Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ

Cukurova Med J 2025;50(3):872-882
DOI: 10.17826/cumj.1717960

RESEARCH

Protective effects of engeletin on doxorubicin-induced cardiotoxicity via NF-ϰB/iNOS and Cyt-c/CASP-3 signaling pathways

Engeletin'in NF-ϰB/iNOS ve Cyt-c/CASP-3 sinyal yolları aracılığıyla doksorubisin kaynaklı kardiyotoksisiteye karşı koruyucu etkileri

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Abstract

Purpose: The goal of this study was to assess the effects of Engeletin (ENG) on apoptosis via the Cytochrome c/ Caspase 3 (Cyt-c/CASP-3) pathway, fibrosis via the Transforming Growth Factor Beta 1 (TGF-β1) pathway, and oxidative status via the Nuclear Factor kappa-light-chain-enhancer of activated B cells/ Inducible Nitric Oxide Synthase (NF-ϰB/iNOS) signaling cascade, in doxorubicin (DOX)-induced cardiotoxicity.

Materials and Methods: The study sample included five groups: Control, DOX (1 μM), DOX + ENG 10 μM, DOX + ENG 20 μM, and DOX + ENG 40 μM. Gene expression levels in the proinflammatory, apoptotic, and fibrotic signal cascades were quantified by real-time reverse transcription-quantitative polymerase chain reaction analysis. Oxidative stress parameters were determined by spectrophotometric analysis.

Results: Data demonstrate that ENG substantially improved H9c2 cell viability, diminished lactate dehydrogenase (LDH) levels (52%), and attenuated DOX-induced ROS generation. Furthermore, ENG down-regulated proinflammatory cytokines and inflammatory enzymes through NF-ϰB inactivation. The data also showed that ENG inhibited cardiomyocyte apoptosis by downregulating Cyt-c, CASP-3, and B-cell lymphoma 2/ Bcl-2-associated X protein (Bcl2/BAX) expression in the apoptotic pathway.

Conclusion: These observations suggest the cardioprotective effect of ENG on DOX-induced cardiotoxicity by attenuating oxidative stress, cardiomyofibrosis, and apoptosis.

Keywords: Engeletin, doxorubicin, cardiotoxicity, apoptosis

Öz

Amaç: Bu çalışmanın amacı, Engeletin'in (ENG) Sitokrom c/ kaspaz 3 (Cyt-c/CASP-3) yoluyla apopitoz, Transforme Edici Büyüme Faktörü Beta 1 (TGF-β1) yoluyla fibrozis ve Doksorubisin (DOX) kaynaklı kardiyotoksisitede Nükleer Faktör Kappa B/ İndüklenebilir Nitrik Oksit Sentaz (NF-ϰB/iNOS) sinyalleme kaskadı yoluyla oksidatif durum üzerindeki etkilerini değerlendirmektir.

Gereç ve Yöntem: Çalışma beş gruptan oluşmaktadır: Kontrol, DOX (1 μM), DOX + ENG 10 μM, DOX + ENG 20 μM ve DOX + ENG 40 μM. Proinflamatuvar, apopitotik ve fibrotik sinyal kaskadlarındaki gen ekspresyon seviyeleri gerçek zamanlı ters transkripsiyon-kantitatif polimeraz zincir reaksiyonu analiz ile kantifiye edildi. Oksidatif stres parametreleri spektrofotometrik analiz ile belirlendi.

Bulgular: Bulgular ENG'nin kardiyomiyosit (H9c2) hücre canlılığını önemli ölçüde iyileştirdiğini, laktat dehidrogenaz (LDH) seviyelerini azalttığını (%52) ve DOX kaynaklı reaktif oksijen türleri (ROS) oluşumunu zayıflattığını göstermektedir. Dahası, ENG NF-ϰB (DOX: 2.22±0.09; DOX+ENG 10: 2.00 ±0.02; DOX+ENG 20: 1.80±0.06; DOX+ENG 40:1.23±0.05), inaktivasyonu yoluyla proinflamatuvar sitokinleri ve inflamatuvar enzimleri aşağı düzenledi. Veriler ayrıca ENG'nin apopitotik yolda Cyt-c, CASP-3, ve B-hücre Lenfoma 2/ Bcl-2 İlişkili X Proteini (Bcl2/BAX) (ekspresyonunu aşağı düzenleyerek kardiyomiyosit apopitozunu engellediğini gösterdi.

Sonuç: Genel olarak, bu gözlemler ENG'nin oksidatif stresi, kardiyomiyofibrozu ve apopitozu zayıflatarak DOX kaynaklı kardiyotoksisite üzerinde kardiyoprotektif etkisi olduğunu göstermektedir.

Anahtar kelimeler: Engeletin, doksorubisin, kardiyotoksisite, apopitozis

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Received: 16.06.2025 Accepted: 15.08.2025

INTRODUCTION

Cancer mortality has steadily dropped for two decades, but it is still one of the leading global killers[1]. While advances in diagnostic strategies and new anticancer drugs have improved the longevity of cancer patients, numerous agents have severe side effects, including short- and long-term cardiotoxicity. While anthracycline derivatives such as doxorubicin (DOX) are effective in cancer treatment, they pose a significant risk of side effects such as cardiotoxicity[2,3].

DOX is most widely used as an effective chemotherapeutic agent for several cancers. The dose-dependent toxic effect of DOX on cardiomyocytes, however, is a major concern[3]. A major obstacle to utilizing DOX in clinical settings is its cardiotoxicity. The heart damage caused by DOX is marked by irreversible deterioration of the heart muscle and the development of congestive heart failure[4,5]. The exact cause of DOX-induced cardiotoxicity is not completely known. Still, growing evidence suggests that the production of free radicals and heart muscle cell death play crucial roles in this process[6]. DOX-induced cardiotoxicity primarily occurs through generating reactive oxygen species (ROS), which can trigger oxidation[7]. Moreover, previous research has linked ROS production with the programmed cell death of cardiomyocytes[2,8]. Studies have demonstrated that increasing the levels of enzymes like superoxide dismutase (SOD) and catalase can mitigate DOX-induced cardiotoxicity[2,6]. These findings underscore the significance of ROS production and apoptosis in DOX-induced cardiotoxicity. Consequently, reducing ROS production and preventing apoptotic cell death could significantly alleviate DOX-induced cardiotoxicity. Therefore, developing strategies to prevent DOX-induced cardiotoxicity would greatly enhance the quality of life for cancer patients.

Rat embryonic cardiomyoblast (H9c2) cells originated as a free animal substitute for adult cardiomyocytes that fail to proliferate. Additionally, their origin from embryonic rat cardiac ventricular muscle is clinically relevant. The similarities of H9c2 cells with adult cardiomyocytes, including morphology, protein expression, signaling, and hypertrophy-associated characters, have been demonstrated in the literature[9,10].

Nowadays, research on natural and plant-derived components has gained importance in reducing the side effects of chemotherapeutic drugs, such as DOX. Flavonoid species, in particular, attract attention. Flavonoids are compounds found naturally in plants and have antioxidant properties[11]. Studies show that flavonoids play a potential role in reducing DOX-induced cardiotoxicity. Flavonoids are suggested to attenuate the cardiotoxic effects of DOX by reducing oxidative stress caused by reactive oxygen species. In particular, the anti-inflammatory and anti-apoptotic properties of flavonoids can also contribute to the protection of heart tissue. For instance, some flavonoids are reported to have protective activities by preserving mitochondrial functions against DOX mitochondrial damage[12].

Engeletin (dihydrokaempferol 3-rhamnoside) (ENG) is a flavonol glycoside isolated from the leaves of *Engelhardia Roxburghiana*. Recent findings suggest ENG has important anti-inflammatory, anticarcinogenic, and antioxidant features[13,14]. It has attracted increasing interest in recent years due to its anti-inflammatory effect. ENG has been reported to inhibit lipopolysaccharide (LPS) and D-galactosamine-induced liver injury in mice[15]. ENG attenuated LPS-induced acute lung injury, as demonstrated by dramatically diminished levels of inflammatory cytokines in bronchoalveolar lavage fluid[16]. ENG displayed potent anti-inflammatory activities in mouse models of LPS-induced endometritis by suppressing toll-like receptor 4-mediated activation of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-$\varkappa$B)[17]. The effects of ENG on DOX-induced cardiotoxicity, however, have not been investigated. The present study is the first to investigate ENG against DOX-induced cardiotoxicity on H9c2 cells. Here, this study investigated whether ENG could offer protective efficacy during DOX-induced cardiotoxicity, as well as whether ENG can attenuate oxidative stress by Nuclear Factor kappa-light-chain-enhancer of activated B cells/ Inducible Nitric Oxide Synthase (NF-$\varkappa$B /iNOS) signaling pathways and inhibit apoptosis by regulating the Cytochrome c/ Caspase 3 (Cyt-c/CASP-3) signaling pathway. By uncovering the dual modulatory effect of ENG on oxidative stress and apoptotic pathways in DOX-induced cardiotoxicity, this study not only fills a critical gap in the literature but also lays the groundwork for future in vivo and clinical investigations of flavonoid-based cardioprotective strategies in oncology. We

hypothesize that ENG protects H9c2 cells from DOX-induced cardiotoxicity by attenuating oxidative stress via the NF-ϰB/iNOS pathway and inhibiting apoptosis through the Cyt-c/CASP-3 pathway.

MATERIALS AND METHODS

Cell culture and treatment

The present study utilized the H9c2 cell line obtained from the American Type Culture Collection (ATCC). The cells stored in a Cryotube within a liquid nitrogen tank were thawed in a water bath at 37°C for brief dissolution. The resulting soluble cells were transferred to a T75 cm2 flask. Cultivation of the H9c2 cell line was performed in a standard suitable medium supplemented with 20% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin-Amphotericin B (PSA), and 2 mmol Glutamine within a 95% humidity, 5% Carbon Dioxide (CO_2), and 37°C incubator. All compounds were dissolved in the cell culture medium. Following a 24-hour treatment period with DOX (1 μM)[18] for 30 minutes, various concentrations of ENG (572-31-6 - sigma) at 10, 20, and 40 μM were administered[13]. The investigation was divided into five groups: Control, DOX (1 μM), DOX + ENG 10, DOX + ENG 20, and DOX + ENG 40.

Procedure

This study did not involve human participants or animals and was conducted exclusively using H9c2 cells obtained from ATCC. Therefore, ethical approval was not required in accordance with institutional and international guidelines.

All experimental procedures were carried out at the Molecular and Cell Culture Laboratory Department of Pharmacology, Kastamonu University, under the standard laboratory practices and data integrity principles of the institution. All cell culture, treatments, and assays were performed by the contributing authors, who were trained in relevant techniques by the department. This study was supported by the Scientific Research Projects Coordination Unit of Kastamonu University under project number: KÜBAP-01/2022-26.

Lactate dehydrogenase (LDH) assessment

Cells were plated with 100 μl of a cell suspension ($5x10^3$ cells/well) in 96-well plates and subjected to treatments upon reaching 50% confluency. Initially, doxorubicin was administered, and then ENG doses were administered after a half-hour interval, with concentrations of 10, 20, and 40 μM. LDH release measurements were performed on both control and sample wells using an Enzyme-Linked Immunosorbent Assay (ELISA) reader to measure absorbance, employing the relative LDH assay kit (Roche, Cat. No. 04 744 926 001, Mannheim, Germany) following the manufacturer's instructions.

Evaluation of SOD Activity and levels of malondialdehyde (MDA) and reduced glutathione (GSH)

Cells were seeded at a rate of 200.000/well in plates and incubated at 37°C in a humidified environment containing 5% CO_2. They were removed from the plates by scraping. Cell lysates were prepared in a homogenate buffer and homogenized with a Tissue Lyser. Subsequently, the samples were subjected to centrifugation in accordance with the instructions provided in the kit. SOD, MDA, and GSH levels were measured from each sample by means of manual measurement methods to conduct biochemical studies. Concentrations of protein were determined using commercial protein standards according to the Lowry method.

SOD activities were measured by the Winterbourn et al. method[19]. SOD activities are calculated based on the change in absorbance values resulting from the reduction of nitroblue tetrazolium used as a substrate. MDA and GSH levels were measured using the method reported by Tangjitjaroenkun et al[20]. The spectrophotometric measurement method was conducted using an ELISA reader (Epoch Microplate Spectrophotometer, BioTek, USA). Each sample was tested in triplicate. The chemicals for all the biochemical assays were purchased from Sigma.

Real-time polymerase chain reaction (PCR) analyzes

Analysis of gene expressions in cell lines

Cells were seeded at 200,000 cells per well in plates and subsequently cultivated at 37°C in a humidified environment with 5% CO_2. Following incubation, the cells were detached from the 6-well plates using trypsinization and subsequently lysed in the Tissue Lyser II (Qiagen) device. Specifically, 350 μl of RNA Lysis Tissue buffer (RLT) buffer was added to 1x105 cells for homogenization. Ribonucleic Acid (RNA) extraction procedures were then performed using the

QIAcube RNA isolation device following the manufacturer's guidelines.

Reverse transcriptase reaction and cDNA synthesis

The synthesis used the High-Capacity Complementary DNA (cDNA) Reverse Transcription Kit enzyme to work on total RNA. Each reaction utilized 10µl of RNA, and the cDNA synthesis was carried out using the Veriti 96 Well Thermal Cycler (Applied Biosystems), following specific temperature parameters. The quantity of cDNA synthesized was assessed using nanodrop spectrophotometry (EPOCH Take3 Plate, Biotek), and the resulting samples were stored at -20°C.

Real-time quantitative PCR

The genes NF-ϰB (Rn00595794_m1), iNOS (NOS-2) (Rn00561646_m1), Cyt-c (Rn00822088_g1), CASP-3 (Rn00563902_m1), Tumor Necrosis Factor alpha (TNF-α) (Rn01525859_g1), Interleukin-1 beta (IL-1β) (Rn00580432_m1), Interleukin-6 (IL-6) (Rn00561420_m1), BAX (Rn02532082_g1), Bcl-2 (Rn99999125_m1) were assessed using the TaqMan Gene Expression Master Mix kit. Amplification and quantification were performed using the StepOne Plus Real-Time PCR System (Applied Biosystems). For each reaction with 100 ng of cDNA, the genes NF-ϰB, iNOS, Cyt- c, CASP-3, TNF-α, IL-1β, IL-6, BAX, and Bcl-2, along with β-actin (ACTB) as the housekeeping gene (Rn00667869_m1), were pipetted. The PCR ran for 40 cycles, with Ct values automatically converted to ΔCt within the system. Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS) 20.00 software.

Statistical analysis

All data are presented as mean ± standard deviation (SD). The normality of the data distribution was assessed for each group using the Shapiro–Wilk test, and $p > 0.05$ was considered to indicate a normal (Gaussian) distribution. Skewness values were also examined to verify normality. Homogeneity of variances was evaluated with Levene's test, and $p > 0.05$ was accepted as an indication of homogeneous variances. For comparisons among multiple groups, one-way analysis of variance (ANOVA) was applied, followed by Tukey's multiple comparison test to determine pairwise differences. These analyses were performed separately for each parameter measured, including cell viability (MTT assay), LDH activity, antioxidant enzyme activities and levels (SOD, MDA, GSH), and gene expression levels from Real-time Quantitative PCR (NF-ϰB, iNOS, Cyt-c, CASP-3, TNF-α, IL-1β, IL-6, BAX, and Bcl-2. All statistical tests were conducted using GraphPad Prism 9 software (GraphPad Software, Inc., San Diego, CA, USA), and results were considered statistically significant at $p<0.05$.

RESULTS

LDH is an enzyme commonly used as a marker of cell damage. The observation of DOX-induced LDH leakage indicated a protective effect of ENG, which was notably greater compared to untreated cells, in contrast to the control group. Specifically, LDH leakage decreased by 52% in the ENG-treated group when cells were exposed to a concentration of ENG 40 µM and incubated with a DOX concentration of 1 µM (Figure 1). To investigate the potential of ENG to protect cardiomyoblasts from DOX-induced cytotoxicity over time, H9c2 cells were treated with DOX at a concentration of 1 µM. Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 2)

Significant cytotoxic effects were noted in the groups treated with DOX compared to the control group. Notably, considerable toxicity was evident in the DOX-treated group, particularly at the 72-hour mark, indicating a time-dependent effect. Conversely, the application of ENG demonstrated a protective effect against DOX-induced cytotoxicity in H9c2 cells compared to the respective control groups, with the protection showing a time-dependent pattern.

SOD activity, GSH, and MDA levels were assessed in lysates from the H9c2 cell line. Figure 3 illustrates the impact of DOX/ENG on antioxidant enzyme activities. DOX administration resulted in notable reductions in both SOD and GSH activities. Additionally, an elevation in MDA activities, a recognized marker of oxidative stress, was observed in H9c2 cells. Treatment with ENG at a concentration of 40 µM led to a significant increase in SOD activity and GSH levels, bringing them closer to levels observed in the control group. Moreover, a decrease in MDA levels was evident alongside these results. The effect closely resembled that of the control group, indicating that ENG effectively mitigated the oxidative damage induced by DOX. These findings unequivocally demonstrated the antioxidant properties of ENG.

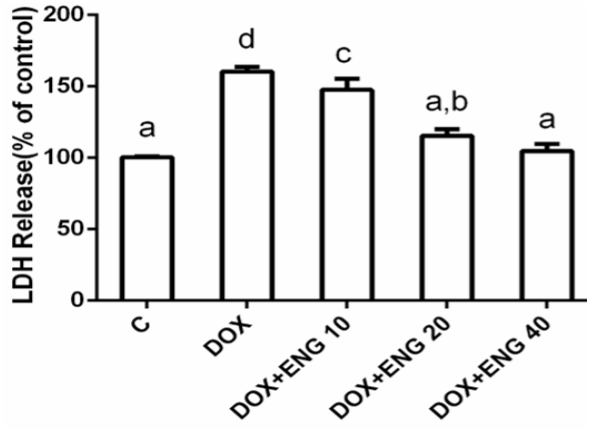

Figure 1. Effect of ENG on LDH release levels in Doxorubicin-induced cardiotoxicity.

(a, b, c, d; Different letters in the same column represent a statistically significant difference (a: significantly different from the DOX group; b: significantly different from the DOX+ENG 10 group; c: significantly different from the control group; d: significantly different from all other groups, $p<0.05$) (DOX: Doxorubicin; ENG: Engeletin; LDH: Lactate Dehydrogenase)

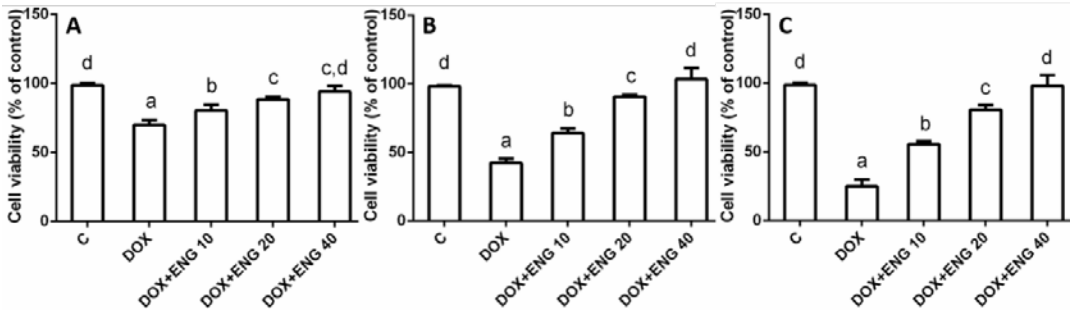

Figure 2. Effect of Engeletin on cell viability in Doxorubicin-induced cardiotoxicity.

(a, b, c, d; Different letters in the same column represent a statistically significant difference (a: significantly different from the control group; b: significantly different from the DOX group; c: significantly different from the DOX+ENG 10 group; d: significantly different from the DOX+ENG 20 group, $p<0.05$). (DOX: Doxorubicin; ENG: Engeletin).

In assessing the efficacy of ENG in DOX-induced H9c2 cells, we examined the expression levels of inflammatory parameters TNF-α, IL-1β, IL-6, TGF-β1, NF-ϰB, and iNOS mRNA in cDNAs derived from H9c2 lysates using real-time PCR. Figure 4 depicts the mRNA expression of TNF-α, IL-1β, IL-6, TGFβ1, NF-ϰB, and iNOS across different groups.

Compared to the healthy control group, the groups treated with DOX exhibited a significant increase in TNF-α, IL-1β, IL-6, TGF-β1, NF-ϰB, and iNOS mRNA expression. However, treatment with ENG 40, compared to the DOX group, led to a significant reduction in mRNA expression levels of TNF-α, IL-1β, IL-6, TGF-β1, NF-ϰB, and iNOS.

To evaluate the efficacy of ENG in DOX-induced cardiotoxicity, the expression levels of the apoptotic parameters Cyt-c, CASP-3, and Bcl2/BAX ratio mRNA were assessed in cDNAs extracted from H9c2 using real-time PCR. Figure 5 depicts the mRNA expression of Cyt-c, CASP-3, and Bcl2/BAX across the various experimental groups. Cyt-c and CASP-3 mRNA expression significantly increased in the DOX-treated groups compared to the healthy control group, whereas the Bcl2/BAX ratio decreased. However, treatment with ENG 40 significantly decreased Cyt-c and CASP-3 mRNA expression relative to the DOX-treated group while notably increasing the Bcl2/BAX ratio.

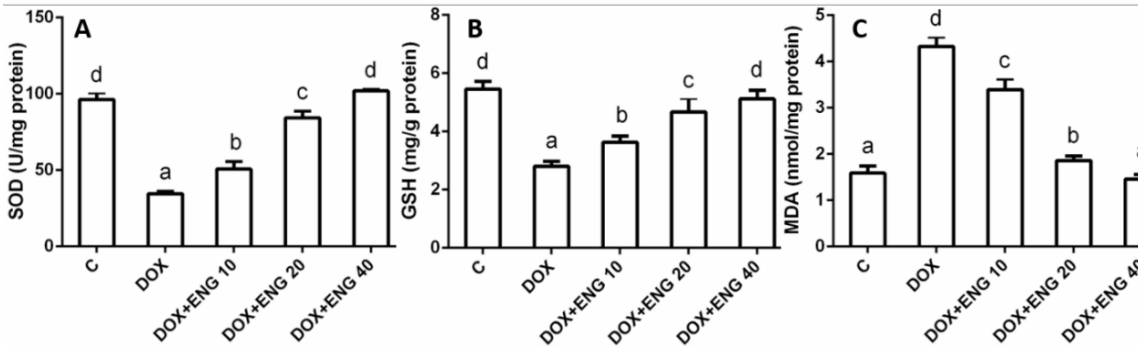

Figure 3. Effect of Engeletin on antioxidant marker levels in Doxorubicin-induced cardiotoxicity.

(a, b, c, d; Different letters in the same column represent a statistically significant difference (a: significantly different from the control group, b: significantly different from the DOX group, c: significantly different from the DOX+ENG 10 group, d: significantly different from the DOX+ENG 20 group, $p<0.05$). (DOX: Doxorubicin; ENG: Engeletin; GSH: Reduced Glutathione; MDA: Malondialdehyde; SOD: Superoxide dismutase).

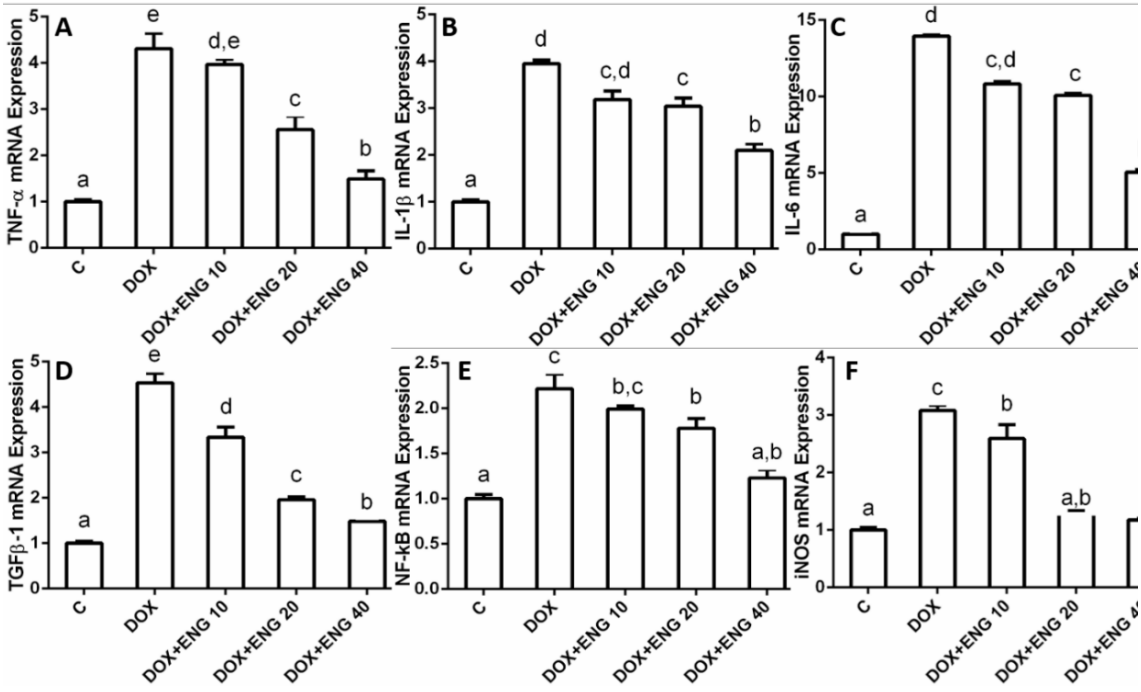

Figure 4. Effect of ENG on inflammation marker levels in cardiotoxicity with Doxorubicin.

(a, b, c, d; Different letters in the same column represent a statistically significant difference (a: significantly different from the DOX group; b: significantly different from the DOX+ENG 10 group; c: significantly different from the DOX+ENG 20 group; d: significantly different from the DOX+ENG 40 group; e: significantly different from the control group, $p<0.05$). (DOX: Doxorubicin; ENG: Engeletin; IL-1β: Interleukin-1 beta; TNF-α: Tumor Necrosis Factor alpha; IL-6: Interleukin-6; TGF-β1: Transforming Growth Factor Beta 1; NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells; iNOS: Inducible Nitric Oxide Synthase).

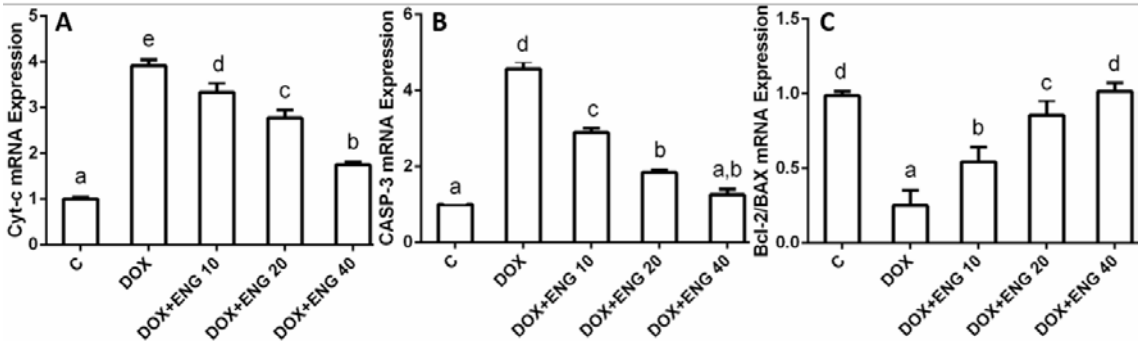

Figure 5. Effect of Engeletin on apoptosis marker levels in Doxorubicin-induced cardiotoxicity.

(a, b, c, d; Different letters in the same column represent a statistically significant difference (a: significantly different from the DOX group; b: significantly different from the DOX+ENG 10 group; c: significantly different from the DOX+ENG 20 group; d: significantly different from the DOX+ENG 40 group; e: significantly different from the control group, $p<0.05$). (Cyt-c: Cytochrome c; CASP-3: Caspase 3; Bcl2/BAX: B-cell lymphoma 2/ Bcl-2-associated X protein).

DISCUSSION

DOX, a representative drug from the anthracycline antibiotics, is a potent chemotherapeutic that is widely used in cancer treatment. Although DOX is an effective chemotherapeutic agent used in cancer treatment, its cardiotoxic side effects are a serious concern. DOX-induced cardiotoxicity can lead to damage and dysfunction of heart tissue, making it a limiting factor in treatment. In recent years, the search for new therapeutic strategies to reduce or prevent such cardiotoxic side effects has increased[6-8]. In particular, using compounds derived from natural sources is gaining more attention. Natural products such as flavonoids have antioxidant, anti-inflammatory, and cytoprotective properties. These properties may be effective in reducing the side effects caused by chemotherapeutic agents used in cancer treatment[12].

ENG is a flavonoid compound that possesses a multifaceted array of biological functions, encompassing antioxidant, anti-inflammatory, and cytoprotective properties[13,14]. In the cardiovascular system, recent reports have shown that ENG inhibits the oxidative stress pathway, and also reduces cardiac electrical remodeling and the ventricular fibrillation risk[21]. Another study showed that ENG inhibited NF-κB, signaling-mediated inflammatory responses, eventually attenuating atherosclerosis progression[22].

This study examined the protective effect of ENG against DOX-induced cardiotoxicity in vitro. The study findings showed that ENG can protect against cardiotoxicity by regulating the Cyt-c/CASP-3 pathway, modulating iNOS balance, and improving oxidative stress parameters.

Many biomarkers are used to predict toxicity, such as a cardiotoxic event. LDH is one of the enzymes used as a marker of cardiac toxicity. When the body is under normal conditions, LDH levels are low in serum and body fluids, but after the cell membrane is damaged, large amounts of LDH are released into the cells [23]. In our study, high LDH levels in the DOX group indicate a cardiotoxic event. A dose-dependent decrease in LDH levels was observed in ENG-treated groups. ENG decreased serum levels of myocardial damage markers such as LDH in an isoproterenol-induced cardiac remodeling study, and reduced isoproterenol-induced myocardial damage[21], which is parallel with the results of our research. High LDH activity is attributed to a significant increase in free radicals and the subsequent damage they cause to the cellular membrane.

Numerous studies have demonstrated that DOX-induced cardiotoxicity mechanisms are closely related to oxidative stress in myocardial cells. DOX administration can induce oxidative stress in myocardial cells by increasing free radical production. This can lead to lipid peroxidation, protein oxidation, and cell DNA damage[24]. Increased oxidative stress can impair the function of myocardial cells and contribute to cardiotoxicity[6]. Cells have intrinsic antioxidant systems to protect themselves against the harmful effects of ROS. These systems reduce ROS levels and increase the cell's ability to survive. Compared to other tissues, myocardium is more prone to oxidative stress, possibly due to low levels

of antioxidant enzymes[25]. Therefore, the oxidant-antioxidant balance is disrupted due to excessive ROS production and an impaired antioxidant defense system. In this context, preserving or enhancing antioxidant defense mechanisms may be a potential strategy for preventing or treating DOX-induced cardiotoxicity. In this study, we examined the role of important antioxidant parameters such as SOD, MDA, and GSH to understand the mechanisms underlying DOX-induced cardiotoxicity. SOD plays a critical role in reducing oxidative stress and protects cells by neutralizing ROS[24]. GSH is one of the main antioxidant defense mechanisms in cells. However, a decrease in SOD activity and GSH levels was observed due to DOX exposure, which may indicate increased oxidative stress and weakened protective mechanisms of cells[6]. In the present study, ENG caused a significant dose-dependent decrease in SOD activity and GSH levels. Furthermore, ENG exerted cardioprotective effects against DOX-induced cardiotoxicity in H9c2 cell lines by reducing MDA, a lipid or protein peroxidation biomarker, and restoring the various inflammation parameters in this study. Many studies have shown that flavonoids and their derivatives have cardioprotective activity against DOX in both in vitro and in vivo models, while myocardial antioxidant enzymes such as SOD and GSH increase, and oxidative stress markers such as MDA decrease[12,26]. Therefore, these results showed that ENG significantly alleviated DOX-induced myocardial oxidative damage.

In cardiotoxicity, many signals have been shown to trigger oxidative stress in conjunction with each other. Nitric oxide (NO) is one of the primary sources of DOX-induced oxidative stress. NO levels are increased in DOX-mediated cardiotoxicity. iNOS regulates the production of cardiac NO levels. The expression of iNOS is often associated with inflammation and malignant diseases. DOX administration increases iNOS transcription and protein expression[27]. Previous work confirmed the cardioprotective effect of iNOS inhibition in DOX-induced cardiotoxicity[28]. It was consistently found that DOX decreased iNOS expression in the study. It was also demonstrated that the protective effect of ENG on DOX-induced cardiotoxicity was mediated through the reduction of iNOS expression. The results of the present study also suggest that DOX-induced cardiotoxicity is associated with increased iNOS expression and elevated oxidative stress parameters.

In oxidative stress states, iNOS activation increases NO production, leading to effects that further increase cellular damage and inflammation[29]. This also activates the NF-κB signaling pathway, resulting in a mutually reinforcing inflammation and oxidative stress cycle. NF-κB is a transcription factor that plays an important role in cells. Internal and external stimuli activate and control several biological processes by regulating cellular responses. Activated NF-κB can increase gene expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) [30]. In our study, DOX increased the expression of NF-κB and related pro-inflammatory cytokines. According to the present study results, ENG prevented the occurrence and progression of cardiotoxicity in a dose-dependent manner by down-regulation of TNF-α, IL-6, and IL-1β in the NF-κB pathway. ENG inhibited the inflammatory response mediated by the NF-κB pathway and LPS-induced release of interleukin IL-1β in the previous study[31]. Another study confirmed that ENG could suppress phosphorylation of the NF-κB pathway in an in vitro sepsis model [32]. In a more recent study, Wei et al. demonstrated that ENG attenuated the inflammatory response in a Crohn's disease-like colitis model by inhibiting the TLR4–NF-κB signaling pathway[33]. Similarly, another study reported that ENG regulated microglial inflammation after spinal cord injury through modulation of the NF-κB pathway[34].

DOX treatment disrupts the balance in ROS and NO dynamics, causing oxidative damage to biological macromolecules and impairing cell integrity. This disrupts the regulation of Bcl-2/BAX expression in the apoptotic pathway, which leads to the release of Cyt-c, and causing cardiomyocyte apoptosis. The activation process continues with CASP-3, the end product of the apoptotic pathway[35]. In our study, ENG inhibited DOX-induced cardiomyocyte apoptosis by suppressing ROS and downregulating proinflammatory cytokines (TNF-α, IL-1β, IL-6) and inflammatory enzymes (iNOS)), and reducing Cyt-c expression through NF-κB inactivation. Furthermore, ENG inhibited cardiomyocyte apoptosis by downregulating Bcl2/BAX expression in the apoptotic pathway. One study revealed that ENG improved TNF-α-induced apoptosis and reduced ROS generation in chondrocytes[21]. Another study showed that ENG reduced the expression of inflammatory factors and apoptosis-related genes[36]. In accordance with the previously mentioned findings, another study demonstrated that ENG suppressed NF-κB-mediated inflammation,

antioxidant pathway, reduced oxidative stress markers (MDA, CAT, GSH, SOD), and attenuated epithelial apoptosis by restoring the balance between pro- and anti-apoptotic proteins (Bax/Bcl-2, c-caspase-3), thereby supporting the observation that ENG mitigates DOX-induced apoptotic signaling through regulation of oxidative stress and apoptosis-related proteins[37].

Recent studies have shown that oxidative stress promotes myocardial fibrosis and the evolution of cardiac electrical remodeling[38]. Dysregulated cardiomyocyte contraction increases ROS levels and promotes pro-fibrotic signal transduction, thus leading to oxidative damage and myocardial fibrosis[39]. TGF-β1, a key profibrogenic cytokine secreted by fibroblasts, drives fibrosis by promoting cell proliferation, cardiac apoptosis, and hypertrophy. Numerous reports indicated that DOX upregulates TGF-β1 and provided documented evidence for the role of the TGF-β1 signal cascade in DOX-induced cardiac fibrosis and cardiotoxicity[40]. Previous work has demonstrated the ability of ENG to treat pulmonary fibrosis by suppressing the expression of fibrosis-associated markers[41]. Another research indicated that ENG has a significant potential therapeutic impact on cardiac remodeling [21]. Our research showed that DOX-induced, upregulated TGF-β1 expression was downregulated by ENG treatment, indicating an anti-fibrotic effect.

One of the main limitations of this study is the lack of in vivo validation. While the current in vitro findings are promising, further studies using animal models are warranted to confirm the efficacy and safety of ENG in a physiological context. Additionally, although only TGF-β1 was analyzed in this study, future research should include additional fibrotic markers such as collagen I, collagen III, and α-SMA to provide a more comprehensive understanding of ENG's effects on fibrosis.

Furthermore, in the present study, we focused solely on ENG to investigate its effects in vitro. Future comparative studies with other flavonoids will be essential to determine whether ENG offers any specific advantages or shares similar properties with structurally related compounds.

Given the dual challenge of preserving DOX's potent anticancer activity while minimizing its cardiotoxic side effects, the study highlights ENG as a promising adjunct candidate. Future research should focus on evaluating whether ENG not only protects cardiac tissue but also enhances or supports the chemotherapeutic efficacy of DOX in a synergistic manner. Such investigations could open the door to combination strategies that maintain anticancer potency while significantly improving patient safety and tolerability. Ultimately, this approach may help bridge the gap between efficacy and toxicity in anthracycline-based cancer therapies**.**

In conclusion, this study demonstrates that ENG defends against DOX-induced myocardial injury by alleviating oxidative stress by inhibiting the NF-κB signaling pathway, preventing apoptosis by regulating the Cyt-c/CASP-3 pathway, and reducing fibrosis by down-regulating TGF-β1 expression. As a promising new therapeutic drug candidate, ENG offers a potential strategy for DOX-induced cardiotoxicity, as it is associated with multiple anti-apoptotic, antioxidant, and anti-inflammatory pathways.

Author Contributions: Concept/Design: İÇ; Data acquisition: İÇ; Data analysis and interpretation: SA; Drafting manuscript: BD; Critical revision of manuscript: SA; Final approval and accountability: İÇ, SA, BD; Technical or material support: İÇ, SA; Supervision: BD; Securing funding (if available): n/a.
Ethical Approval: This study did not involve human participants or animals. Therefore, ethical approval was not required in accordance with institutional and international guidelines.
Peer-review: Externally peer-reviewed.
Conflict of Interest: The authors declare that they have no conflict of interest.
Financial Disclosure: The authors declared no financial support.
Acknowledgement: This study was supported by the Scientific Research Projects Coordination Unit of Kastamonu University (KÜBAP-01/2022-26).

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