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Protective effects of naringin against oxidative stress, inflammation, apoptosis, and DNA damage in rats with doxorubicin−induced hepatotoxicity

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ABSTRACT

Objective: To investigate the protective effects of naringin on doxorubicin (DOX)-induced liver injury.

Methods: A total of 50 male rats were allocated into five groups: the control group, the DOX group, the DOX groups treated with 50 mg/kg and 100 mg/kg of naringin by gastric lavage for 10 days, as well as the group treated with 100 mg/kg of naringin alone. Liver and serum samples were collected for biochemical, histopathological, and molecular analyses, including liver enzyme activity, oxidative stress markers, inflammation, apoptosis-related proteins, and DNA damage indicators.

Results: Naringin attenuated DOX-induced elevation in liver enzyme activity and inflammation markers while enhancing antioxidant activities. Naringin also activated the Nrf2-HO-1 signaling pathway, with the most pronounced effect in the high-dose naringin group. In addition, naringin modulated apoptotic signaling by downregulating the expression of PI3K-AKT and BAX, and upregulating Bcl-2, as well as reduced the level of 8-OHdG. Histopathological evaluation showed that DOX-induced structural liver alterations, such as cellular degeneration and necrosis, were notably attenuated by naringin treatment.

Conclusions: Naringin treatment exerts protective effects against DOX-induced liver injury through its antioxidative, anti-inflammatory, and anti-apoptotic effects.

KEYWORDS: Doxorubicin; Hepatotoxicity; Inflammation; Naringin; Oxidative stress

1. Introduction

Cancer is one of global causes of mortality[1]. Numerous therapeutics have been developed for the treatment of this life-threatening disease. One such critical therapeutic compound is doxorubicin (DOX), a powerful anticancer drug commonly employed in the treatment of numerous malignancies, such as leukemia, lymphoma, lung cancer, and cancers of the breast, ovaries, and thyroid. Owing to its broad-spectrum efficacy, DOX plays

Summary

Question: Does naringin protect against doxorubicin-induced liver injury in rats?

Findings: Naringin significantly alleviated doxorubicin-induced hepatotoxicity, as evidenced by reduced liver enzyme levels, oxidative stress, and inflammatory cytokines, with the higher dose showing more pronounced effects.

Meaning: Naringin has potential hepatoprotective effects, which could be explored further for therapeutic use in mitigating doxorubicin-induced liver toxicity.

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a vital role in combating these life-threatening malignancies[2]. Despite its chemotherapeutic efficacy, DOX is limited by significant side effects, including cardiotoxicity, hepatotoxicity, nephrotoxicity, diabetic cardiomyopathy, and infertility[3], with reported hepatotoxicity at acute and subacute doses[4]. Hepatotoxicity primarily arises from reactive oxygen species (ROS) generation during hepatic transformation, which, in combination with oxidative stress and impaired antioxidant defense mechanisms, exacerbates low activity of antioxidant enzymes with consequent inflammation, mitochondrial dysfunction, and apoptosis[5,6]. Furthermore, hepatic damage is evidenced by serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) overactivities, which indicate tissue injury[4,7].

DOX decreases the level of glutathione (GSH), and the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) as potent antioxidants through an increase in ROS production that inhibits the activity of defense mechanisms[4,5]. DOX markedly upregulated the expression of both intrinsic and extrinsic apoptotic markers. It contributes to liver cell damage by triggering the activation of caspase-3, mediated through pro-apoptotic proteins such as BAX and Fas[8].

In this regard, multiple critical cellular signaling pathways are involved in the hepatotoxic effects of DOX and the protective actions of natural antioxidants such as naringin. Notably, the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) pathway is essential for maintaining the cellular antioxidant response, while the nuclear factor kappa-B (NF-κB) pathway is a major regulator of inflammation. Additionally, apoptotic processes are governed by key proteins such as Bcl-2 and BAX, which modulate cell survival and death mechanisms[5]. Alterations in the activity or expression of these pathways are closely linked to oxidative stress, inflammation, and programmed cell death, and thus serve as crucial markers in evaluating liver injury and protection.

In chemotherapy, increasing the therapeutic effect on cancer cells and minimizing the toxic effects on normal cells[9] are very important. To minimize the side effects of anticancer drugs on organs and tissues, research has been conducted on their natural active ingredients[10,11]. Flavonoids, which have antioxidant properties, are considered powerful bioactive components against free radicals and oxidative stress[12,13]. Flavonoids are reported to have protective effects against toxic substances by activating antioxidant enzymes in cells or through the inhibition of ROS[14,15]. The intake of these natural compounds is also known to prevent the development of important diseases such as cancer. Naringin (4',5,7-trihydroxy flavanone 7-rhamnoglucoside) is the main and active flavanone glycoside of grapes and many citrus fruits[12,14–16]. When given orally, naringin is hydrolyzed *via* β-glucosidase and α-rhamnosidase enzymes to yield naringenin, the main metabolite and absorbable form of the compound[17]. It has many active pharmacological properties, such as antioxidant, anticancer, antiulcer, anti-inflammatory, anti-apoptotic, and hypolipidemic effects[12,14–16]. Since naringin is lipophilic, it easily binds to the cell membrane and minimizes the formation of free radicals, thus protecting the cell membrane[3]. Naringin has been reported to have protective effects on many tissues both *in vitro* and *in vivo*[7,10,12,14,15].

Although numerous studies have addressed DOX-induced hepatotoxicity, there is a lack of comprehensive investigations evaluating the protective effects of naringin using an integrated approach involving histopathological, immunohistochemical, immunofluorescence, and biochemical assessments. Therefore, this study was designed to investigate the potential hepatoprotective effects of naringin against DOX-induced liver damage using these combined methods.

2. Materials and methods

2.1. Chemicals

DOX was purchased from Koçak Farma, and naringin (CAS Number: 10236-47-2) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Malondialdehyde (MDA), SOD, GSH, GPx, CAT, tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), interferon-γ (IFN-γ), cyclooxygenase-2 (COX-2), endothelial nitric oxide synthase (eNOS), NF-κB, peroxisome proliferator-activated receptor gamma (PPARγ), and caspase-3 were analyzed *via* commercial ELISA kits (Sunred Biological Tecnology, Shanghai, China). 8-Hydroxy-2'-deoxyguanosine (8-OHdG), Bcl-2-associated X protein (BAX), B-cell lymphoma 2 (Bcl-2), Nrf2, phosphoinositide 3-kinase (PI3K), HO-1 and phospho-AKT (p-AKT) antibodies were purchased from Abcam (United Kingdom).

2.2. Animals

This study utilized twelve-week-old male Sprague-Dawley rats, each weighing between 250 and 300 g, which were obtained from the Atatürk University Medical Experimental Research and Application Centre. The experiments were conducted at the Atatürk University Medical Experimental Research and Application Centre. The rats were housed in an environment with a temperature of 25℃, 65%-70% humidity, and a 12-hour light-dark cycle. They had unrestricted access to food (Bayramoglu Feed and Flour Industry Trade A.Ş., Erzurum) and water, with continuous ventilation in the facility. The animals were allowed a one-week acclimatization period before the treatments commenced. Ethical approval for the study was granted

by the Atatürk University Local Ethics Committee (Ethical Decision Number: 2020/52).

2.3. Experimental design

The rats were weighed prior to the experiment to ensure that their weights fell within the specified range. Once this was confirmed, the rats were randomly assigned to five groups, with 10 rats in each group: Control, DOX, NA50+DOX, NA100+DOX and NA100. The rats in the control group received 1 mL of distilled water intragastrically (*i.g.*) for 10 d. The DOX, NA50+DOX, and NA100+DOX groups received distilled water orally for 10 d, with a single intraperitoneal dose of DOX (40 mg/kg) administered on the 8th day[18]. The NA50+DOX, NA100+DOX and NA100 groups received naringin at doses of 50 and 100 mg/kg *i.g.* for 10 d[15]. On the 11th day, the rats were euthanized under sevoflurane anesthesia.

2.4. Liver and body weights and serum analyses of the rats

All the rats were weighed on the 11th day of the experiment before euthanasia. At the end of euthanasia, intracardiac blood samples were taken from the rats. The liver tissues of the rats were then removed and weighed. Serum samples were analyzed for alkaline phosphatase (ALP), ALT, AST, and lactate dehydrogenase (LDH) levels *via* an autoanalyzer (RanDOX Monaco-United Kingdom) at Atatürk University, Faculty of Veterinary Medicine, Veterinary Diagnosis and Analysis Laboratory.

2.5. Analyses of oxidative stress, inflammation, and apoptosis parameters in liver tissue

Oxidant and antioxidant markers (MDA, SOD, GSH, GPx, CAT), inflammation markers (TNF-α, IL-1β, IL-6, IL-4, IL-10, IFN-γ, COX-2, eNOS, NF-κB, PPARγ) and apoptosis markers (caspase-3) in liver tissue supernatants were analyzed with ELISA kits according to the manufacturer's protocol. The readings of the plates were measured in a BioTek ELISA reader with the EPOCH Ⅱ program at a wavelength of 450 nm.

2.6. Histopathological examination

At the end of the experiment, liver samples taken from the rats were fixed in 10% formaldehyde solution for 48 h. The tissues were embedded in paraffin blocks after follow-up procedures. Then, 4 μm thick sections were taken from each block. The tissue sections were stained with hematoxylin-eosin and examined under a light microscope (Leica DM1000, Germany) at 40× magnification. Sections were evaluated as none (-), mild (+), moderate (++) and severe (+++) according to their histopathological findings[10].

2.7. Immunohistochemical investigation

After deparaffinizing and dehydrating the sections obtained from the liver, primary antibodies (8-OHdG, cat. no: sc-66036, reconstitution ratio: 1/100, US; BAX, cat. no: sc-7480, reconstitution: 1/100, US; and Bcl-2, cat. no: sc-7382, reconstitution ratio: 1/100, US) were added, and the samples were incubated according to the instructions for use. The 3-3' diaminobenzidine (DAB) was used as a chromogen in the tissues. The stained sections were examined *via* light microscopy (Zeıss AXIO GERMANY). The intensities of immunopositivity were calculated with ZEISS Zen Imaging Software[19].

2.8. Immunofluorescence examination

After deparaffinizing and dehydrating the sections obtained from the liver, the tissues were treated with primary antibodies (Nrf2, cat no: ab89443, reconstitution ratio: 1/100, UK; and PI3K, cat no: ab225720, reconstitution: 1/100, UK) and incubated according to the instructions for use. An immunofluorescent secondary antibody was used as a secondary marker (FITC cat no: ab6785, dilution ratio: 1/1 000) and incubated in the dark for 45 min. A secondary antibody targeting HO-1 (catalog no: ab189491, dilution 1∶100, UK) and protein kinase B (p-AKT) (catalog no: ab8805, dilution 1∶100, UK) was applied and incubated following the manufacturer's instructions. For detection, an immunofluorescent secondary antibody (Texas Red, catalog no: ab6719, dilution 1∶1 000, UK) was used and incubated in the dark for 45 min. Subsequently, DAPI with mounting medium (catalog no: D1306, dilution 1∶200, UK) was added to the sections, which were then kept in the dark for 5 min before being covered with coverslips. The stained sections were observed using a fluorescence microscope (Zeiss AXIO, Germany), and the ZEISS Zen Imaging Software was utilized to quantify the immunopositive intensity[20].

2.9. Statistical analysis

GraphPad Prism 8.0.1 software was used for quantitative values, and SPSS 13.0 software was used for histopathological examinations. The ZEISS Zen Imaging Software program was used for immunohistochemical and immunofluorescence methods. Statistical analyses were performed *via* one-way ANOVA followed by Tukey's test. A value of $P<0.05$ was considered significant, and the data are presented as mean ± SD.

3. Results

3.1. Effects of naringin on the body and liver weights of rats

The body and liver weights of the rats are given in Supplementary Table 1. No significant differences in body and liver weights were detected between the groups (P>0.05).

This finding indicates that neither naringin nor DOX significantly influenced the overall growth and liver mass, suggesting that these parameters were not directly affected by the treatments under the experimental conditions.

3.2. Effects of naringin on liver enzymes in rats with DOX–induced hepatotoxicity

As shown in Table 1, serum ALP, AST, ALT, and LDH levels in the DOX group were significantly increased compared with the control group. Treatment with 50 mg/kg of naringin significantly lowered the levels of ALT and LDH (P<0.05). In addition, 100 mg/kg naringin markedly reduced the levels of ALP, ALT and LDH (P<0.05). However, AST level was not noticeably decreased after naringin treatment at both doses (P>0.05). There was no significant difference in ALP, AST, ALT, and LDH levels between the naringin-treated alone group and the control group (P>0.05).

Table 1. Effects of naringin on liver enzymes and oxidative stress parameters in rats with doxorubicin-induced hepatotoxicity.

Parameters	Control	DOX	NA50+DOX	NA100+DOX	NA100
ALP (U/L)	83.00±13.55	277.80±26.91**	218.40±43.83*	133.80±32.87$^{*\#}$	108.00±36.06
AST (U/L)	135.50±28.85	384.06±83.05**	348.44±66.30*	288.10±77.43*	133.12±18.96
ALT (U/L)	26.00±4.53	122.00±19.38**	72.80±15.59$^{*\#}$	51.00±18.10$^{*\#}$	39.40±9.76
LDH (U/L)	1 408.76±85.43	1 685.40±76.63**	1 545.78±61.86$^{\#}$	1 436.84±90.35$^{\#}$	1 411.40±76.94
MDA (pg/g tissue)	3.08±0.30	5.83±0.82**	4.25±0.38$^{*\#}$	3.37±0.47$^{\#}$	3.04±0.34
SOD (ng/g tissue)	46.17±5.68	17.92±2.41**	19.01±4.90*	30.93±2.28$^{*\#}$	44.02±6.07
GSH (ng/g tissue)	280.18±43.78	118.85±1.85**	145.88±20.14*	219.78±19.08$^{\#}$	267.84±31.13
GPx (pg/g tissue)	11.45±1.69	5.96±0.92**	7.63±0.87*	8.47±1.23$^{*\#}$	10.18±0.87
CAT (katal/g protein)	33.52±3.95	16.19±2.41**	19.35±3.29*	26.37±2.89$^{*\#\$}$	31.18±3.96

The data are presented as mean ± SD (n=10) and analyzed by one-way ANOVA followed by Tukey's test. $^{*}P$<0.05, $^{**}P$<0.001 compared with the control group; $^{\#}P$<0.05 compared with the doxorubicin only; $^{\$}P$<0.05 compared with naringin 50 mg/kg plus doxorubicin. DOX: doxorubicin; NA50: 50 mg/kg naringin; NA100: 100 mg/kg naringin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; MDA: malondialdehyde; SOD: superoxide dismutase; GSH: reduced glutathione; GPx: glutathione peroxidase; CAT: catalase.

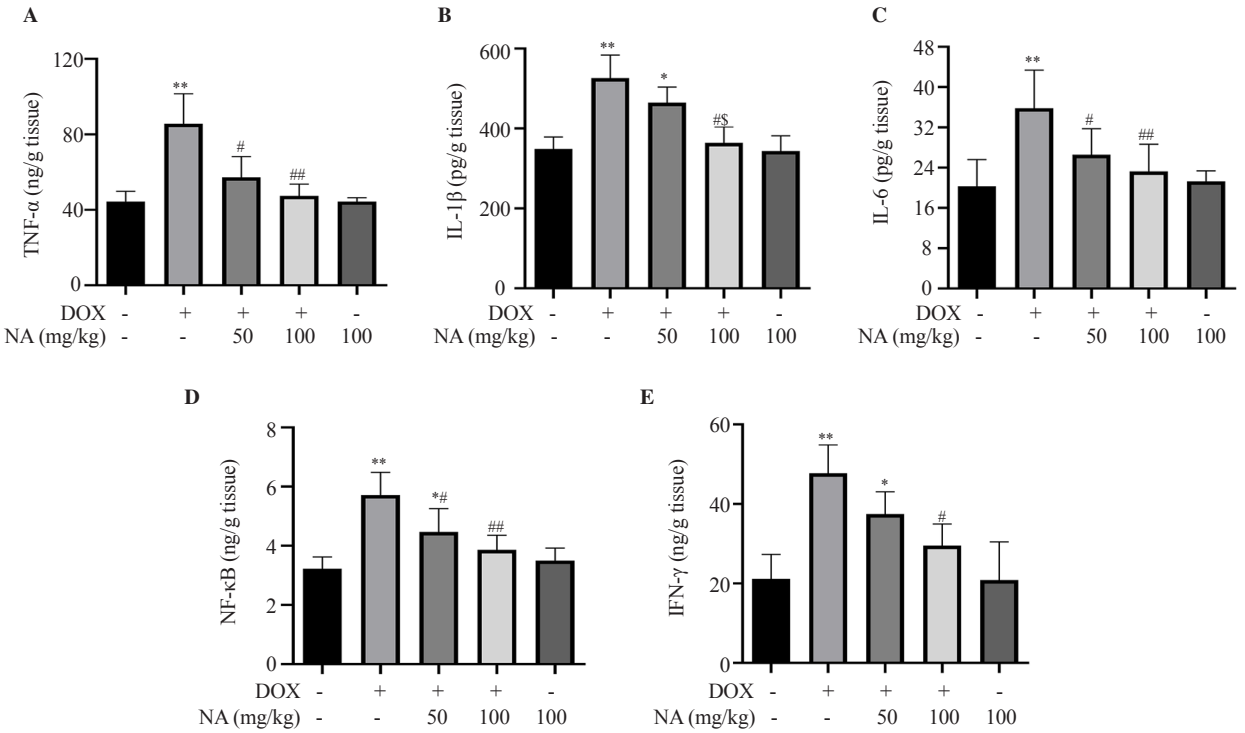


Figure 1. Effects of naringin on TNF-α (A), IL-1β (B), IL-6 (C), NF-κB (D), and IFN-γ (E) levels in rat liver tissue. The data are presented as mean ± SD (n=10) and analyzed by one-way ANOVA followed by Tukey's test. $^{*}P$<0.05, $^{**}P$<0.001 compared with the control group; $^{\#}P$<0.05, $^{\#\#}P$<0.001 compared with the doxorubicin only; $^{\$}P$<0.05 compared with naringin 50 mg/kg plus doxorubicin. DOX: doxorubicin; NA: naringin.

3.3. Effects of naringin on oxidant and antioxidant parameters in rats with DOX–induced hepatotoxicity

As shown in Table 1, the MDA level was significantly elevated by DOX administration (P<0.05). Naringin pretreatment prevented DOX-induced increases in the MDA level. Moreover, liver SOD, GPx, and CAT activities as well as GSH levels were significantly lower in the DOX-administered group than in the control group (P<0.05). However, naringin pretreatment significantly increased the levels of these antioxidants (P<0.05). Moreover, treatment with naringin alone did not affect the levels of oxidant and antioxidant parameters (P>0.05). This suggests that naringin can partially restore antioxidant enzyme activities in the liver, which may contribute to the attenuation of oxidative stress induced by DOX.

3.4. Effects of naringin on DOX–induced hepatic inflammation

The effects of naringin on DOX-induced hepatic inflammation are shown in Figure 1. The levels of TNF-α, IL-1β, IL-6, NF-κB, and IFN-γ were significantly higher in the DOX group compared with the control group (P<0.05). Naringin pretreatment markedly lowered the levels of these inflammation-related parameters in rats with DOX-induced hepatotoxicity (P<0.05).

3.5. Effects of naringin on anti–inflammatory cytokines in DOX–induced hepatic inflammation

The DOX group exhibited significantly lower IL-4 levels compared with the control group (P<0.05). Treatment with 50 mg/kg of naringin induced a slight increase in IL-4 level but with no significant difference compared with the DOX group (P>0.05), whereas 100 mg/kg naringin significantly enhanced IL-4 level (P<0.05). In the naringin-treated alone group, IL-4 level was similar to that in the control group (P>0.05) (Figure 2A). A similar trend was observed in IL-10 (Figure 2B). Only 100 mg/kg of naringin prevented DOX-induced reduction in anti-inflammatory cytokines (P<0.05).

3.6. Effects of naringin on COX–2, eNOS and PPARγ levels

The effects of naringin on COX-2, eNOS and PPARγ levels in rats with DOX-induced hepatotoxicity are shown in Figure 2. Compared with the control group, COX-2 and eNOS levels in the DOX group were significantly increased, and the PPARγ level was decreased (P<0.05). A low dose of naringin did not affect DOX-induced changes in these parameters, whereas 100 mg/kg of naringin significantly prevented the increase in COX-2 and eNOS levels and the decrease in PPARγ level (P<0.05).

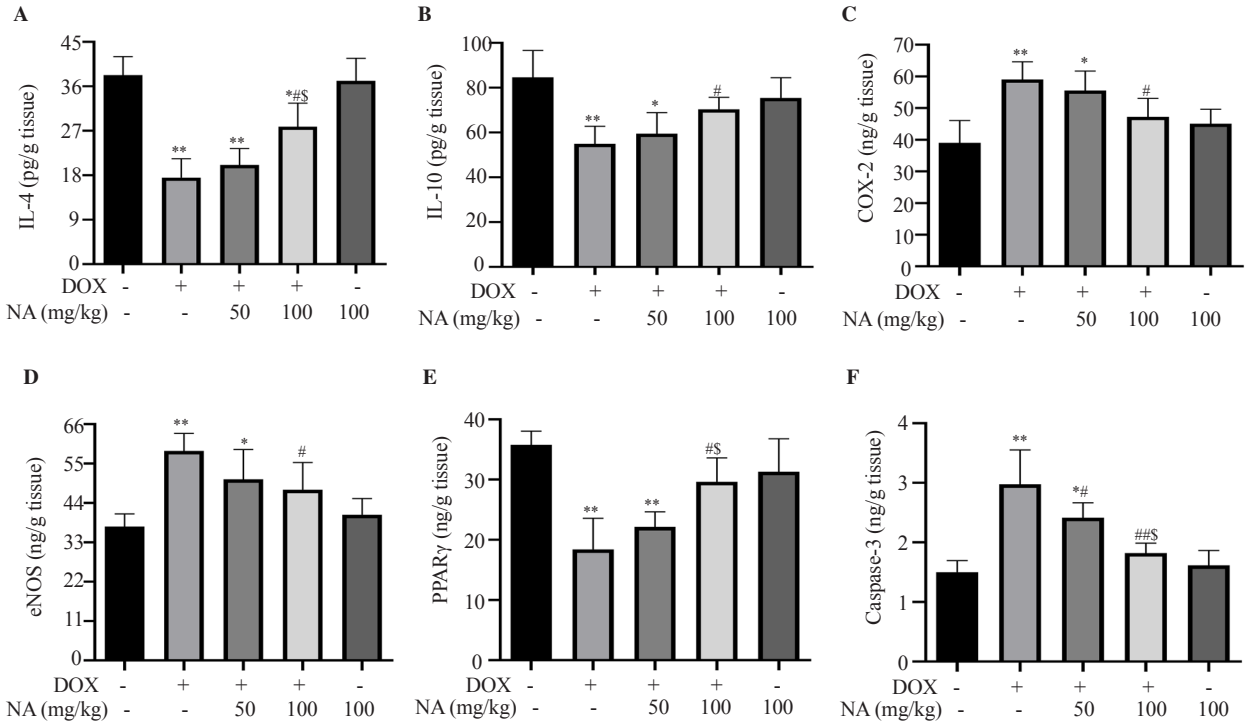


Figure 2. Effects of naringin on IL-4 (A), IL-10 (B), COX-2 (C), eNOS (D), PPARγ (E), and caspase-3 (F) levels in rat liver tissue. The data are presented as mean ± SD (n=10) and analyzed by one-way ANOVA followed by Tukey's test. $^{*}P$<0.05, $^{**}P$<0.001 compared with the control group; $^{\#}P$<0.05, $^{\#\#}P$<0.001 compared with the doxorubicin only; $^{\$}P$<0.05 compared with naringin 50 mg/kg plus doxorubicin. DOX: doxorubicin; NA: naringin.

3.7. Effects of naringin on caspase-3 levels

A marked rise in caspase-3 level was found in the DOX group (P<0.05). Both doses of naringin significantly attenuated the DOX-induced increase in caspase-3 in a dose-dependent manner (P<0.05). There was no significant difference in caspase-3 levels between the control group and the group treated with 100 mg/kg of naringin alone (P>0.05) (Figure 2F).

3.8. Histopathological findings

The histopathological findings are shown in Figure 3 and

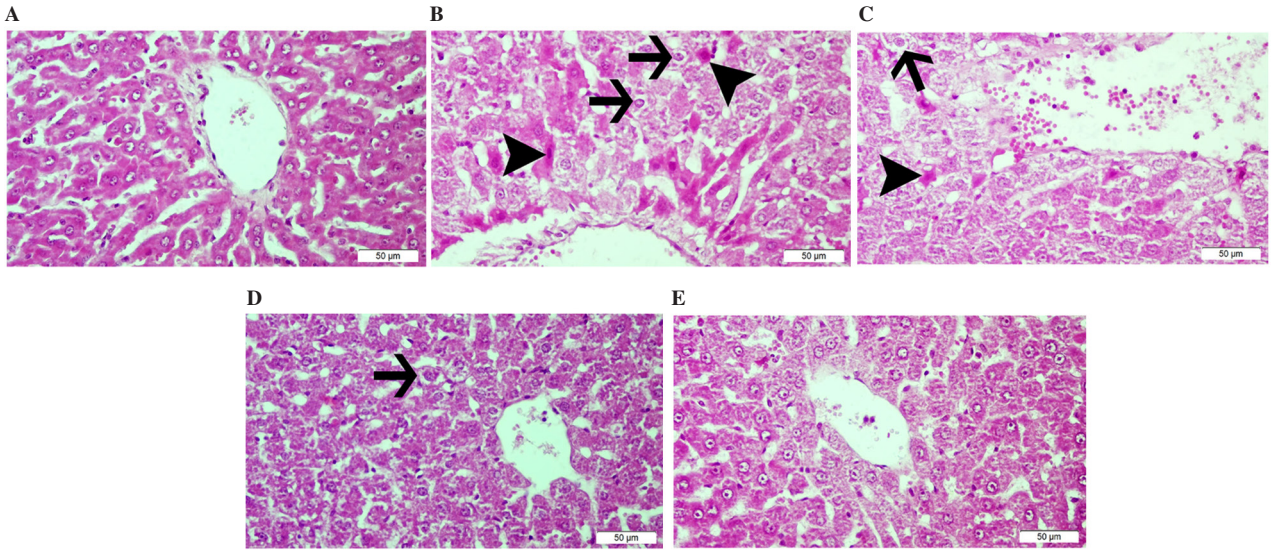
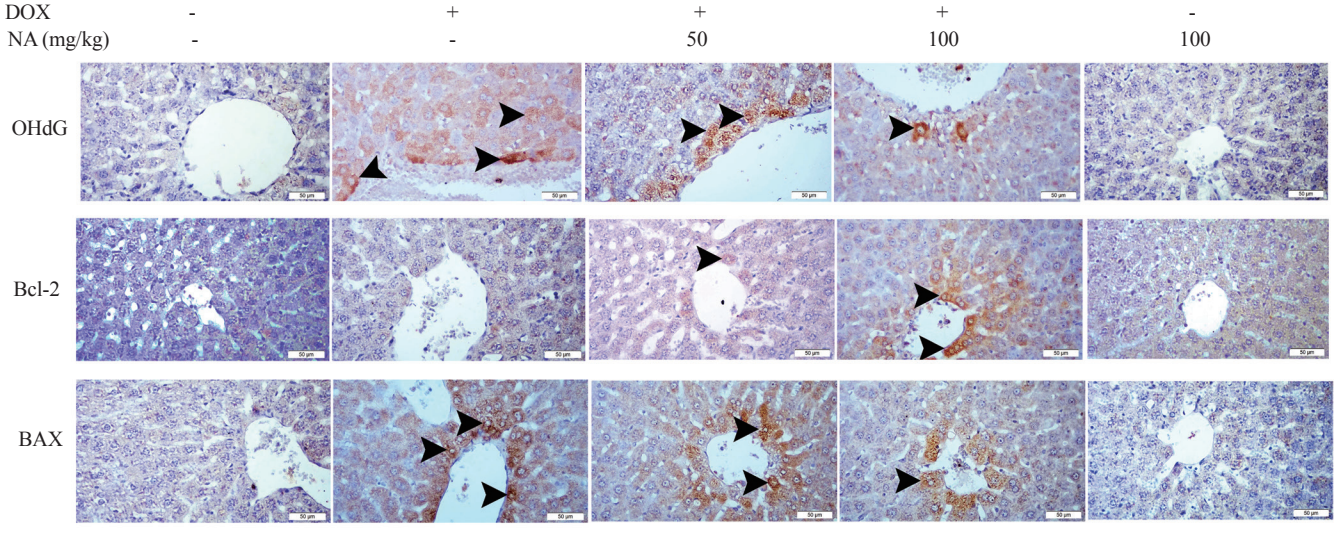


Figure 3. Histopathological results in liver tissue (H&E, Bar: 50 μm). Control (A), doxorubicin (B), naringin 50 mg/kg plus doxorubicin (C), naringin 100 mg/kg plus doxorubicin (D), naringin 100 mg/kg (E). Degeneration in hepatocytes is shown with arrows, necrosis with arrowheads.

A



B

Parameters	Control	DOX	NA50+DOX	NA100+DOX	NA100
8-OHdG	20.80±0.31	81.33±2.91**	52.92±1.89$^{*\#}$	32.73±1.25$^{*\#\$}$	20.45±0.59
Bcl-2	23.96±1.78	24.99±1.60	37.11±1.83$^{*\#}$	57.21±2.33$^{**\#\#\$}$	25.39±1.18
BAX	18.91±0.38	73.59±2.33**	63.75±1.57$^{*\#}$	29.19±0.76$^{*\#\#\$}$	19.02±0.45

Figure 4. Expressions of 8-OHdG, Bcl-2 and BAX in liver tissue are determined by immunohistochemical analysis. (A) The expressions of 8-OHdG, Bcl-2 and BAX are shown with arrowheads (IHC-P, scale bar: 50 μm). (B) Quantitative results. The data are presented as mean ± SD (n=10) and analyzed by one-way ANOVA followed by Tukey's test. $^{*}P$<0.05, $^{**}P$<0.001 compared with the control group; $^{\#}P$<0.05, $^{\#\#}P$<0.001 compared with the doxorubicin only; $^{\$}P$<0.05 compared with naringin 50 mg/kg plus doxorubicin.

Supplementary Table 2. Liver tissues in the control group and the group treated with 100 mg/kg of naringin alone presented a normal histological structure. In the DOX group, severe degeneration and necrosis in hepatocytes, severe hyperemia in veins, and severe dilatation in sinusoids were detected. In the DOX group treated with 50 mg/kg of naringin, moderate degeneration and mild necrosis in hepatocytes, severe hyperemia in veins, and moderate dilatation in sinusoids were observed. In the DOX group treated with 100 mg/kg of naringin, mild degeneration in hepatocytes, moderate hyperemia in veins, and mild dilatation in sinusoids were observed. Histopathological analysis further corroborates the biochemical findings, showing that naringin, especially at the higher dose, helps preserve the liver structure and minimizes DOX-induced damage.

3.9. Effects of naringin on DNA damage and apoptosis

Immunohistochemical staining of liver tissues was performed to evaluate the effects of naringin on DNA damage and apoptosis. As shown in Figure 4, the DOX group exhibited significant DNA damage, evidenced by elevated 8-OHdG expression, and increased apoptotic activity, as indicated by higher BAX levels (P<0.05). In contrast, the DOX group treated with 50 mg/kg of naringin showed moderate reductions in both 8-OHdG and BAX expression, with an increase in Bcl-2 expression, whereas 100 mg/kg of naringin demonstrated the most pronounced protective effects, as evidenced by significantly lower 8-OHdG and BAX levels and markedly higher Bcl-2 expression (P<0.05).

3.10. Naringin–mediated modulation of oxidative stress and cell survival pathways in DOX–induced hepatotoxicity

The immunofluorescence staining results (Table 2, Figures 5-6) demonstrate that naringin modulates critical antioxidant and cell survival signaling pathways in the liver under DOX-induced hepatotoxicity. In the DOX group, no significant changes were observed in Nrf2 and HO-1 expression, while PI3K and p-AKT levels were markedly elevated. Treatment with 50 mg/kg of naringin provided moderate improvements, with increased expression of Nrf2 and HO-1 and a reduction in PI3K and p-AKT levels. However, the most notable effects were observed in the DOX group treated with 100 mg/kg of naringin, where Nrf2 and HO-1 expressions were significantly enhanced, and PI3K and p-AKT levels were substantially reduced. These findings suggest that naringin, particularly at the higher dose, enhances antioxidant defenses and restores survival signaling pathways, offering further evidence for its potential hepatoprotective effects on DOX-induced liver damage.

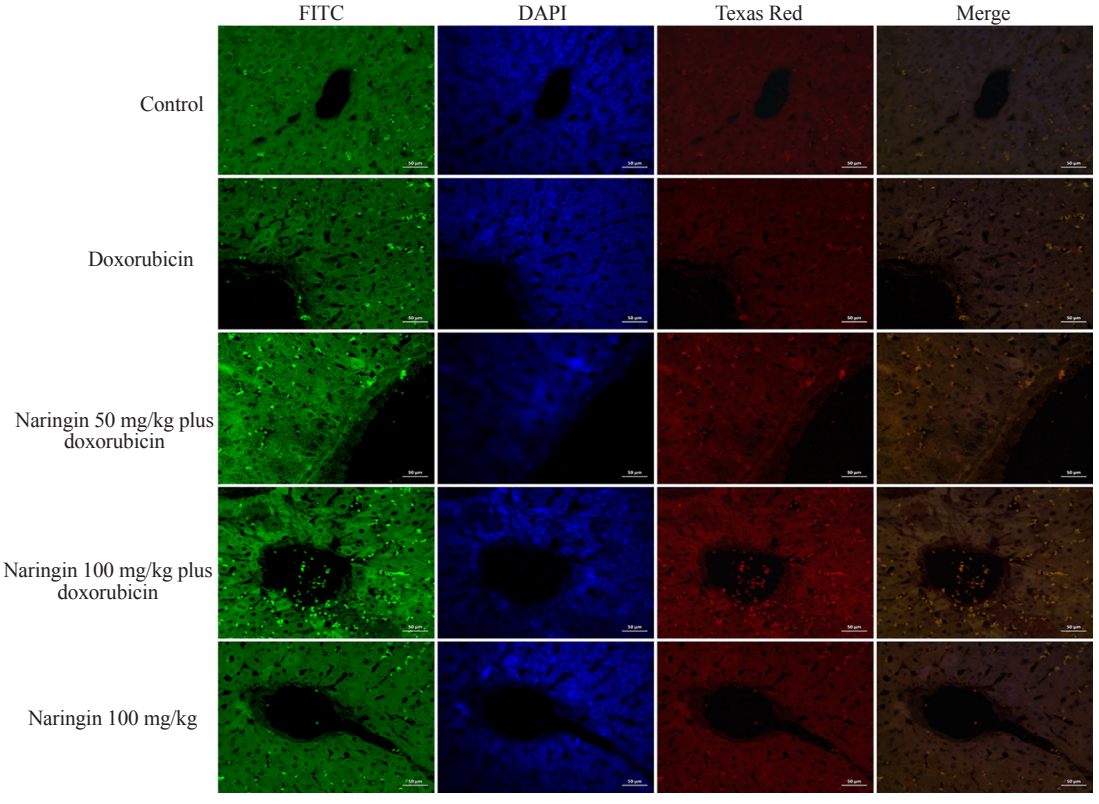


Figure 5. Effect of naringin on Nrf2 and HO-1 in liver tissue by immunofluorescence examination [Nrf2 immunopositivity (FITC) and HO-1 immunopositivity (Texas Red), scale bar: 50 µm].

Table 2. Effect of naringin on the expression levels of Nrf2, HO-1, PI3K, and p-AKT by immunofluorescence staining.

Parameters	Control	DOX	NA50+DOX	NA100+DOX	NA100
Nrf2	22.81±1.59	20.12±1.39	33.03±0.97$^{*\#}$	60.60±2.19$^{*\#\$}$	20.92±1.38
HO-1	20.49±1.48	21.93±1.65	33.11±1.23$^{*\#}$	65.38±2.91$^{*\#\$}$	20.27±1.59
PI3K	22.82±0.58	87.59±3.13**	58.61±1.99$^{*\#}$	35.69±1.33$^{*\#\$}$	23.30±0.71
p-AKT	20.57±0.69	81.59±2.48**	55.93±1.81$^{*\#}$	37.66±0.94$^{*\#\$}$	21.03±0.80

The data are presented as mean ± SD (n=10) and analyzed by one-way ANOVA followed by Tukey's test. $^{*}P$<0.05, $^{**}P$<0.001 compared with the control group; $^{\#}P$<0.05 compared with the doxorubicin only; $^{\$}P$<0.05 compared with naringin 50 mg/kg plus doxorubicin.

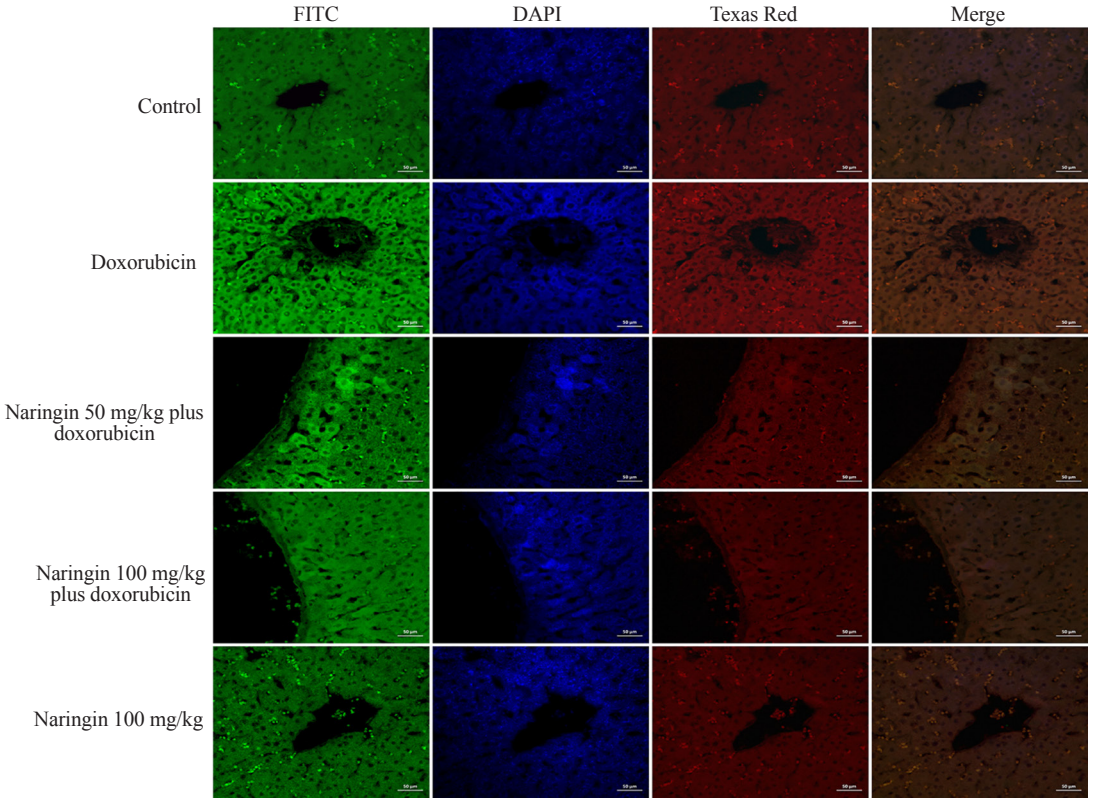


Figure 6. Effect of naringin on PI3K and p-AKT in liver tissue by immunofluorescence examination [PI3K immunopositivity (FITC) and p-AKT immunopositivity (Texas Red), scale bar: 50 μm].

4. Discussion

DOX is a widely used chemotherapeutic agent, but its application is limited by its hepatotoxic effects, particularly at acute and subacute doses. The hepatotoxicity caused by DOX primarily results from oxidative stress due to the generation of ROS during drug metabolism in the liver. This imbalance leads to cellular dysfunctions, including apoptosis, inflammation, and mitochondrial damage. Previous studies have established these molecular mechanisms as the primary contributors to DOX-induced hepatotoxicity[4–6].

In the current study, while DOX administration resulted in typical markers of liver injury, including increases in serum liver enzymes such as ALP, AST, ALT, and LDH, no significant changes were observed in the body or liver weights of the rats. This contrasts with previous studies, where DOX administration was linked to alterations in liver-to-body weight ratios[21]. The lack of significant changes in our study could be due to the specific dosing regimen or the experimental model used.

Regarding liver enzyme levels, we observed a significant increase in the ALP, AST, ALT, and LDH levels in response to DOX administration, consistent with other reports[22]. These enzymes are widely used biomarkers for liver injury, and their elevated levels suggest DOX-induced hepatotoxicity in our model. Importantly, the higher dose of naringin (100 mg/kg) effectively mitigated this increase, indicating a protective effect. These results are in line with studies showing the hepatoprotective potential of flavonoids like naringin[7].

Oxidative stress plays a central role in DOX-induced hepatotoxicity by enhancing ROS production and lipid peroxidation, leading to the generation of MDA, which in turn disrupts antioxidant defense mechanisms such as GSH, SOD, GPx, and CAT. In this study, DOX

administration significantly increased MDA levels while decreasing antioxidant enzyme levels, consistent with the oxidative stress pathway associated with DOX toxicity[5,10]. Interestingly, naringin administration effectively prevented the increase in MDA and restored antioxidant enzyme levels, suggesting that its protective effects are mediated through its antioxidant properties. This oxidative stress not only disrupts cellular redox balance but also contributes to amino acid oxidation and subsequent DNA damage[16]. A key biomarker for oxidative DNA damage is 8-OHdG[11]. Previous studies have demonstrated that DOX induces hepatic DNA damage, marked by elevated 8-OHdG expression[23,24]. Notably, naringin has been shown to mitigate such damage in models of oxaliplatin-induced hepatotoxicity[18]. In agreement with these findings, our results revealed that DOX significantly increased 8-OHdG expression in liver tissues, whereas naringin administration markedly reduced this elevation, suggesting the protective effect of naringin against DOX-induced genotoxic stress.

In addition to oxidative stress, DOX-induced liver injury involves inflammation mediated by the activation of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6. This study confirmed that DOX administration led to increased levels of these cytokines, as well as NF-κB activation. Naringin significantly reduced the levels of TNF-α, IL-1β, IL-6, and NF-κB, indicating that its hepatoprotective effects may involve the modulation of inflammatory pathways. These findings are consistent with previous reports on the anti-inflammatory effects of naringin in liver injury models[25,26].

The anti-inflammatory response is further supported by the observed effects on IL-4 and IL-10, which are important anti-inflammatory cytokines. DOX reduced the levels of IL-4 and IL-10, while naringin administration prevented this decrease. This suggests that naringin can help maintain the balance between proinflammatory and anti-inflammatory cytokines, contributing to its protective role against liver inflammation.

Regarding signaling pathways, we observed that DOX treatment did not significantly decrease the levels of Nrf2 and HO-1, which are key regulators of cellular antioxidant response, while naringin treatment significantly increased the levels of Nrf2 and HO-1. These results suggest that this compound may exert its hepatoprotective effects through activation of the Nrf2/HO-1 signaling pathway. Conversely, the PI3K/AKT signaling pathway did not show the expected changes. While previous studies suggested that this pathway is crucial for liver protection, our findings indicate that the PI3K/AKT pathway may not be a central mechanism in our model. This discrepancy could be attributed to the specific conditions of our study, such as the dosage and duration of DOX exposure. The results suggest that naringin may engage in a more context-dependent modulation of this pathway, potentially stabilizing cell survival signals while mitigating excessive apoptotic cascades. Moreover, the activation of the COX pathway contributes to liver injury, with elevated COX-2 expression linked to chronic hepatitis, cirrhosis, and hepatocellular carcinoma[27]. DOX administration has been shown to upregulate COX-2 in hepatic tissue[28]. Similarly, nitric oxide produced by eNOS plays a protective role in hepatic physiology; however, increased eNOS levels have also been observed in response to DOX-induced inflammation[29,30]. Peralta *et al*. demonstrated that eNOS deficiency exacerbates hepatic ischemia and reperfusion injury, confirming its protective role[29]. PPARγ, which negatively regulates COX-2 and iNOS expression, is downregulated in response to DOX exposure[31,32]. Naringin has been reported to counteract these effects by suppressing COX-2 and eNOS while restoring PPARγ levels[16,33,34]. Our findings are consistent with these reports: DOX increased hepatic COX-2 and eNOS and decreased PPARγ, all of which were ameliorated by naringin treatment.

Furthermore, the study examined the expression of apoptotic markers[35–38]. DOX administration led to increased levels of BAX and caspase-3, indicating the activation of apoptotic pathways. However, naringin treatment reduced the expression of BAX and caspase-3, while increasing Bcl-2 levels, suggesting that naringin possesses anti-apoptotic properties. This finding is consistent with other studies showing the ability of naringin to modulate apoptosis in various liver injury models[16,18].

In line with previous research, the data presented here show that naringin exerts a dose-dependent hepatoprotective effect. The higher dose of naringin (100 mg/kg) provided more robust protection compared to the lower dose (50 mg/kg), as evidenced by improvements in liver enzyme levels, oxidative stress markers, and inflammation. These effects were also confirmed by histopathological and molecular analyses, where the higher dose of naringin showed significantly better preservation of liver tissue structure and function. These findings suggest that naringin offers a dose-dependent hepatoprotective effect in DOX-induced liver injury. However, it is important to note the limitations of this study. The investigation was limited to only two doses of naringin, which restricts our ability to fully characterize the dose-response relationship. Additionally, the study did not include functional liver assessments, pharmacokinetic data, or mechanistic pathway validation, which limits the depth of interpretation. Future research should explore a broader range of doses, longer observation periods, and more detailed mechanistic studies to further elucidate the protective effects of naringin and its underlying mechanisms.

In conclusion, our study provides evidence that naringin has hepatoprotective effects against DOX-induced liver toxicity. These effects are likely mediated by its antioxidant, anti-inflammatory, and anti-apoptotic properties, and the higher dose of naringin appears to confer more significant protection. These findings contribute to the growing body of literature on naringin's therapeutic potential in liver diseases, and future studies should aim to expand on these results with more comprehensive experimental designs.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

PDA conceived and designed the study, performed data analysis and interpretation, and drafted the manuscript. SY, ES, MW, and ST contributed to data analysis and interpretation and participated in manuscript drafting. FA contributed to data analysis. AC planned the methodology, conducted statistical analysis, critically revised the manuscript, and approved the final version for publication.

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Publisher's note

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Supplementary Table 1. Effects of NA on the body and liver weights of rats.

Parameters	Control	DOX	NA50+DOX	NA100+DOX	NA100
Initial body weight (g)	277.00±14.90	272.00±15.99	276.00±17.85	277.00±13.46	277.00±9.93
Final body weight (g)	295.00±22.40	288.00±16.60	289.00±8.80	286.00±19.40	301.00±14.27
Liver weight (g)	12.03±1.40	10.85±1.10	11.15±0.60	11.83±0.80	11.99±1.40

Supplementary Table 2. Histopathological findings and scoring in rat liver tissues.

Parameters	Control	DOX	NA50+DOX	NA100+DOX	NA100
Degeneration in hepatocytes	-	+++	++	+	-
Necrosis in hepatocytes	-	+++	+	-	-
Hyperemia in the veins	-	+++	+++	++	-
Dilatation of sinusoids	-	+++	++	+	-

It was evaluated as none (-), mild (+), moderate (++)and severe (+++).