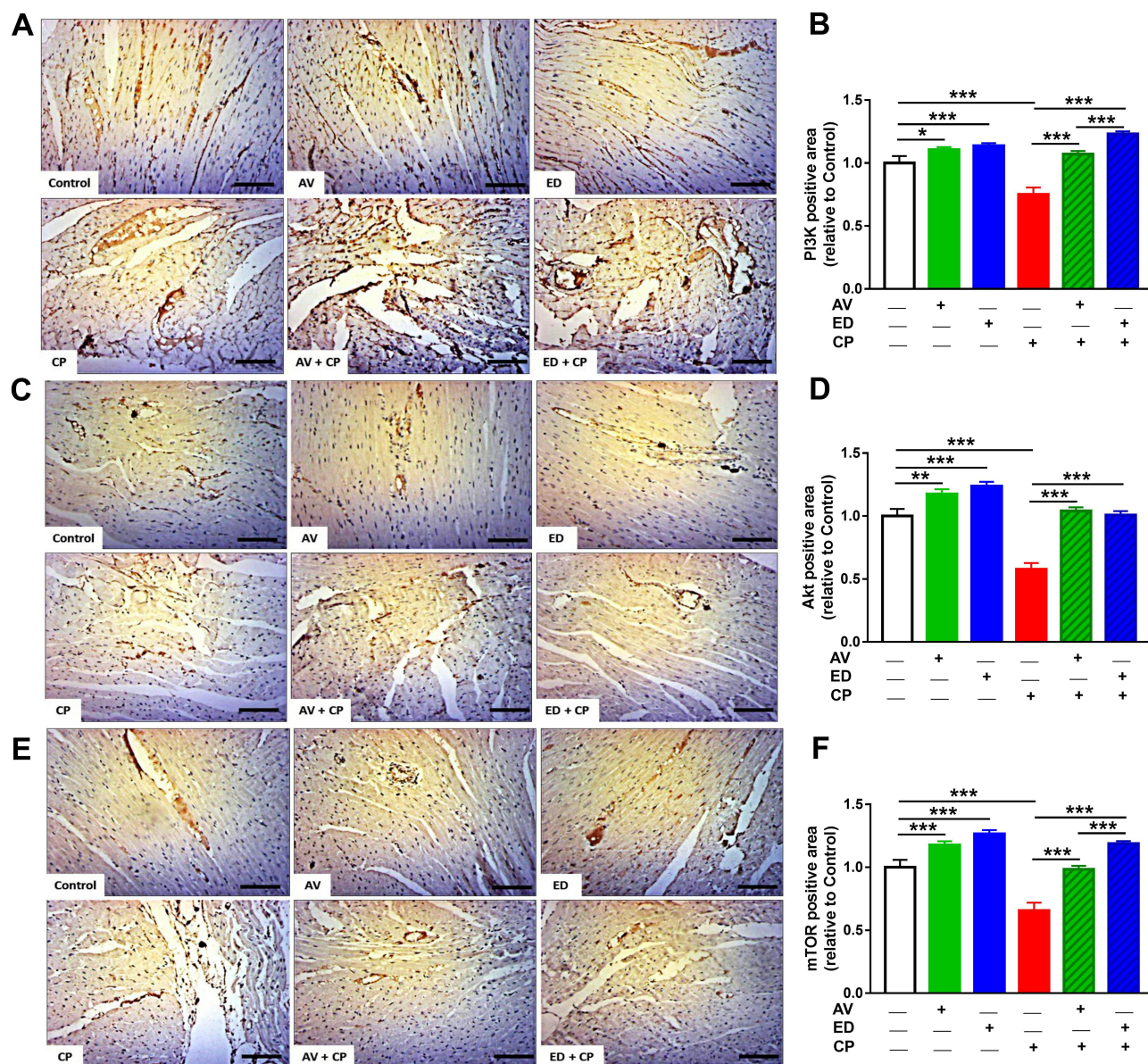


Figure 6 AV and ED upregulate PI3K/Akt/mTOR signaling in the heart of CP-induced rats. Treatment with AV or ED increased the expression of PI3K (A, B), Akt (C, D) and mTOR (E, F) in the heart of rats. [Scale bar = 100µm]. Data are mean ± SEM, (n = 8). *P<0.05, **P<0.01 and ***P<0.001.

and doxorubicin-induced cardiotoxicity.⁴⁴ In these studies, the cardioprotective effects of AV and ED were attributed mainly to their ability to attenuate oxidative stress. Therefore, we investigated the effect of AV and ED on LPO and the antioxidants GSH and SOD in the heart of CP-intoxicated rats. The LPO marker MDA was significantly increased following CP administration whereas GSH and SOD were declined, demonstrating oxidative stress. The cellular mechanism of CP toxicity is so far mediated by oxidative stress caused by excessive ROS generation and the principle alkylating metabolites phosphoramidate mustard and acrolein.¹² Acrolein binds

covalently to lipids and proteins, leading to the formation of free radicals.¹² In addition, elevated ROS levels induce cell injury through oxidizing the cellular macromolecules and depleting antioxidant defenses by disrupting their protein conformation.⁴⁵ Accordingly, ROS and MDA were increased and cellular antioxidants were decreased in different tissues of rats received CP,^{8,9,11,46} adding support to the role of oxidative stress in the adverse effects of CP. AV and ED supplementation effectively decreased LPO and boosted the antioxidant defenses in the heart of CP-intoxicated rats. The ability of AV and ED to attenuate oxidative stress in different models of cardiac injury has

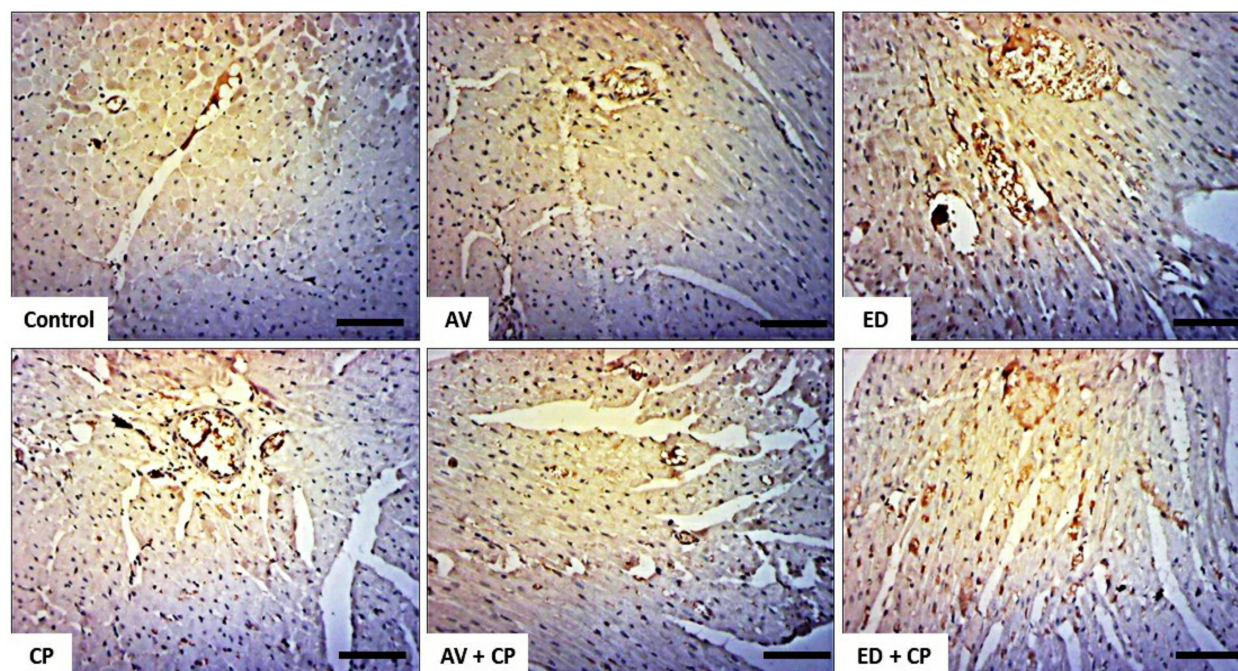
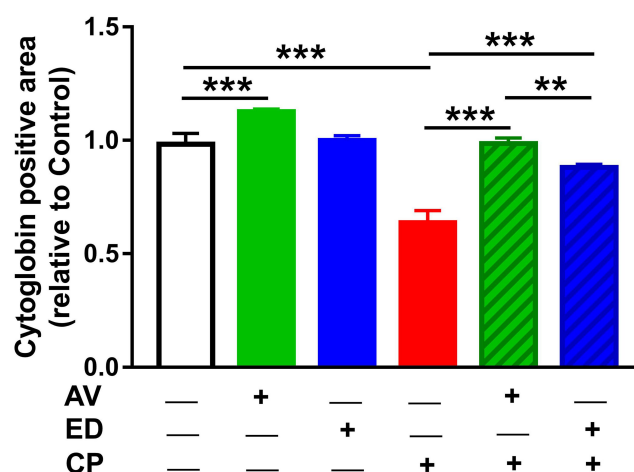
A**B**

Figure 7 AV and ED increase cardiac cytoglobin expression in CP-induced rats. Treatment with AV or ED increased the expression of cytoglobin in the heart of rats. **(A)** Representative images showing immunohistochemical staining of cytoglobin in the heart of rats. [Scale bar = 100 μ m]. **(B)** image analysis of the expression of cytoglobin in the heart of rats. Data are mean \pm SEM, (n = 8). **P<0.01 and ***P<0.001.

been previously reported.^{20,40–44} In cisplatin-intoxicated rats, oral supplementation of 600 mg/L AV for 5 days decreased cardiac LPO and increased GSH.⁴⁰ AV increased cardiac GSH in isoproterenol-induced rats⁴¹ and serum SOD and catalase in diabetic rats.²⁰ Similarly, ED decreased ROS and MDA and increased the activity of antioxidant enzymes in the heart of valproic acid-⁴³ and methamphetamine-induced rats.⁴² These studies along with the findings of this study demonstrate the potent antioxidant activities of both AV and ED.

NOX is a major enzymatic source of ROS generation in the cardiovascular system.⁴⁷ NOX-derived ROS, such as superoxide, contributes to cardiovascular pathogenesis through activating other enzymatic systems and triggering ROS production. Besides the oxidation of cellular macromolecules and depletion of antioxidants, ROS produced by NOX can cause mitochondrial DNA damage and oxidation of components of the membrane permeability transition pore and opening of the redox-sensitive mitochondrial ATP-sensitive K⁺ channel, resulting in mitochondrial

uncoupling and more ROS production.^{47–49} Given the role of NOXs in cardiovascular disease, it is noteworthy assuming that inhibition of NOX-derived ROS plays a role in the cardioprotective effect of AV and ED.

Nrf2, a redox-sensitive transcription factor controlling the expression of antioxidant enzymes, has been reported to decrease in different tissues of CP-intoxicated rats.^{8–11} In addition, Nrf2 deficiency exaggerated cardiotoxicity induced by the anticancer drug doxorubicin in mice.⁵⁰ In contrast, upregulation of Nrf2 protected against CP hepato- and nephrotoxicity,^{8–11} and negatively regulated oxidative stress and cardiac dysfunction induced by transverse aortic constriction.⁵¹ We assumed that Nrf2 is involved in the cardioprotective effect of AV and ED. Therefore, we evaluated the effect of AV and ED on Keap1 and Nrf2 expression in the heart of rats and conducted an *in silico* investigation of their binding modes with Keap1. mRNA abundance of Keap1 was increased whereas Nrf2 gene and protein expression levels were downregulated in the heart of CP-intoxicated rats. Although activated by oxidative stress, Nrf2 was declined in CP-intoxicated rats which could be attributed to sustained and prolonged ROS generation. Accordingly, previous studies have demonstrated suppressed Nrf2 signaling under conditions of excessive ROS generation and oxidative stress both *in vitro* and *in vivo*.^{52–54} Both AV and ED decreased Keap1 mRNA and upregulated the expression of Nrf2 in the heart of CP-induced rats, pointing to the role of Keap1/Nrf2 signaling in their cardioprotective effect. These findings added support to previous studies showing the possible role of Nrf2 upregulation in the beneficial effects of AV and ED against different disease conditions. In rat models of cisplatin cardiotoxicity⁴⁰ and quinolinic acid neurotoxicity,⁵⁵ AV has been reported to upregulate Nrf2 mRNA levels and suppress oxidative stress. ED has also shown an ability to upregulate Nrf2 signaling and subsequently attenuate oxidative injury in experimental models of traumatic brain injury⁵⁶ and chlorpyrifos neurotoxicity.⁵⁷ Our study introduces new information that Nrf2 signaling mediated, at least in part, the protective effects of AV and ED against CP cardiotoxicity. Interestingly, both agents upregulated Nrf2 expression in the heart of normal rats. Additionally, the molecular docking simulations revealed the ability of AV and ED to bind to Keap1. Both agents exhibited binding affinities and form hydrogen bonds with the side chains of the charged amino acids in vicinity of the binding pocket of Keap1. The benzene ring of AV and ED promotes the hydrophobic

interaction with the hydrophobic residues of the binding sites of Keap1. These polar and hydrophobic interactions permit a tight fit of AV and ED in the binding site of Keap1. Thus, these *in silico* findings suggest the value of AV and ED as promising modulators of Keap1 activity.

The PI3K/Akt/mTOR signaling is another pathway whose activation was associated with attenuation of oxidative stress.¹⁴ Activation of the PI3K/Akt/mTOR pathway has been reported to activate Nrf2 signaling, resulting in upregulation of antioxidants. For instance, PI3K/Akt/mTOR-mediated Nrf2/HO-1 signaling protected against oxidative stress induced by oxidized low-density lipoprotein in endothelial cells.⁵⁸ mTOR is an atypical serine/threonine kinase belongs to the family of the PI3K related kinases and exerts its cellular functions through assembly with adaptor proteins to form mTORC1 and mTORC2.⁵⁹ mTOR is involved in the regulation of the embryonic development of the cardiovascular system and maintenance of cardiac structure and function. In this context, mTOR knockout in mice provoked derangements in cardiac fatty acid metabolism, cardiac dysfunction, massive apoptosis, fibrosis and mitochondrial abnormalities and dysfunction.^{60,61} The Akt is one of the main regulators of mTOR where its activation activates mTOR or inhibits the endogenous mTORC1 inhibitor PRAS40.⁶² Previous studies have demonstrated the inhibitory effect of the anticancer drugs on the PI3K/Akt/mTOR pathway.^{63,64} Acute cardiac dysfunction induced by doxorubicin was associated with the inhibition of mTOR signaling.⁶³ In addition, doxorubicin-induced myocardial apoptosis was associated with the downregulation of the PI3K/Akt/mTOR pathway.⁶⁴ On the other hand, activation of this signaling pathway contributed to the protection against drug-induced cardiac injury.^{63,64} The current study showed the downregulation of PI3K, Akt and mTOR expression in the heart of rats administered with CP. In accordance, the study of Albayrak et al⁶⁵ demonstrated the downregulation of PI3K, Akt and mTOR expression in the kidney of mice received CP every 2 days for 3 consecutive weeks. Given the role of the PI3K/AKT/mTOR pathway in maintenance of the cardiac structure and function and its suppression by CP, it would be intriguing to know whether the modulation of this pathway is involved in the cardioprotective effects of AV and ED. Our results revealed the positive effect of AV and ED on the PI3K/Akt/mTOR signaling pathway where both agents increased the cardiac levels of PI3K, Akt and mTOR in normal and CP-intoxicated rats. These findings clearly support the notion that upregulation of the

PI3K/Akt/mTOR signaling plays a role in the protective effect of AV and ED against CP cardiotoxicity.

The upregulation of cytoglobin expression is another effect we thought to participate in the cardioprotective activity of AV and ED. Cytoglobin is an intracellular respiratory globin ubiquitously expressed almost in all tissues.⁶⁶ It has been shown to prevent oxidative stress through scavenging excess ROS,⁶⁷ thereby maintaining physiological ROS levels.⁶⁸ Cytoglobin protected the cardiac progenitor cells against oxidative stress and promoted their survival.⁶⁹ The direct interaction between cytoglobin and PI3K/Akt/mTOR signaling in the heart has not been presented before. Given the role of PI3K/Akt/mTOR signaling in tumorigenesis and the progression of cancer,²² numerous studies have demonstrated its interaction with cytoglobin.^{70,71} In L929 fibroblast cell line, overexpression of cytoglobin downregulated cell proliferation, migration and tumor growth through repressing the PI3K/Akt/mTOR pathway and inducing apoptosis.⁷¹ In addition, positive correlation between PI3K/Akt activation and low expression of cytoglobin has been associated with tumor progression in glioma.⁷⁰ Here, CP administration resulted in downregulation of cytoglobin expression in the heart of rats which might be attributed to surplus and sustained ROS generation. Treatment with AV and ED resulted in a pronounced upregulation of cytoglobin in the cardiac tissue. Therefore, the positive regulation of cytoglobin is involved in the cardioprotective effect of AV and ED against CP toxicity. It is worth mentioning that numerous studies have discussed the antioxidant activity of AV and ED, but our study introduced a new information on their effect on cardiac cytoglobin expression, and shed light on the possible role of cytoglobin in the pathogenesis of CP cardiotoxicity.

Conclusion

The present findings provide a compelling evidence for the protective effect of AV and ED against CP cardiotoxicity. The cardioprotective efficacy of AV and ED was associated with their ability to upregulate Nrf2 and PI3K/Akt/mTOR signaling, and cytoglobin, resulting in attenuation of oxidative stress and tissue injury. Therefore, AV and ED might represent promising cardioprotective agents in patients on chemotherapy, pending further investigations and clinical studies to explore the exact mechanism(s) underlying their beneficial effects.

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Disclosure

The authors declare that there are no potential conflicts of interest.

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