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ORIGINAL ARTICLE

The effect of ellagic acid on caspase-3/bcl-2/Nrf-2/NF-kB/TNF-α / COX-2 gene expression product apoptosis pathway: a new approach for muscle damage therapy

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Abstract

The goal of this study was to determine the protective role of ellagic acid (EA) against CCl_4-induced muscle injury in rats. In this study, 36 Wistar albino rats (n = 36, 8 weeks old) were used. The rats were divided into 4 groups and 9 rats were included each group. Groups: (i) control Group: standard diet; (ii) EA Group: standard diet + EA group; (iii) CCl_4 group: standard diet + CCl_4 group; (iv) EA + CCl_4 group: standard diet + EA + CCl_4. The animals were decapitated after 8 weeks, and their muscle tissues were received and investigated. In the muscle tissue, TNF-α, COX-2, Nrf-2, NF-kB, caspase-3 and bcl-2 expression levels were analyzed by the western blotting technique, lipid peroxidation was detected by MDA (malondialdehyde), and catalase and GSH levels were determined by a spectrophotometer. In our findings, in comparison to the CCl_4 group, in the EA + CCl_4 group, the Nrf-2 and caspase-3 protein expression levels, GSH and catalase activities increased, while the NF-kB, bcl-2, TNF-α and COX-2 protein expression levels and MDA levels decreased. These results suggest that EA reduces muscle tissue damage rate in rats and that EA may also be used as a potential drug to protect against muscle tissue damage in the future.

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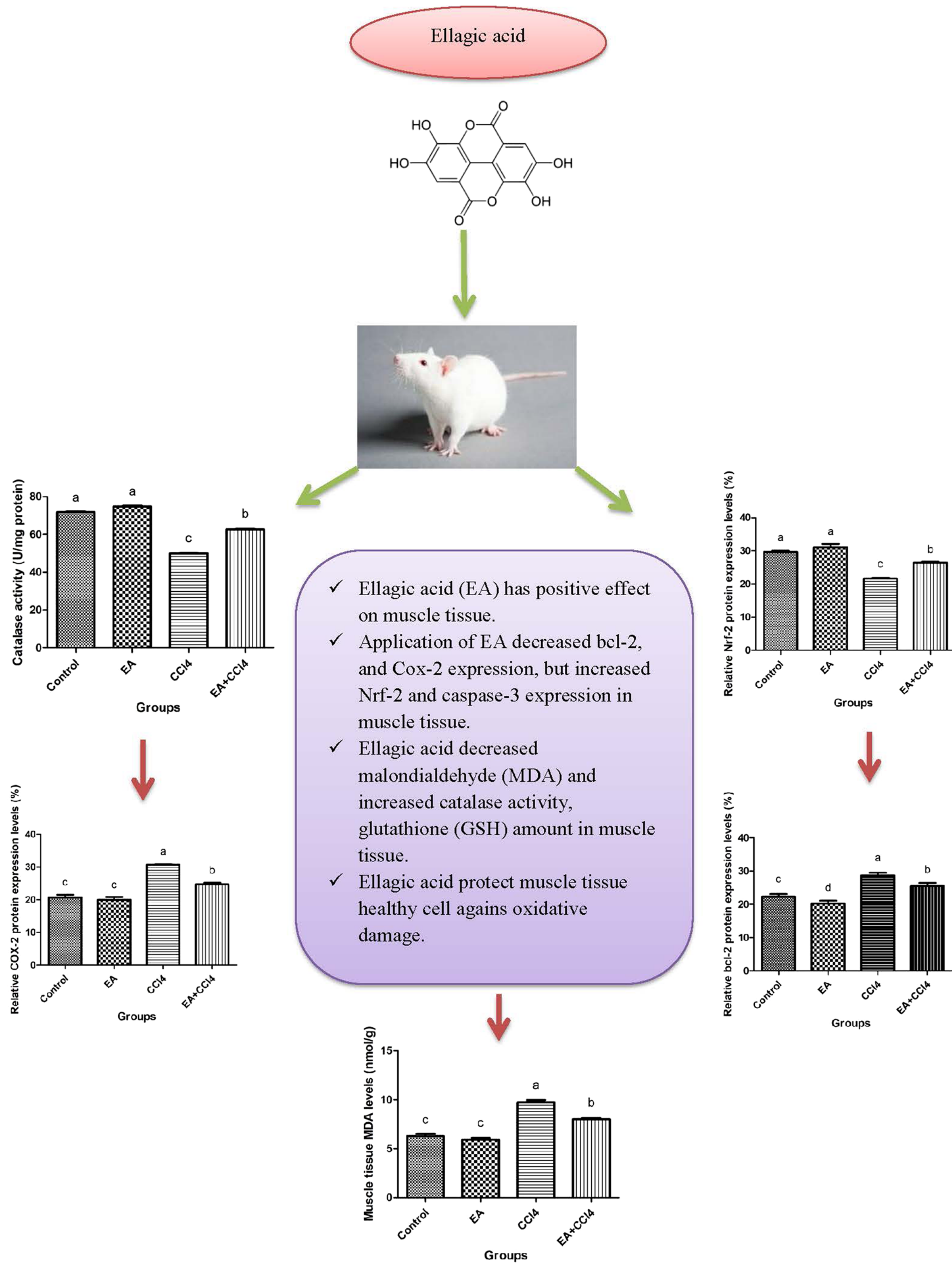
Graphic abstract



Keywords Bcl-2 · COX-2 · Ellagic acid · Muscle damage · NF-kB · Nrf-2 · TNF-α

Abbreviations

AO	Azoxymethane
CAT	Catalase activity
CCl_4	Carbon tetrachloride
EA	Ellagic acid
EDTA	Ethylenediaminetetraacetic acid
ETs	Ellagitannins
FUDAM	Firat University Experimental Animals Research Institute
HaCaT	Human keratinocyte cell
HEPP	Hesperidin
I/R	Ischemia/reperfusion
Ip	Intraperitoneal
MDA	Malondialdehyde
NF-Kb	Nuclear factor kappa B
O_3	Ozone
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulphonyl fluoride
PR1P	Prominin-1
RGC	Retinal ganglion cell
ROS	Reactive oxygen species
TGF-β1	Growth factor-β1
TNF-α	Tumor necrosis factor-α
TP	Tomato powder
TRF	Tocotrienol-rich fraction
COX-2	Cyclooxygenase -2
SDS	Sodium dodecyl sulphate
HBMVECs	Microvascular endothelial cells
VPF	Vascular permeability factor

Introduction

We think that the result of this study will bring important knowledge to the literature and we did not find any studies on the contents of this article. A muscle functions as an organ that regulates and secretes metabolism. Muscle and bone interact not only mechanically but also with secreted biochemical signaling molecules [1]. Carbon tetrachloride (CCl_4) is a strong carcinogenic agent that leads to production of reactive free radicals and initiates cell damage. CCl_4 has been shown to be a pulmonary toxic compound that causes deterioration of the alveolar septa by accumulation of countless neutrophils, fibroblasts and macrophages in blood vessels [2]. It is also a potent hepatotoxic agent commonly used to induce liver degeneration in vivo with oxidative stress [3]. Besides the liver, oxidative damage has been determined in the kidneys, brain, lung, testes and muscles. CCl_4 induces oxidative stress through free radical formation which causes tissue damage [4]. Under normal conditions, many internal and external stress factors constantly change the cellular balance. Antioxidants play an important role in protecting cells against these factors [5]. In recent studies, EA, which is known as a polyphenol compound with its antioxidant properties, has been found to play a protective role against lipid peroxidation and damage to skeletal muscles. It was expressed that the antioxidant effect of EA against skeletal muscle ischemia and reperfusion injury [6] inhibits vascular smooth muscle cell proliferation and prevents atheroma formation in streptozotocin-induced diabetic rats [7]. It was also found to significantly inhibit the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 induced by oxidized LDL [8]. Caspase-3 is a member of the caspase family. It has a central role on the apoptotic pathway [9].

Caspases, which are localized in the cytosol in the cell, are divided into two as initiator and effector caspases and are also called cysteine proteases. In apoptosis, they break down peptides especially from aspartate residues. The main task of DNA polymerase enzyme activity is to block the apoptosis of the cell to prevent cell damage [10]. Bcl-2 is an internal mitochondrial membrane protein. It is also one of the pro-apoptotic, anti-apoptotic or anti-autophagic proteins. It serves anti-apoptotic function [11]. The nuclear factor erythroid 2-associated factor 2 (Nrf-2) is an essential regulator for antioxidation. Its main function is to improve the expression of anti-oxidative genes such as HO-1 [12]. NF-kB is a nuclear transcription factor that arranges the expression of multiple genes that are critical for cellular apoptosis, proliferation, tumorigenesis, inflammation and various autoimmune diseases. It is a dimeric subunit complex of the NF-kB/Rel family [13]. It is a family of DNA-binding proteins that play a role in transcriptional regulation of many gene products. Activation of NF-kB may be induced in response to lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), interleukins, radiation and other stimulating agents [14]. Cyclooxygenase (COX) enzymes have two isoforms: COX-1 and COX-2. It is known that the COX-2 enzyme catalyzes conversion of arachidonic acid into prostaglandins, which play an important role in proliferation of cancer cells [15]. TNF-α is an inflammatory cytokine and has a role in initiation and growth of cancer. TNF-α modulates various aspects of cancer cell phenotypes such as cell proliferation, migration, invasion and metastatic potential [16].

Materials and methods

Preparation and application of carbon tetrachloride

CCl_4 was applied to the rats twice a week for 8 weeks intraperitoneally (1,5 ml/kg) syringed with olive oil at a rate of 1:3 [17].

Ellagic acid preparation and application to rats

100 mg of EA (A15722) was dissolved in 10 ml of DMSO (dimethyl sulfoxide) [18], after EA from this stock solution was administered to the animals intraperitoneally (10 mg/kg body weight) five times a week. The study continued for 8 weeks [19, 20].

In vivo experimental design

All animals were housed, cared and used experimentally according to the 'Guide for the Care and Use of Experimental Animals' approved by the Institutional Animal Ethics Committee, Firat University, Elazig (Registration Number: 27.02.2019/2019-04). 36 male Wistar albino (n = 36, 8 weeks) rats were used in this study, and the rats were distributed into four groups. The groups were:

Group (i) Control: feed with only standard diet,
Group (ii) CEA: standard diet + EA group (10 mg/kg, ip),
Group (iii) CCl_4: standard diet + CCl_4 group (1,5 ml/kg, ip),
Group (IV) EA + CCl_4: standard diet + CCl_4 (1,5 ml/kg, ip) + EA (10 mg/kg, ip).

Feed and water were given ad libitum for the process of the study. When work was finished, the rats were sacrificed and the muscle tissue was received and kept at − 80 °C until analysis.

Muscle tissue MDA analysis

0,5 gr muscle tissue samples were sliced into small sections after which they were homogenized in 4.5 ml, 1.15% KCl solution MDA analysis was carried out on this homogenate according to the method described by Ohkawa et al. [21] and some minor changes were made on this method [21]. The results were recorded as nmol/g muscle tissue MDA [17, 22].

Muscle tissue homogenization and SDS-PAGE and western blotting

Muscle tissue samples were cut into small sections and lysed in a lysis buffer (0.5 M Tris [pH 8], EDTA, β-Mercaptoethanol, Phenyl methyl sulfonyl fluoride [PMSF]) in a glass homogenizer. The cut tissue samples were centrifuged at 15,000 rpm for 45 min. The supernatant was collected and kept at − 80 °C until usage [17]. The protein samples of the muscle tissues were run on 12% gel via the SDS-PAGE method and transported to a nitrocellulose membrane. The samples were then incubated overnight at 4 °C with specific primary antibodies against TNF-α, Nrf-2, NF-kB, COX-2, caspase-3 and bcl-2 (dilution: 1/500 for all antibodies). Later, the samples were incubated by the HRP-conjugated secondary antibody (1/1000) for 60 min at room temperature, the nitrocellulose membrane was tested with DAB staining and the protein levels measured with an imaging program [17, 23, 24].

Catalase activity assay

Hydrogen peroxide was added into the 1/15 M Na-phosphate buffer (Na_2HPO_4–NaH_2PO_4, pH 7) solution. 1 ml of this solution was incubated, the supernatant was then added at different concentrations and then, the absorbance changes were read [17].

GSH analysis

GSH measurement was made according to the method reported by Yigit et al. [25]. This protocol describes a procedure for determining glutathione (GSH) concentrations in different cells and tissues. The absorbance was read on a spectrophotometer at 412 nm against distilled water [25].

Statistical analysis

All results of this study were analyzed by the SPSS 22 package program. One-Way ANOVA, Post Hoc Tukey, Games Howell, Duncan and LSD tests were performed to detect intragroup differences. In terms of the reliability of the statistics, the analyses were conducted by at least three replications.

Results

MDA analysis

Table 1 and Fig. 1 show that the MDA amount decreased in the EA and EA + CCl_4 groups in comparison to the CCl_4 groups ($p < 0.05$).

Table 1 Muscle tissue MDA results

Groups	Muscle tissue MDA levels (nmol/g)
Control	6.28 ± 0.24^{c}
EA	5.92 ± 0.07^{c}
CCl_4	9.73 ± 0.05^{a}
EA + CCl_4	8.01 ± 0.03^{b}

[a–c] Differences between groups with different letters are statistically significant ($p < 0.05$). One-Way ANOVA Post Hoc LSD Test

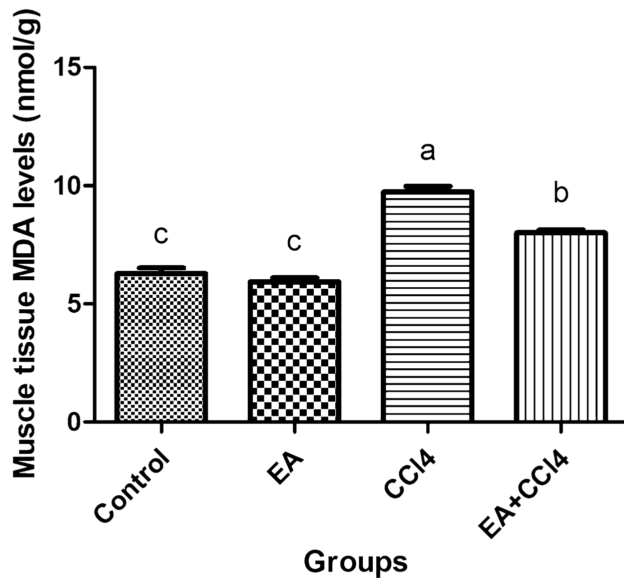


Fig. 1 Muscle tissue MDA levels between groups. (*a–c*) Differences between groups with different letters are statistically significant ($p < 0.05$). One-way ANOVA, Post Hoc LSD test

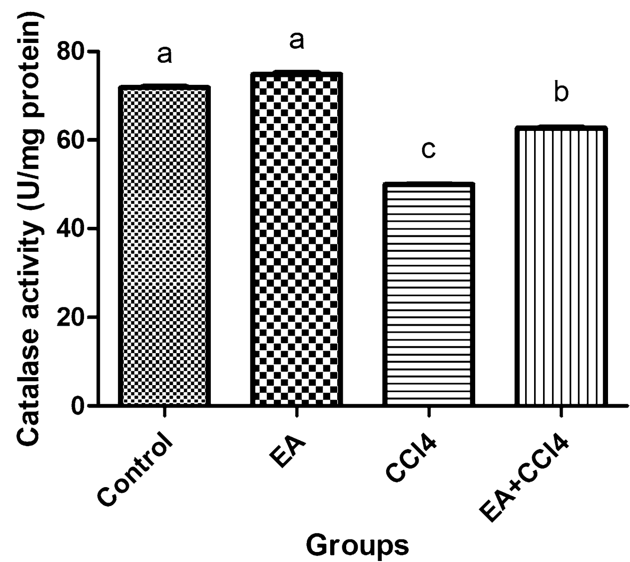


Fig. 2 Catalase activity between groups. (*a–c*) Differences between groups with different letters are statistically significant ($p < 0.05$). One-way ANOVA, Post Hoc LSD test

Table 2 Catalase activity results

Groups	Muscle tissue catalase activity (U/mg protein)
Control	71.81 ± 0.3^{a}
EA	74.81 ± 0.05^{a}
CCl_4	49.99 ± 0.04^{c}
CCl_4 + EA	62.72 ± 0.06^{b}

$^{a–c}$Differences between groups with different letters are statistically significant ($p < 0.05$). One-way ANOVA Post Hoc LSD Test

Table 3 GSH analysis results

Groups	Muscle tissue GSH levels (μmol/mg protein)
Control	67.04 ± 0.5^{a}
EA	67.96 ± 0.03^{a}
CCl_4	33.14 ± 0.04^{c}
EA + CCl_4	55.54 ± 0.04^{b}

$^{a–c}$Differences between groups with different letters are statistically significant ($p < 0.05$). One-way ANOVA Post Hoc Games-howell Test

Catalase activity results

Table 2 and Fig. 2 show that the catalase activity of the EA + CCl_4 group was observed higher than the CCl_4 group.

GSH analysis

In our findings, the GSH amount of the EA + CCl_4 group was observed higher than the CCl_4 group (Table 3 and Fig. 3).

TNF-α, Nrf-2, NF-kB, COX-2, Caspase-3 and Bcl-2 protein expression levels

Figure 4a reveals that the lowest rate of caspase-3 was calculated in the CCl_4 group, whereas the highest rates were in the control and EA groups ($p < 0.05$). Figure 4b shows that the bcl-2 protein expression levels of the CCl_4 group was determined higher in comparison to the EA and EA + CCl_4 groups ($p < 0.05$). Figure 4c points out that the Nrf-2 protein expression in the CCl_4 group was obtained as lower than the EA and EA + CCl_4 groups ($p < 0.05$). Accordingly, it may be stated that EA increases these biomarkers' protein synthesis. When the protein expression level of NF-kB was evaluated as shown in Fig. 4d, a statistically significant distinction was obtained between the groups ($p < 0.05$). The NF-kB amount in the EA + CCl_4 group was detected to be lower than that of the CCl_4 group. As seen in Fig. 4e, f, the highest COX-2 amount was obtained in the CCl_4 group, but with EA application, the COX-2 expression level decreased in the EA + CCl_4 group. The TNF-α amount in the EA + CCl_4 group was determined to be lower than that of the CCl_4 group (Fig. 4g).

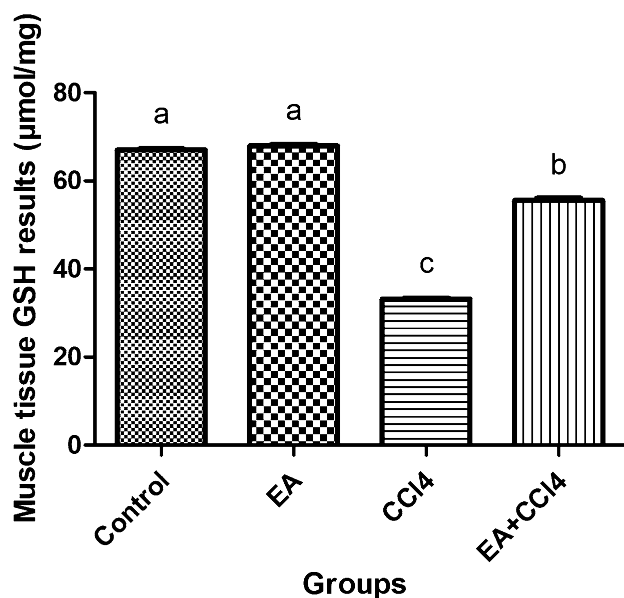


Fig. 3 Muscle tissue GSH levels between groups. (*a*–*c*) Differences between groups with different letters are statistically significant ($p<0.05$). One-way ANOVA, Post Hoc Games-Howell test

Discussion

The aim of our study was to investigate the protective role of ellagic acid (EA) on caspase-3, bcl-2, Nrf-2, NF-kB, TNF-α and COX-2 protein expressions in case of CCl_4-induced muscle injury in rats. In apoptosis, which is a controlled mechanism for physiological cell death, several proteins play an essential role [26]. Activation or inhibition of these proteins leads to not only apoptosis but also a non-apoptotic differentiation process [27, 28]. Our results showed that high expression of caspase-3 and Nrf-2 and low expression of bcl-2, NF-kB, TNF-α and COX-2 are associated with decreased muscle tissue damage. If the synthesis of caspase-3 and Nrf-2 protein is increased by the effect of ellagic acid in the cell, if the synthesis of NF-kB, TNF-α, COX-2 and bcl-2 protein is decreased by the effect of ellagic acid in the cell, then we may state that ellagic acid reduces muscle tissue by directing damaged cells to apoptosis. When we look at caspase-3 and Nrf-2 protein expressions in especially the EA + CCl_4 group, we may easily see these proteins' over expressions in comparison to the CCl_4 group. We think that, despite the suppression of carbon tetrachloride on expressions of this protein, ellagic acid was able to induce the synthesis of these proteins by inhibiting CCl_4 inhibition. Similarly, when we look at the bcl-2, NF-kB, TNF-α and COX-2 protein expressions, we may see that ellagic acid can reduce the synthesis of these proteins in the EA + CCl_4 group despite CCl_4. Moreover, our results showed that a high amount of MDA can damage DNA. In this context, when we look at our results, especially after EA application, the MDA level decreased in the EA + CCl_4 group in comparison to the CCl_4 group. These results showed that ellagic acid protects muscle tissue DNA against CCl_4 damage. Additionally, high glutathione and increased catalase enzyme activity indicate that ellagic acid protects muscle tissue against CCl_4 damage. We consider that the results of this study will contribute essential knowledge for future academic studies and according to our knowledge, the effect of ellagic acid on expression of these proteins (caspase-3, bcl-2, Nrf-2, NF-kB, TNF-α and COX-2) in muscle tissue damage was studied for the first time in this article. According to many in vitro and in vivo studies, EA has an anti-tumorigenic antiproliferative drug action [29]. Lee et al. researched the effect of tocotrienol-rich fraction (TRF) on skeletal muscle damage in diabetic mice. In the TRF groups, caspase-3 and Nrf-2 expressions increased, but bcl-2, NF-kB and TNF-α expressions decreased [30]. Similarly, when we look at our results, after EA supplementation, the caspase-3 and Nrf-2 expressions increased, but the bcl-2, NF-kB and TNF-α expressions decreased in the EA + CCl_4 group in comparison to the CCl_4 group. These results show that ellagic acid induces Nrf-2 and caspase-3 biomarker synthesis in CCl_4-induced muscle damage and inhibits the synthesis of the NF-kB, TNF-α and COX-2 biomarkers, resulting in apoptosis of the damaged cells. Li et al. explored the effects of hypoxia-induced pulmonary artery smooth muscle proliferation and they concluded that NF-kB expression levels decreased in the hypoxia-treated groups [31]. Gok investigated the protective effect of EA in rats with liver injury with CCl_4. They reported that MDA levels decreased in the EA + CCl_4 groups with EA application in comparison to CCl_4 groups. Additionally, they also observed that EA treatment increased caspase-3 expression and decreased bcl-2 expression in comparison to the CCl_4 group [32]. According to our results with EA application, the MDA levels decreased in the EA + CCl_4 group in comparison to the CCl_4 group. Aslan investigated the protective effect of tomato powder (TP) in rats with colorectal cancer to azoxymethane. When compared to the azoxymethane (AO) group, TP increased caspase-3 expression and decreased Cox-2 expression levels in the TP + AO group [10]. Rani et al. stated that EA inhibits atherosclerosis and intracellular ROS formation in streptozotocin-induced diabetic rats [7]. Yu et al. investigated the anti-oxidative and protective effects of linarin, a natural compound, on myocardial (heart muscle) cells. In linarin groups, they stated that MDA and ROS levels decreased. Additionally, they also observed that linarin treatment increased Nrf-2 expression and reduced NF-kB and Bcl-2 expression [12]. Akdemir et al. investigated the antioxidant effects of EA and hesperidin (HEPP) against skeletal muscle ischemia/reperfusion injury (I/R). In

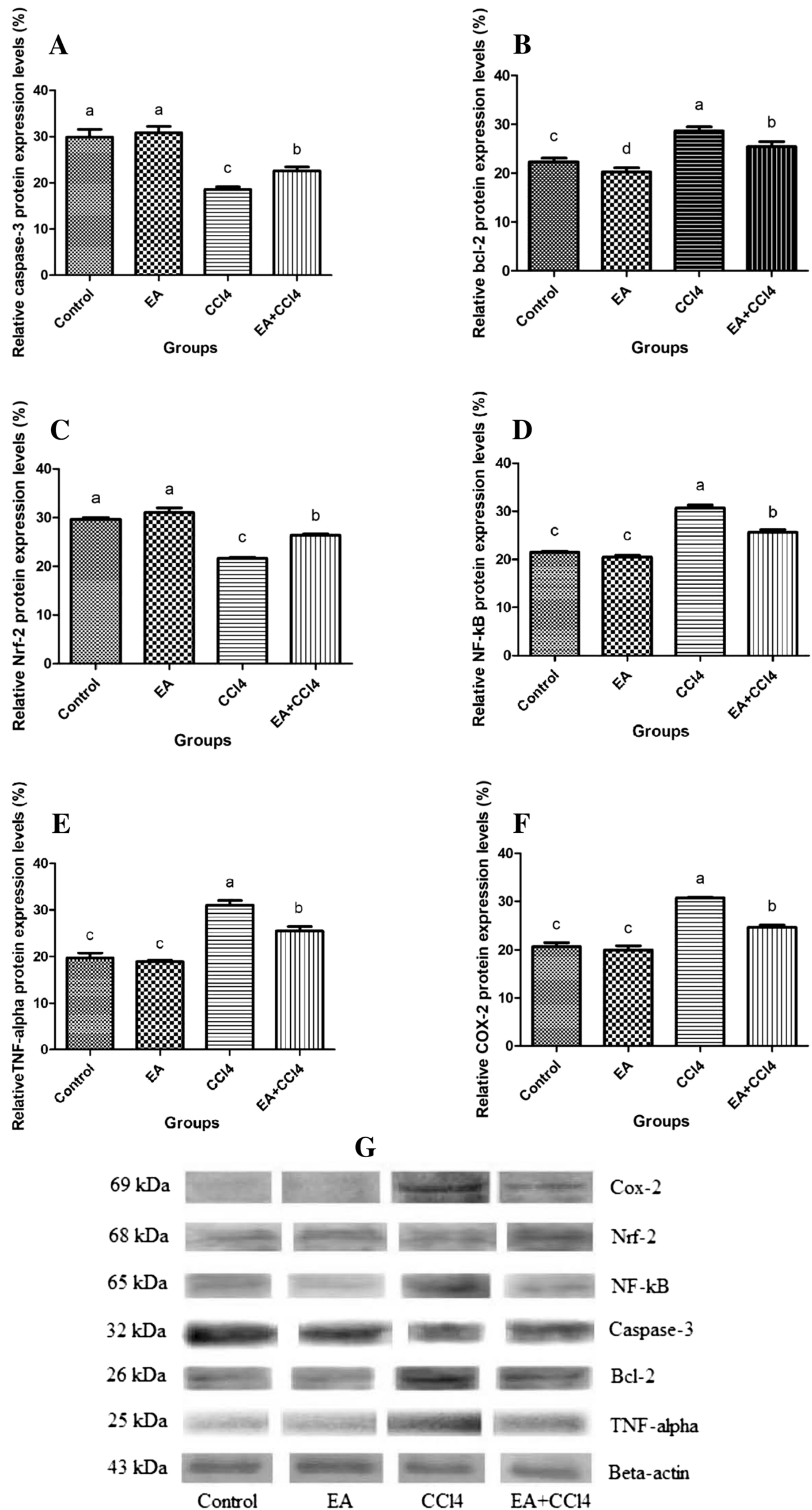


Fig. 4 Western blotting mean protein expression results; **A** caspase-3, **B** bcl-2, **C** Nrf-2, **D** NF-kB, **E** TNF-α, **F** COX-2, **G** western blotting protein bands. (*a*–*d*) Differences between groups with different letters are statistically significant ($p < 0.05$). One-Way ANOVA, Post Hoc Duncan Test

EA + HES groups, CAT activity increased, but MDA levels decreased in comparison to the case in ischemia and I/R groups. Additionally, they also reported that EA and HEPP with antioxidant properties had cell-protective effects against lipid peroxidation [6]. In our study, after EA application, the catalase activity increased in the EA + CCl_4 group, while the MDA levels decreased. These results show that ellagic acid increases catalase enzyme activity and reduces muscle tissue damage.

Hayes et al. investigated the effect of lutein, sesamol, ellagic acid and olive leaf extract on lipid oxidation and oxymyoglobin oxidation. They concluded that lutein, sesamol, ellagic acid and olive leaf extract decreased MDA levels [33]. Ekinci et al. researched the potential benefits of quercetin on skeletal muscle ischemia–reperfusion injury, and they reported that MDA levels decreased, but CAT activity significantly increased in the quercetin-treated groups [34]. Hadi et al. studied the effects of green tea on oxidative stress and muscle damage. They reported that the levels of MDA significantly decreased in green tea groups [35]. Onal et al. examined the effects of hypothermia and ozone (O_3) on skeletal muscle ischemia–reperfusion injury in rats. They reported that hypothermia and ozone down-regulated the tourniquet-induced ischemic reperfusion injury in the musculoskeletal system by decreasing the levels of oxidative and nitrosative stress parameters and increasing antioxidant enzymes. Additionally, they also concluded that CAT activity was increased in the hypothermia and ozone groups and the MDA levels decreased significantly [36].

Yang et al. investigated the protective effects of kaempferol-3′-sulfonate on hydrogen peroxide introduced into vascular smooth muscle cells. In the H_2O_2-treated group in comparison to the control group, GSH levels decreased and MDA levels significantly increased. Furthermore, they found that kaempferol-3′-sulfonate increased caspase-3 expression and decreased Bcl-2 expression in comparison to H_2O_2 [37]. Seigner et al. studied the anti-inflammatory effects of the plant *Symphytum officinale* on muscle and joint pain. They concluded that there was a significant decrease in NF-kB and COX-2 expression levels in the plant-given group [38]. When our results were evaluated, the bcl-2 and COX-2 decreased in the EA + CCl_4 group in comparison to the CCl_4 group and thus, we may easily state that EA has a preventive effect on muscle damage. Hsu et al. investigated the effects of ginseng steroids on the oxidative stress and antioxidant capacity of rat skeletal muscles. They reported that the GSH levels and CAT activities significantly increased in the ginseng steroids groups [39]. Guan et al. examined the antioxidant, anti-inflammatory and antitumor effects of celastrol on the skeletal muscle in diabetic rats and in the celastrol-given group, they concluded that the levels of MDA decreased and the level of GSH increased [40]. According to our results, the GSH levels increased, but MDA decreased after EA supplementation in the EA + CCl_4 group in comparison to the CCl_4 group. Wang et al. reported that, in lung injury caused by skeletal muscle ischemia and reperfusion in rats, COX-2 expression levels decreased in the skeletal reperfusion group in comparison to the NS-398 (COX-2 inhibitor) group [41]. Cheng et al. investigated the protective effect of lutein against ischemia/reperfusion injury in rat skeletal muscles. They also concluded that Nrf-2 expression levels significantly increased, while NF-kB and COX-2 expression levels significantly decreased in the lutein-treated groups in comparison to the skeletal reperfusion group [42].

Aslan et al. researched the preventive effects of EA in rats with liver damage induced by CCl_4. They found that NF-kB and Bcl-2 expression levels decreased, but Nrf-2 and caspase-3 expression levels increased in the EA treatment group in comparison to the CCl_4 group [17]. Hseu et al. searched the role of EA in human keratinocyte (HaCaT) cells on oxidative stress and apoptosis and they revealed that EA administration reduced ROS production and MDA levels. Additionally, they concluded that caspase-3 expression levels increased and Bcl-2 expression levels significantly decreased in the EA-treated groups [43]. Basham et al. investigated the effects of curcumin supplementation on oxidative stress, inflammation, muscle damage and muscle pain caused by exercise. According to their results, oxidative stress and inflammation decreased in the curcumin group. Moreover, they also concluded that there was a significant decrease in TNF-α expression levels and MDA levels in the curcumin groups [44]. El-Shitany et al. stated that nanoparticle ellagic acid protected against cisplatin-induced hepatotoxicity in rats [45]. Ahire and Mishra revealed that oral supplementation of EA significantly inhibited the induction of micronuclei aberrations against gamma radiation (1.5–3 Gy) and EA has a preventive effect on some cancer cells [46]. Accordingly, our results indicated that, with EA application, the TNF-α protein expression decreased in the EA + CCl_4 group in comparison to the CCl_4 group. These results show that ellagic acid decreases TNF-α protein expression and reduces muscle tissue damage.

Conclusion

Especially when the results obtained from this study were evaluated, it was concluded that ellagic acid had a significant effect on the mechanism of apoptosis. It protects the muscle tissue against CCl_4 damage, induces caspase-3 and Nrf-2 protein signaling pathways, suppresses Bcl-2, NF-kB, TNF-α and COX-2 protein signaling pathways and directs damaged cells to apoptosis. Thus, it is seen that it causes destruction of damaged cells in a controlled manner. Additionally, it is seen that EA reduces MDA levels and prevents damage to the DNA of healthy cells. In particular, it is seen

that EA contributes significantly to reduction of muscle tissue damage by increasing catalase enzyme activity and glutathione levels. When all these results are evaluated, we conclude that EA may have similar effects when ingested by humans and animals.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

Ethical approval All applicable international, national and institutional guidelines for the care and use of experimental animals were followed. The animal section of this study was performed by the approval of the Animal Experiments Ethics Committee of Firat University with the approval number of 27.02.2019 /2019-04.

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