sciendo
10.2478/AMB-2025-00061

ORIGINAL ARTICLE

PROTECTIVE EFFECTS OF QUERCETIN, CURCUMIN AND RESVERATROL IN AN IN VITRO MODEL OF DOXORUBICIN-INDUCED CARDIOTOXICITY

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Abstract. Introduction: *Over the past two decades, drug-induced cardiotoxicity has resulted in the withdrawal of several drugs, including prenylamine, rofecoxib, and levomethadyl acetate, while others like rosiglitazone remain available only under restricted conditions. However, some cardiotoxic drugs, like doxorubicin (DOX), are still used due to their potent antitumor activity despite their dose-dependent cardiotoxicity. This cardiotoxicity, often caused by lipid peroxidation and reactive oxygen species (ROS), can be mitigated by natural substances like quercetin (QR), curcumin (CRC), and resveratrol (RES), which have notable antioxidant and cardioprotective effects.* ***Aim:*** *This study aimed to evaluate the potential of QR, RES, and CRC to enhance the viability of H9c2 cardiomyocytes in an in vitro model of doxorubicin-induced cardiotoxicity.* ***Materials and Methods:*** *H9c2 cells were treated with doxorubicin (0.25 μM and 1 μM) and varying concentrations of QR, RES, and CRC (0.01 μM, 0.1 μM, 1 μM, 2.5 μM, 5 μM, 10 μM) for 24 and 48 h. Cell viability was assessed using the MTT assay to determine the protective effects of the natural antioxidants on H9c2 cell line.* ***Results:*** *Our data demonstrated that QR and CRC significantly improved the viability of H9c2 cells in the DOX-induced cardiotoxicity model of treatment with 0.25 μM DOX (24 h). At these conditions, RES also showed protective cell viability effects, but it was not effective at the injury with higher DOX concentration (1 μM, 24 h).* ***Conclusions:*** *This study highlights the in vitro protective effects of QR and CRC in reducing DOX-induced cardiotoxicity in H9c2 cardioblast cells, most probably attributed to their well-established antioxidant effects.*

Key words: *doxorubicin, cardiotoxicity, curcumin, quercetin, resveratrol, Rat H9c2 cardiomyoblasts*

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Received: *14 January 2025;* ***Revised/Accepted:*** *09 May 2025*

INTRODUCTION

Doxorubicin (DOX), an anthracycline anticancer antibiotic, is widely used in the treatment of hematological malignancies and various solid tumors. However, its clinical application is significantly limited by its well-established dose-dependent cardiotoxic adverse effects, which often culminate in congestive heart failure [1]. Recent studies have demonstrated that DOX-induced myocardial damage involves

multiple biological pathways, including oxidative stress, lipid peroxidation, DNA damage, mitochondrial dysfunction, apoptosis, and autophagy [2-8]. Among these mechanisms, oxidative stress is considered a central contributor to DOX-induced cardiac injury [9-11]. Specifically, DOX generates high levels of superoxide anion radicals ($\bullet O_2^-$) and other reactive oxygen species (ROS), leading to mitochondrial dysfunction and extensive cellular damage [12, 13]. Therefore, targeting oxidative stress represents a promising strategy for the prevention and treatment of DOX-induced cardiotoxicity. Several antioxidant and anti-inflammatory agents have demonstrated potential in mitigating cardiac damage associated with DOX treatment [9, 14]. In recent years, considerable research efforts have focused on identifying and characterizing cardioprotective agents capable of preventing anthracycline-induced cardiotoxicity. Natural antioxidants, including apigenin, dihydromyricetin, salidroside, melatonin, glutathione, coenzyme Q10, vitamins, omega-3 fatty acids, quercetin (QR), isorhamnetin, cannabidiol (CBD), resveratrol (RES), curcumin (CRC), and catechins, among others, have emerged as promising candidates for preventing or reducing anthracycline-induced cardiac damage without compromising the anticancer efficacy of these agents [15, 16]. Additionally, dexrazoxane, a synthetic chemoprotective agent, has received FDA approval for the prevention of anthracycline-induced cardiotoxicity [17]. Despite these advances, further clinical studies are needed to rigorously evaluate the efficacy and safety of these cardioprotective agents. Although encouraging results have been reported in preclinical models, clinical evidence in humans remains limited, emphasizing the necessity for more comprehensive and well-designed trials [18].

Quercetin (QR; 3,3',4',5,7-pentahydroxyflavone) is one of the most abundant flavonoids found in a variety of fruits and vegetables [19]. Its potent antioxidant properties have been demonstrated in numerous animal studies, showing protection against ischemia-reperfusion injury, exposure to toxic compounds, and oxidative stress-related damage in the heart, brain, and other tissues [20-23]. Moreover, QR has been identified as a chemosensitizer in breast cancer chemotherapy when co-administered with DOX, suggesting its dual role in enhancing anticancer efficacy and reducing cardiotoxicity [24]. A recent study in rats showed that QR could potentiate the protective effects of losartan against chronic DOX-induced cardiotoxicity [24-29].

Similarly, resveratrol (RES), a naturally occurring polyphenol predominantly found in grapes and berries, has been shown to attenuate ROS production and reduce oxidative damage in the heart across various animal models [30, 31]. In murine models of chronic DOX exposure, RES supplementation significantly alleviated DOX-induced cardiac injury [32-37]. Curcumin (CRC), a polyphenol extracted from the rhizome of turmeric (Curcuma longa), has also demonstrated protective effects against DOX-induced cardiotoxicity in experimental models [38-42]. Numerous studies have confirmed the anti-inflammatory, antioxidant, and anti-apoptotic effects of CRC, supporting its potential as a therapeutic agent for cardiac protection in DOX-treated cancer patients [43]. Moreover, recent evidence suggests that RES inhibits DOX-induced autophagy and cardiomyocyte apoptosis, proposing that autophagy suppression is a crucial mechanism underlying RES-mediated cardioprotection [44].

Given these findings, the exploration of plant-derived polyphenols as adjunctive therapies alongside DOX is an area of growing interest [45]. Accumulating experimental evidence highlights that polyphenols, particularly flavonoids, may effectively counteract DOX-induced cardiotoxicity due to their combined anticancer and cardioprotective properties [35, 46-49]. However, the limited number of in vitro studies investigating the combined administration of free DOX with free QR, CRC, or RES has prompted further research into these potential interactions.

In this context, the aim of our current study was to investigate the cardioprotective effects of three natural antioxidants, quercetin (QR), curcumin (CRC), and resveratrol (RES), with a particular focus on their potential to attenuate DOX-induced cardiotoxicity in an in vitro model. We employed the H9c2 rat cardiomyoblast cell line, treating cells with DOX to establish a reliable model of chemotherapy-induced cardiac injury. Subsequently, we evaluated whether pretreatment with QR, RES, and CRC could mitigate DOX-induced cellular damage, thereby providing insights into their possible therapeutic roles in protecting cardiovascular health during chemotherapy.

MATERIALS AND METHODS

Materials

Doxorubicin hydrochloride (CAS number: 25316-40-9), QR (CAS Number: 117-39-5), CRC (CAS number: 458-37-7), RES (CAS Number: 501-36-0), DMEM high glucose medium, heat-inactivated FBS,

and MTT were all purchased from Sigma-Aldrich (Darmstadt, Germany). All materials used were of high chemical grade and did not require additional purification. Deionized water was prepared using the ion exchange method.

Cell lines and culture conditions

Rat H9c2 cardiomyoblast cells were obtained from ECACC and cultured in DMEM modified high glucose medium (4500 mg/l) supplemented with 10% fetal calf serum (FBS) and 2 mM glutamine (they were cultured as monolayers in Dulbecco's modified Eagle's medium in an atmosphere of 5% CO_2 in air saturated with water vapor at 37°C). The medium was replaced with fresh medium every 2 days [50].

MTT cell viability assay

The cell suspension from the H9c2 line was adjusted to the required density and seeded into 96-well plates at a density of 1x104 per well (100 µl per well). The plates were then incubated for 24 h to ensure proper cell attachment. The treatment solutions were prepared as follows:

- DOX solution at concentrations of 0.25 µM and 1 µM.
- QR solution at concentrations of 0.01 µM, 0.1 µM, 1 µM, 2.5 µM, 5 µM, and 10 µM.
- CRC solution at concentrations of 0.01 µM, 0.1 µM, 1 µM, 2.5 µM, 5 µM, and 10 µM.
- RES solution at concentrations of 0.01 µM, 0.1 µM, 1 µM, 2.5 µM, 5 µM, and 10 µM.

For each treatment type and concentration, a minimum of 8 wells per plate were treated. The plates were then incubated under standard conditions (37°C, 5% CO2_22, and maximum humidity) for an additional 24 h.

Subsequently, an MTT dye solution was prepared in the appropriate culture medium at a final concentration of 0.5 mg/ml. The solution was added to each well. For 3 h, the mitochondrial systems of viable cells metabolized the yellow MTT salt into purple formazan crystals. These crystals were solubilized by adding 100 µl of DMSO per well.

The treated plates were subjected to spectrophotometric measurement using a Synergy 2 multiplate reader (BioTek Instruments). Absorbance was measured at 570 nm, with a background reference wavelength of 690 nm.

DOX-induced cell injury

Cell viability was evaluated using an MTT assay following the experimental protocols outlined below. The H9c2 cell line was treated with DOX at two concentrations: 0.25 µM and 1 µM. The treatments were administered for two different durations: 24 h and 48 h, resulting in four distinct experimental groups.

Group 1: Cells were treated with DOX (0.25 µM and 1 µM) for 24 h. After 24 h, the DOX solutions were aspirated, and antioxidant treatments were applied at the appropriate concentrations. The cells were then incubated with the antioxidants for an additional 24 h before viability assessment using the MTT assay.

Group 2: Cells were treated with DOX (0.25 µM and 1 µM) for 48 h. Following this treatment period, the DOX solutions were aspirated, and antioxidant treatments were administered. The cells were allowed to incubate with the antioxidants for another 24 h before conducting the MTT assay.

This experimental design allows for a comprehensive evaluation of cell viability across both treatment durations and concentrations of DOX, providing insights into the protective effects of antioxidants under these conditions.

Statistical analysis

Statistical analyses of the H9c2 cell line data were performed using GraphPad Prism (version 8, GraphPad Software, La Jolla, California, USA). Data significance was evaluated using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test for multiple comparisons. Statistical differences were considered significant at $p \leq 0.05$, $*p \leq 0.01$, and $**p \leq 0.001$. Cytoprotective data were normalized as a percentage relative to the DOX-treated control, which was set at 0% viability.

RESULTS

The cytotoxicity of DOX was accessed across a concentration range of 0.01-80 µM. Following incubation periods of 24 and 48 h, DOX demonstrated significant cardiotoxic effects on H9c2 cardioblasts, with IC50 values of 6.6 µM and 3.8 µM, respectively. Based on these findings and a thorough review of the literature, we selected DOX concentrations of 0.25 µM and 1 µM for subsequent experiments. To estimate the effect of antioxidants in H9c2 cell line, cell viability was reported relative to two control groups, negative (cells incubated in culture medium alone) and positive (cells damaged by DOX). The negative control did not induce mortality, and the result was taken as 100%, whereas the positive control did induce mortality and the result was taken as 0%.

Cytotoxicity effects of DOX on H9c2 cell line

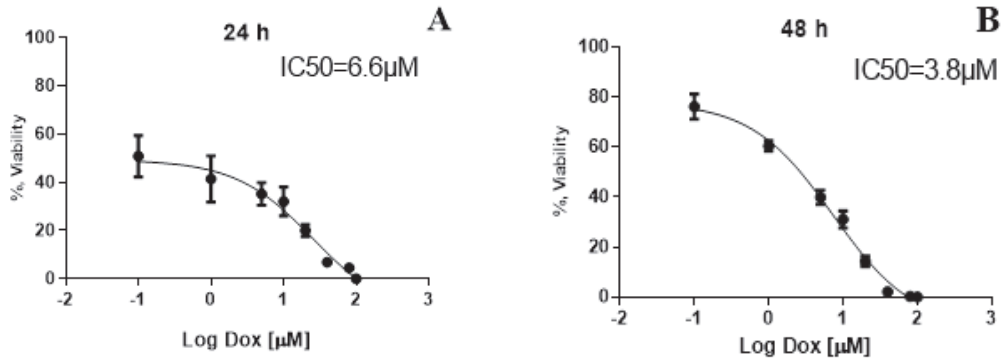

Fig. 1. Cytotoxicity of DOX on H9c2 cell line for 24h (**A**) and 48h (**B**) of treatment

Effects of QR on H9c2 cell line

Our results show that the 24 h treatment with 0.25 μM DOX showed the following protective properties of QR (CAS Number: 117-39-5, ≥95% (HPLC), solid) at concentrations: 0.1 μM – 42.6%, 1 μM – 66.4%, 2.5 μM – 60.8%. Higher DOX concentration – 1 μM determined a higher degree of toxicity for the same time period. Hence, the observed decrease in QR protective property values for the same concentration interval: 0.1 μM – 23.66%, 1 μM – 30.48%, 2.5 μM – 40.52% (Figure 2A and 2B).

For the continuous 48h damage with DOX 0.25 μM, at the same concentration interval, the values of protection were significantly lower as follows: 0.1 μM – 15.78%, 1 μM – 28.28%, 2.5 μM – 32.91%. It was observed that as the concentration of QR increased up to 2.5 μM, the protective effect increased, but then dropped sharply (Figure 2A, 2B, 2C and 2D).

Results show that QR induces cardioprotection against oxidative stress-induced cell death and cardiotoxicity after 24 h and prolonged exposure.

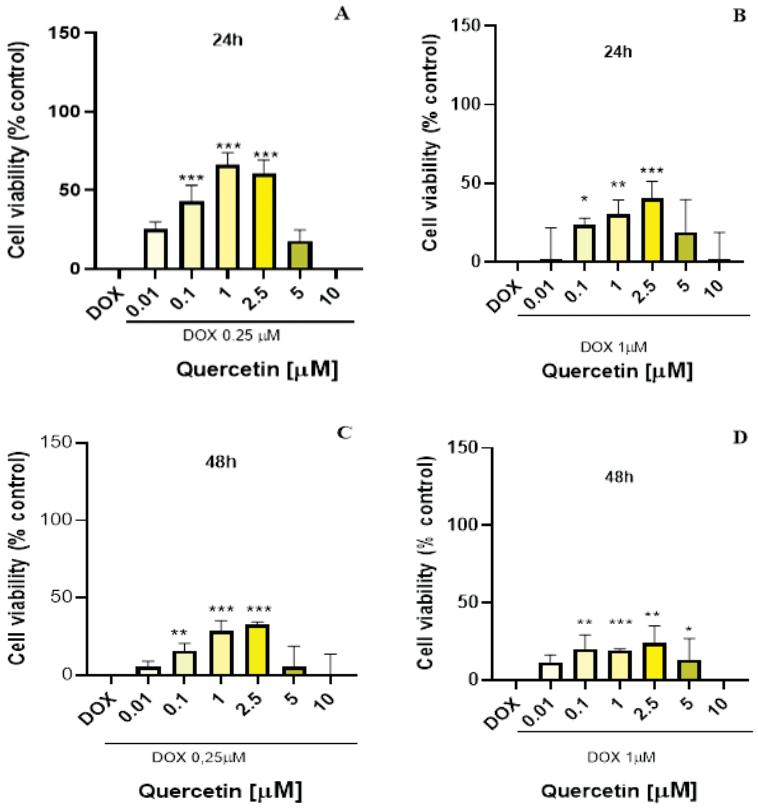

Fig. 2. Protective effects of QR (0.01-10 μM) at 24 and 48 h in a model of DOX-induced damage (0.25 μM and 1 μM) in the H9c2 cell line. All experiments were performed in triplicate, and results are expressed as mean ± standard deviation (SD), with n = 6. Statistical analysis was conducted using one-way ANOVA followed by Dunnett's post hoc test. GraphPad Prism version 8 (GraphPad Software, Inc., La Jolla, CA, USA) was used for the statistical evaluation. Differences were considered statistically significant at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***)

Effects of CRC on H9c2 cell line

Our experiment was conducted in a relatively low concentration range, where the potential protective properties of CRC (0.01 μM, 0.1 μM, 1 μM, 2.5 μM, 5 μM, 10 μM) in an in vitro model of DOX induced cardiotoxicity (0. 25 and 1 μM, after 24 and 48 h of treatment) and showed that the protective properties of CRC, with significant values, were manifested in the 24-h injury with 0.25 μM DOX. It was observed that as the concentration of CRC increased up to 2.5 μM, the protective effect also increased but then dropped sharply (Figure 3A and 3B). In the 48-h injury and DOX concentration of 1 μM, the significant values (14.85%, 30.06%, 23.93%) were observed at 0.1 μM, 1 μM and 2.5 μM concentrations. The peak in protective properties was observed at CRC concentration 1μM, followed by a decline (Figure 3A, 3B, 3C and 3D).

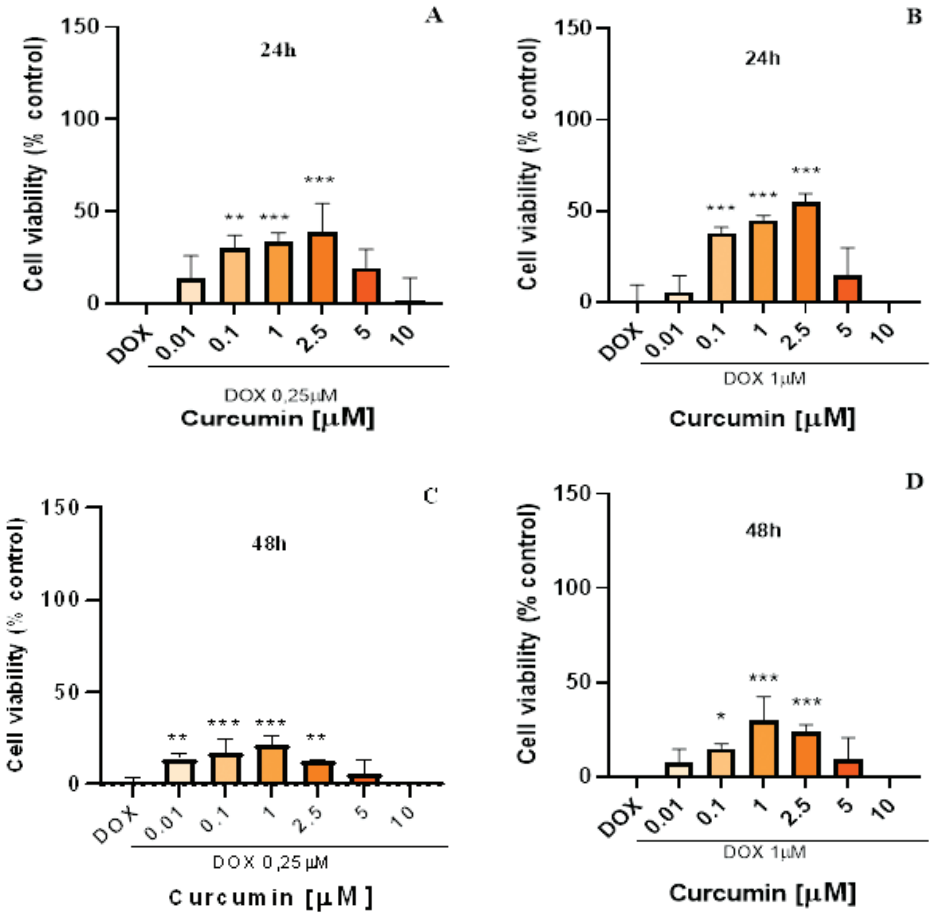

Fig. 3. Protective effects of CRC (0.01–10 μM) at 24 and 48 h in a model of DOX-induced damage (0.25 μM and 1 μM) in the H9c2 cell line. All experiments were performed in triplicate, and results are expressed as mean ± standard deviation (SD), with n = 6. Statistical analysis was conducted using one-way ANOVA followed by Dunnett's post hoc test. GraphPad Prism version 8 (GraphPad Software, Inc., La Jolla, CA, USA) was used for the statistical evaluation. Differences were considered statistically significant at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***)

Effects of RES on H9c2 cell line

The results of the experiment conducted by us, according to the relevant protocol, demonstrate that the 24 h injury with 0.25 μM DOX shows the protective properties of RES, with significant values, at the concentrations: 0.1 μM – 24.25%, 1 μM – 36.79%, 2.5 μM – 29.25%. DOX treatment at 1 μM concentration for 24 h demonstrated that this concentration is too high and damages cells. Accordingly, no protection was observed in this case (Figure 4A and 4B). However, in the 48 h damage with DOX at the low concentration (0.25 μM), protection was observed only at two of the RES concentrations – 0.1 μM – 25.69% and 1 μM – 29.12%. At the same duration of DOX damage in the high concentration – 1 μM it was observed that RES did not exhibit any protecting properties, similar to DOX treatment at 1 μM for 24 h (Figure 4A, 4B, 4C and 4D).

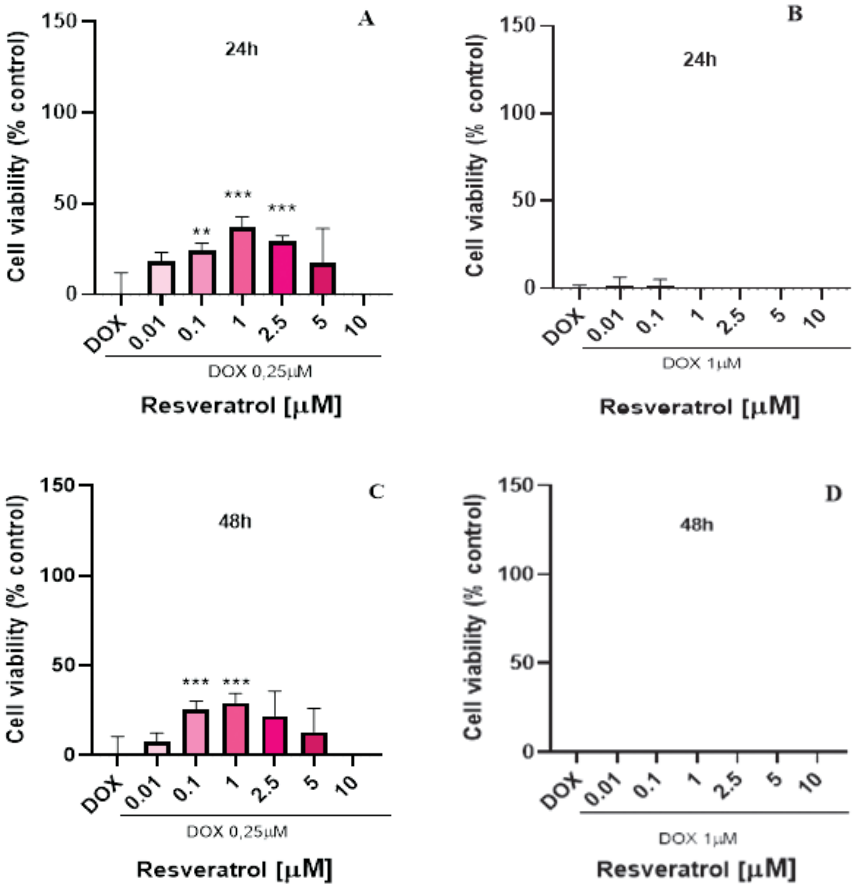

Fig. 4. Protective effects of RES (0.01–10 μM) at 24 and 48 h in a model of DOX-induced damage (0.25 μM and 1 μM) in the H9c2 cell line. All experiments were performed in triplicate, and results are expressed as mean ± standard deviation (SD), with n = 6. Statistical analysis was conducted using one-way ANOVA followed by Dunnett's post hoc test. GraphPad Prism version 8 (GraphPad Software, Inc., La Jolla, CA, USA) was used for the statistical evaluation. Differences were considered statistically significant at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***)

DISCUSSION

The primary adverse effect of doxorubicin (DOX) administration is the development of cardiomyopathy and heart failure. It has been widely suggested that the generation of reactive oxygen species (ROS) plays a central role in mediating DOX-induced cardiotoxicity [51-54]. In our study, we observed a decrease in DOX-induced cytotoxicity in rat H9c2 cardiomyoblast cells when antioxidants were administered sequentially after DOX treatment in vitro. Natural phenolic compounds have previously demonstrated efficacy in protecting against DOX-induced cardiotoxicity [48, 55, 56].

In this investigation, we employed an experimental design aimed at effectively evaluating the in vitro protective effects of antioxidants on H9c2 cell viability following DOX exposure. We established a model of DOX-induced toxicity by treating H9c2 cells with two concentrations of DOX (0.25 μM and 1 μM) for either 24 or 48 hours. Following DOX treatment, cells were exposed to antioxidants for an additional 24 hours, and cell viability was assessed using the MTT assay. This sequential treatment strategy not only demonstrated the cytotoxic effects of DOX but also provided a detailed evaluation of the potential protective roles of antioxidants under varying conditions of exposure.

Over the years, quercetin (QR), curcumin (CRC), and resveratrol (RES) have been extensively studied in various in vitro models for their cardioprotective effects [27, 57-59]. This study applies a modified protocol to further investigate the cardioprotective properties of these natural antioxidants, with particular focus on their potential cardiovascular benefits under DOX-induced oxidative stress conditions.

In our experiments, QR significantly improved cell viability and exhibited strong protective effects in vitro. H9c2 cells were treated with increasing concentrations of QR (0.1, 1.0, and 2.5 μM) for 24 and 48 hours

to evaluate its ability to protect against DOX-induced damage. Our results showed that QR enhanced cardiomyocyte survival against oxidative stress-induced cell death at both time points. The most pronounced protective effect was observed at a QR concentration of 1 μM following 24 hours of treatment with 0.25 μM DOX, where QR preserved 66.4% of cell viability. However, during prolonged 48-hour exposure to 0.25 μM DOX, a reduction in the protective effects of QR was noted. Increasing QR concentration to 2.5 μM restored some of the protective effects, although at concentrations higher than 2.5 μM, protection sharply declined.

It is likely that QR exerted its cardioprotective effects by reducing intracellular ROS production [60], a mechanism supported by previous findings [61, 62]. Similar antioxidant-mediated protective effects have been reported across various cellular models [63]. Nevertheless, additional mechanisms beyond direct ROS scavenging may contribute to QR's cardioprotective actions. QR has been shown to induce phase II detoxification enzymes, increase total glutathione (GSH) content, and elevate the expression of antioxidant enzymes such as gamma-glutamylcysteine ligase, glutathione S-transferase, and NAD(P)H:quinone oxidoreductase [64, 65]. Moreover, flavonoids like QR are known to modulate intracellular signaling pathways, including protein kinases and lipid kinases [66, 67]. For example, Liu et al. demonstrated that QR modulates the ERK and Akt pathways and reduces caspase-3 activity, thereby providing additional cardioprotective effects through modulation of apoptotic and survival signaling [68].

We also evaluated the protective effects of CRC across a concentration range (0.01–10 μM) under the same experimental conditions. CRC exhibited significant cardioprotective effects, particularly after 24-hour exposure to 0.25 μM DOX. As with QR, the protective efficacy of CRC increased with rising concentrations up to 2.5 μM, followed by a sharp decline at higher concentrations. Differences in the protective profiles of QR and CRC may stem from distinct mechanisms of action. For example, Zhu et al. reported that CRC mitigates hypoxia/reoxygenation-induced injury in H9c2 cells by downregulating the Notch signaling pathway [69].

Additionally, our results showed that RES provided notable protection following 24-hour exposure to 0.25 μM DOX, achieving a protective effect of 36.79%. However, at a higher DOX concentration (1 μM), RES did not demonstrate significant protection, suggesting that its cardioprotective effects may be limited under conditions of more severe oxidative stress. Previous studies have suggested that RES alleviates DOX-induced cytotoxicity by modulating the E2F1/mTORC1 and E2F1/AMPKα2 pathways, promoting autophagy and reducing apoptosis [59].

The differences observed among the tested antioxidants in terms of their cardioprotective potential are influenced by factors such as bioavailability, metabolism, and the specific molecular pathways they target. QR and CRC, for instance, undergo extensive metabolism and conjugation, which may affect their bioactivity. RES follows distinct metabolic pathways, which could explain its variable protective efficacy under different experimental conditions. Additionally, the three antioxidants act on different intracellular signaling networks. QR is primarily associated with antioxidant and anti-inflammatory effects, CRC with modulation of inflammation and signaling pathways, and RES with broader metabolic and stress response regulation.

Understanding these differences is crucial for optimizing therapeutic strategies to prevent or mitigate DOX-induced cardiotoxicity. Each antioxidant may offer specific advantages depending on the severity of oxidative injury and the desired therapeutic outcome.

Further studies are warranted to explore the complex interplay of multiple signaling pathways modulated by QR, CRC, and RES, which may contribute to both their cardioprotective and potential cytotoxic effects. The findings presented in this study underscore the potential applications of these natural compounds in the prevention and management of DOX-induced cardiotoxicity.

CONCLUSION

An in vitro model of DOX-induced cardiotoxicity was successfully established using the rat cardiomyoblast cell line H9c2. Within this model, the protective effects of the natural antioxidants quercetin (QR), curcumin (CRC), and resveratrol (RES) were demonstrated. Among these, QR and CRC exhibited the most pronounced cytoprotective effects, underscoring their potential as promising candidates for the prevention of DOX-induced cardiotoxicity. The protective actions of QR are likely attributed to its enhanced cellular uptake, strong antioxidant properties, and its modulation of key signaling pathways involved in inflammation, apoptosis, and cellular stress responses. Similarly, CRC's efficacy appears to derive from its potent anti-inflammatory activity, regulation of intracellular signaling cascades, and ability to mitigate oxidative stress and apoptosis. This study provides new insights into the differential cardioprotective efficacy of these antioxidants, particularly highlighting

the significant effects of QR and CRC at lower DOX concentrations. Overall, the findings support the potential use of QR and CRC as adjunctive therapies to mitigate anthracycline-induced cardiotoxicity, offering a promising strategy for enhancing the safety of chemotherapy regimens.

Abbreviations used: *Doxorubicin – DOX, Reactive oxygen species – ROS, Quercetin – QR, Curcumin – CRC, Resveratrol – RES, Cannabidiol – CBD*

Conflict of Interest Statement: *The authors declare no conflicts of interest related to this work.*

Ethical statement: *This study has been performed in accordance with the ethical standards as laid down in the Declaration of Helsinki.*

Funding: *This study was financed by the Medical Science Council of the Medical University of Sofia under the project "Young Researcher 2024", Decision No. 107/ 29.05.2024.*

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