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RESEARCH ARTICLE

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Protective effects of esculetin against doxorubicin-induced toxicity correlated with oxidative stress in rat liver: In vivo and in silico studies

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Abstract

Doxorubicin (DOX) is widely used in cancer treatment but the dose-related toxicity of DOX on organs including the liver limit its use. Therefore, there is great interest in combining DOX with natural compounds with antioxidant properties to reduce toxicity and increase drug efficacy. Esculetin is a natural coumarin derivative with biological properties encompassing anti-inflammatory and antioxidant activities. In light of these properties, this study was meticulously crafted to investigate the potential of esculetin in preventing doxorubicin (DOX)-induced hepatotoxicity in Sprague-Dawley rats. The rats were divided into a total of six groups: control group, DOX group (administered DOX at a cumulative dose of 5 mg/kg intraperitoneally every other day for 2 weeks), E50 group (administered 50 mg/kg of esculetin intraperitoneally every day), E100 group (administered 100 mg/kg of esculetin intraperitoneally every day) and combined groups (DOX + E50 and DOX + E100) in which esculetin was administered together with DOX. The treatments, both with DOX alone and in combination with E50, manifested a reduction in catalase (CAT mRNA) levels in comparison to the control group. Notably, the enzymatic activities of superoxide dismutase (SOD), CAT, and glutathione peroxidase (GPx) witnessed significant decreases in the liver of rats treated with DOX. Moreover, DOX treatment induced a statistically significant elevation in malondialdehyde (MDA) levels, coupled with a concurrent decrease in glutathione (GSH) levels. Additionally, molecular docking studies were conducted. However, further studies are needed to confirm the hepatoprotective properties of esculetin and to precisely elucidate its mechanisms of action.

KEYWORDS

antioxidant, doxorobicin, hepatoprotective, molecular docking, oxidative stress

1 | INTRODUCTION

Chemotherapy is an effective and widely used treatment method for most malignant tumors. Chemotherapy drugs not only affect cancerous cells, but also affect normal cells, causing side effects such as nausea, hair loss, vomiting and fatigue.[1] Doxorubucin (DOX), an antibiotic from the anthracycline group, is widely used as an anticancer drug against solid and hematologic malignancies. However, the long-term use of DOX in treatments is limited due to its toxic effects on many organs, especially the liver and kidney.[2–4] The molecular mechanisms underlying DOX-induced toxicity are multifactorial and have not been fully characterized to date. However, it is

among the acceptable theories that this toxicity starts with oxidative stress, the formation of the DOX inflammatory cascade and eventually leads to organ toxicity through programmed cellular death.[5]

Esculetin anticancer, antidiabetes, antioxidant, antiapoptotic, neuroprotective, anti-inflammatory, antibacterial, and cardiovascular protective effects and has the potential as a therapeutic drug in non-communicable diseases such as cancer, diabetes, obesity, and neurological disorders.[6–9] Esculetin has been documented to demonstrate both antiapoptotic and proapoptotic activities, mediated through pharmacological mechanisms. Additionally, it has been identified as an inhibitor of reactive oxygen species (ROS) production. Esculetin exhibits the capacity to mitigate the inhibition of antioxidant enzymes and suppress the activation of mitochondria-induced apoptotic pathways initiated by ROS. Consequently, these properties contribute to the protective role of esculetin in shielding cells against apoptosis induced by oxidative stress.

On the other hand, it has been shown to protect cells from inflammatory damage by suppressing the activation of inflammatory pathways.[10] Due to its bioavailable properties, there is an increasing demand for in vivo analyses and pharmacokinetic studies of esculetin. Esculetin prevented ethanol-induced cytotoxicity in HepG2 cells and ethanol-induced liver injury in mice.[11] It also reduced carbon tetrachloride-induced liver apoptosis in rats.[12] On the other hand, it showed a protective effect against N-nitrosodiethylamine-induced hepatotoxicity in rats.[13]

Recently, cancer has become a very common disease worldwide. In the treatment of cancer, powerful chemotherapeutic drugs are used to try to control this disease. However, the chemotherapy drugs including DOX have significant side effects on the people who use them. Therefore, research on the use of protective agents together with chemotherapeutic drugs against side effects has gained importance. Esculetin, found naturally in plants, is known to have hepatoprotective effect.[10] Therefore, it may alleviate DOX-induced hepatotoxicity. In this study, we investigated the therapeutic potential of esculetin against DOX-induced side effects in rat liver via antioxidant systems.

2 | MATERIALS AND METHODS

2.1 | Animal care and experimental design

Male Sprague-Dawley rats (*Rattus norvegicus*, 180 ± 20 g, $n = 48$) were purchased from Atatürk University Medical Experimental Application and Research Center. The animals were kept under standard conditions (12 h light/12 h darkness photoperiod at 22 ± 1°C temperature, 60% relative humidity). Water and standard pellets were supplied ad-libitum. After 1 week of acclimatization, experimental animals were randomly divided into six groups and treated as follows:

Control group: The rats were intraperitoneally injected with normal saline for 14 days.

DOX group: Rats were intraperitoneally injected with DOX (5 mg/kg) every other day for 14 days.

E50 group: The rats were intraperitoneally injected with esculetin (50 mg/kg) daily for 14 days.

E100 group: The rats were intraperitoneally injected with esculetin (100 mg/kg) daily for 14 days.

DOX + E50 group: The rats were intraperitoneally injected with DOX and esculetin as in the DOX and E50 groups. Esculetin was administered 1 h before the DOX treatment.

DOX + E100 group: The rats were intraperitoneally injected with DOX and esculetin as in the DOX and E100 groups. Esculetin was administered 1 h before the DOX treatment.

Cumulative DOX dose and administered esculetin concentrations were determined by considering previous studies.[14–16] After 14 days, the rats of all groups were sacrificed by ketamine/xylazine anesthesia. Liver tissues were collected, frozen immediately in liquid nitrogen and stored at −80°C until analysis. All experimental procedure was carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals following approval by the Atatürk University Local Ethics Council for Animal Experiments (Protocol Number: 2021/4–123).

2.2 | Sample collection and processing

After 2 weeks of application, the animals were sacrificed by cervical dislocation under anesthesia. Liver tissues were quickly removed, frozen in liquid nitrogen and stored at −80°C for the experimental analyses.

2.3 | RNA isolation and gene expression analysis

Total RNA extraction from liver tissues was performed using the RNA isolation kit (EcoPURE total RNA kit, EcoTech) according to the manufacturer's instructions. Purity and concentration of the isolated RNA were determined by the Multiskan GO Microplate Spectrophotometer (Thermo Scientific). cDNA was synthesized from total RNA by iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's procedure. Gene expression levels were determined using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) as described by the manufacturer in Rotor-Gene Q (Qiagen). Gapdh was used as a housekeeping gene to normalize the data. The primer sequences used are described in the previous study.[17] The relative mRNA expression of target genes was assessed the $2^{-\Delta\Delta Ct}$ formula.[18]

2.4 | Preparation of tissue homogenates

Liver tissues were homogenized in 50 mM phosphate buffer (pH:7.4) containing 1 mM EDTA and 1 mM DTT using TissueLyser LT device (Qiagen). The homogenates were centrifuged at 10,000 rpm and 4°C for 30 min. The supernatant was used for biochemical analysis. The protein content of the supernatants was determined according to the Bradford method.[19]

2.5 | Examination of antioxidant enzyme activities

Superoxide dismutase (SOD) activity was assayed according to the method recommended by Sun et al.[20] One unit of SOD was defined as the amount of enzyme inhibited 50% of nitroblue tetrazolium chloride reduction. Catalase (CAT) activity was determined spectrophotometrically using the method described by Aebi.[21] One unit of CAT was expressed as the amount of the enzyme required to decompose hydrogen peroxide per minute. Glutathione peroxidase (GPx) activity was measured according to the method reported by Wendel et al.[22] One unit of GPx was defined as the amount of enzyme required to oxidize NADPH per minute.

2.6 | Measurement of malondialdehyde and glutathione levels

Malondialdehyde (MDA) content was determined using the method described by Ohkawa et al.[23] The level of MDA was calculated using a standard curve of 1,1,3,3-tetramethoxypropane (Sigma-Aldrich) and expressed as nmol/mg protein. Reduced glutathione (GSH) level was measured according to the method of Sedlak and Lindsay.[24] GSH level was determined using a standard curve of L-glutathione reduced (Sigma-Aldrich) and defined as nmol/mg protein.

2.7 | Molecular docking study

The crystal structures of the GPx,[25] SOD,[26] and CAT[27] were acquired from the Protein Data Bank (PDB IDs: 5L71, 4DVH, and 1DGF, respectively). Schrödinger's[28] Protein Preparation Wizard module[29] was utilized to ensure proper preparation of the structures according to our previous studies.[30,31] This process involved adding missing hydrogens, assigning bond orders, and removing all water molecules and heteroatoms as in our previous works.[32,33] Subsequently, a restrained minimization using the OPLS4 force field[34] was implemented to address overlapping hydrogens.[35] The residues of the active site that were ascertained using the SiteMap tool[36,37] were determined in the module for generating the receptor grid.[38,39] Before molecular docking, DOX and esculetin were also subjected to preparation to generate all possible 3D conformations and states under physiological pH conditions,[40] utilizing the LigPrep module.[41] The molecular docking process was carried out using the Glide XP method.[42]

2.8 | Statistical analysis

Experimental data were expressed as mean ± SD and statistically analyzed using Prism software (GraphPad Software, San Diego, CA). Statistical comparison of the data was performed by one-way ANOVA and Tukey's post-hoc test. The statistically significance level was considered as $p < 0.05$.

3 | RESULTS AND DISCUSSION

DOX stands out as a potent anticancer agent, exhibiting a broad spectrum of activities. Nevertheless, its efficacy is accompanied by a plethora of adverse effects, particularly manifesting prominently in various organs, notably the liver.[4] In parallel with its influence on the liver and the heart, DOX, especially in the form of DOX semiquinone, instigates oxidative stress and induces the generation of ROS. This involvement plays a pivotal role in the initiation and progression of hepatotoxicity, as outlined by Rashid et al.[43] DOX undergoes one-electron reduction facilitated by NADPH-cytochrome P450 reductase. This reduction, in turn, is reflected in a diminished activity of antioxidant enzymes, encompassing GSH, GPx, and GR. Concurrently, there is a suppression of SOD and CAT activities, coupled with an escalation in MDA levels.[44] Enduring reservoirs of bioactive compounds, natural products have served as a perennial source of therapeutic agents. The progression in the therapeutic utilization of natural products has evolved from traditional applications involving the use of the entire plant or unprocessed entities to the adoption of standardized extracts.[45] Subsequently, this evolution has advanced to the isolation of specific compounds characterized by well-defined molecular structures.[46] The categorization of compounds exhibiting analogous molecular structures and closely shared pharmacological activities holds the promise of unraveling intricate structure–activity relationships.[47] Such classifications not only elucidate these relationships but also serve as catalysts, prompting chemists to embark on the lead optimization process.[48]

Dietary intervention is one of the anticipation ways for prevention of anticancer-related toxicity to minimize the harmful effects.[49,50] Natural products or natural remedies with a wide range of medicinal properties, including antioxidant, antimutagenic anticancer effects, and anticarcinogenic have drawn researchers' interest.[51,52]

The potential protective role of antioxidants against the adverse effects and toxic manifestations induced by various anticancer drugs, including DOX, has been extensively explored.[53–55] Nonetheless, significant concerns persist regarding the plausible negative impact of these agents on the intrinsic anticancer properties of chemotherapy, a consideration that cannot be dismissed.[56] Within this category of compounds, esculetin has been singled out for in-depth scrutiny.

In this investigation, the study unveils the manifestation of DOX-induced liver damage through a noticeable reduction in CAT/SOD and GPx activity. Moreover, elevated MDA levels were observed subsequent to DOX treatment. Strikingly, rats that were concurrently administered esculetin alongside DOX exhibited diminished levels of oxidative stress. This compelling study underscores the potential therapeutic efficacy of esculetin in mitigating the adverse effects of DOX-induced liver damage. The enzymes SOD, CAT, and GPx play a crucial role in averting cell damage caused by free radicals.[57] SOD functions by reducing the levels of superoxide anion through catalyzing the conversion of superoxide to oxygen and hydrogen peroxide. This process effectively shields the cell from both exogenous and endogenous superoxide formation.[58] Subsequently,

hydrogen peroxide, generated in this process, undergoes detoxification facilitated by CAT and GPx.

The mRNA expression and enzyme activity results of antioxidant enzymes were shown in Figure 1. DOX and DOX + E50 treatments only caused a decrease in CAT mRNA levels compared to the control group (Figure 1B). All other treatments showed no statistically significant difference in mRNA levels as compared to the control (Figure 1A–C). Activities of SOD, CAT and GPx were found to be significantly decreased in the liver of DOX-treated rats compared to the control group (Figure 1D–F). On the other hand, E100 treatment caused a statistically significant increase in SOD and GPx activity compared to control (Figure 1D, F). Treatment of DOX with esculetin at a dose of 50 mg/kg decreased CAT and GPx activities compared to the control group (Figure 1E, F). However, treatment with DOX and esculetin at a dose of 100 mg/kg restored the DOX-induced decrease in SOD and CAT activities by bringing it closer to the values of the control group (Figure 1D, E).

The etiology of DOX-induced toxicity is associated with the release of free radicals, a decline in antioxidant enzyme levels, and an escalation in lipid peroxidation, as documented in the literature.[59] An assessment of the therapeutic influence on GPx, SOD, CAT, enzyme activities, alongside MDA and GSH levels in rats exposed to DOX, unequivocally establishes that esculetin effectively alleviates oxidative damage induced by DOX in the liver. Aksu et al.[60]

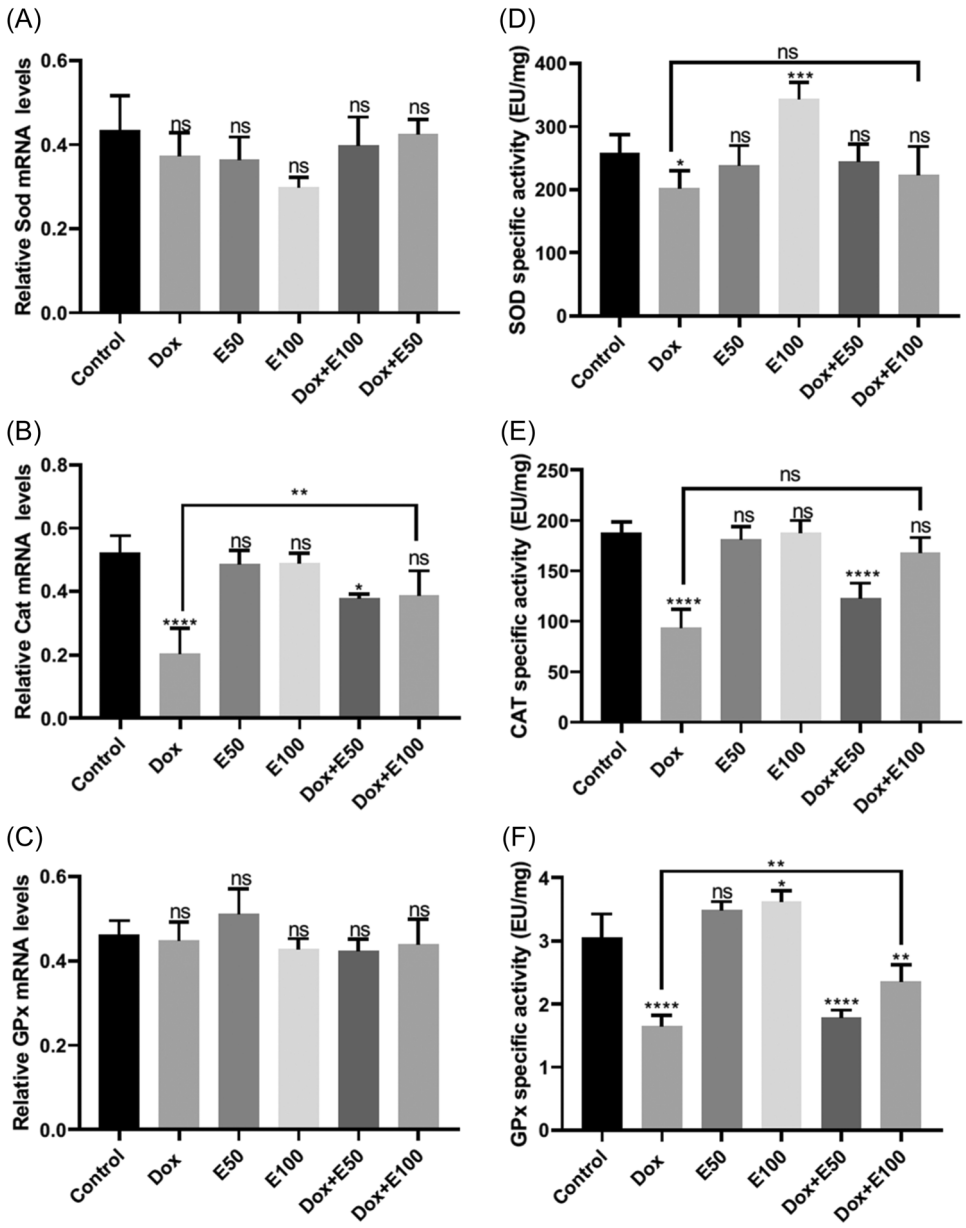

FIGURE 1 Relative mRNA levels (A–C) and activities (D–F) of SOD, CAT and GPx in the liver of rats after the treatments. Each bar represents mean ± SD of five animals in each group. Asterisk indicates statistically significant difference between the means: ns $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

exhibited that in the DOX group, CAT, SOD, and GSH-Px activities, along with GSH levels, were markedly lower compared to the control and curcumin groups. Liao et al.[61] documented a significant increase in oxidative stress parameters, including GPx, GR, CAT, NO and MDA in the hippocampus of rats treated with DOX. Conversely, the levels of antioxidant enzymes exhibited a noteworthy decrease in the same experimental group. In a separate study, the comparison of melatonin-treated cells and mice with models treated with DOX revealed significantly lower levels of MDA. Concurrently, the activities of SOD and GPx were markedly higher in the melatonin-treated group.[62]

MDA, arising from enzymatic processes and lipid peroxidation, as well as prostaglandin biosynthesis, represents an endogenous genotoxic byproduct.[63] The interaction of ROS with poly-unsaturated fatty acids, integral components of cell membranes, instigates lipid peroxidation. This phenomenon induces membrane function degradation, causing a decline in membrane fluidity and compromising the activities of membrane-dependent enzymes and receptors.[64] The tripeptide GSH, when in its reduced state, serves the dual purpose of directly eliminating ROS and acting as a cofactor in detoxification pathways.[65] GSH is instrumental in maintaining cellular components in a reduced form, enzymes and any reduction in GSH levels may precipitate cellular demise.[66]

The levels of MDA and GSH in the liver tissues were represented in Figure 2. DOX treatment caused a statistically significant increase in the level of MDA and a decrease in the level of GSH compared to the control group. Treatment of DOX with esculetin at a dose of 50 mg/kg caused a decrease in MDA levels (Figure 2A), but did not change GSH levels (Figure 2B) compared to the control group. However, treatment of DOX with esculetin at a dose of 100 mg/kg ameliorated DOX-induced changes in both MDA and GSH levels by bringing their levels closer to the level of the control group.

A multitude of researchers have concurred on the significant elevation of MDA levels in tissues following DOX treatment.[67] Kuzu et al.[68] conducted an investigation to assess the protective efficacy of morin pretreatment against DOX-induced kidney and liver damage. The evaluation encompassed liver and kidney function tests, Bcl-2 measurement associated with apoptosis, inflammation markers, antioxidant parameters, immunohistochemical and histo-pathological analyses. In this study, a notable augmentation MDA levels was discerned in both kidney and liver and tissues in comparison to the control group. However, pretreatment with morin demonstrated a substantial preventive effect on this increase, with the extent of mitigation being dose-dependent. In a study undertaken by Tulubas et al.[69] akin to our own investigation, it was reported that DOX led to a significant escalation in MDA levels in liver and kidney tissues, concurrently resulting in a notable decrease in GSH levels. Aksu et al.[61] showed the DOX group exhibited the highest MDA levels, contrasting with the lowest MDA levels observed in the control and curcumin groups. Remarkably, the treatment groups displayed a dose-dependent escalation in MDA levels in comparison to the DOX group. Alzokaky et al.[70] showed the induction of ROS, DOX was effectively curtailed through the intervention of metformin (Met), as evidenced by the mitigation of MDA and nitric oxide (NO) levels. This observation substantiates the assertion that Met exerts a preventive influence on oxidative stress, manifesting in the reduction of ROS and MDA levels, coupled with an augmentation in the synthesis and activity of GSH.

A comprehensive investigation was conducted to obtain additional information regarding the binding modes of DOX and esculetin. This study aimed to explore the behavior of these agents within the binding sites of GPx, SOD, and CAT proteins through a thorough examination of the structure–activity relationship. The results presented in Figures 3–5 demonstrate that DOX and esculetin exhibit similar conformations when situated in the binding pockets of GPx, SOD, and CAT, respectively. The primary interactions established with GPx, SOD, and CAT involve hydrogen bonding. Upon comparing the placement scores of DOX and esculetin (−6.159 and −2.476 kcal/mol for GPx, −5.545 and −3.677 kcal/mol for SOD, and −8.209 and −3.279 kcal/mol for CAT, respectively), it is observed that

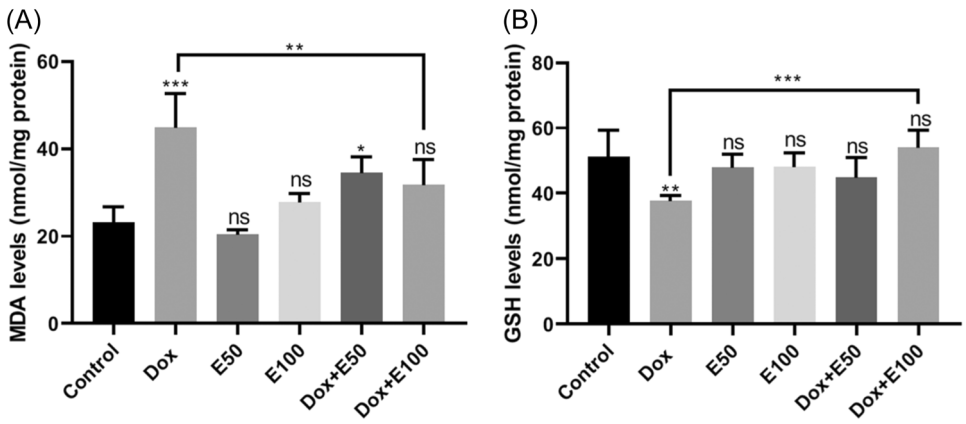

FIGURE 2 Levels of malondialdehyde (MDA) (A) and glutathione (GSH) (B) in the liver of rats following the respective treatments. Each bar denotes the mean ± standard deviation (SD) of five animals in each group. An asterisk signifies a statistically significant difference between the means: ns $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

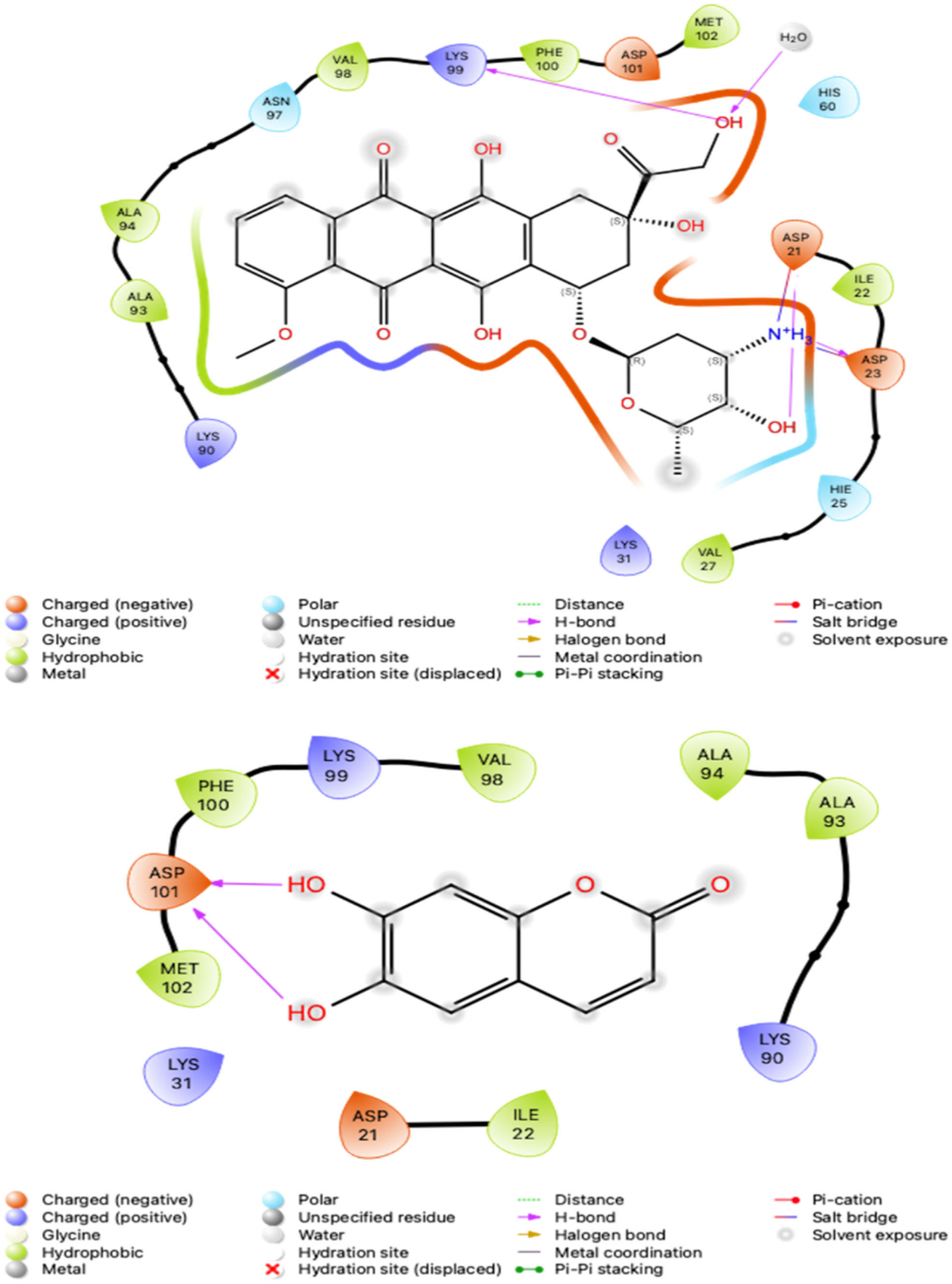

FIGURE 3 The two-dimensional docking positions of DOX, displayed at the top, and esculetin, shown at the bottom, were observed with the key amino acids located within the binding site of GPx, which PDB ID 5L71 identifies. To maintain clarity, only the amino acids that interacted were depicted.

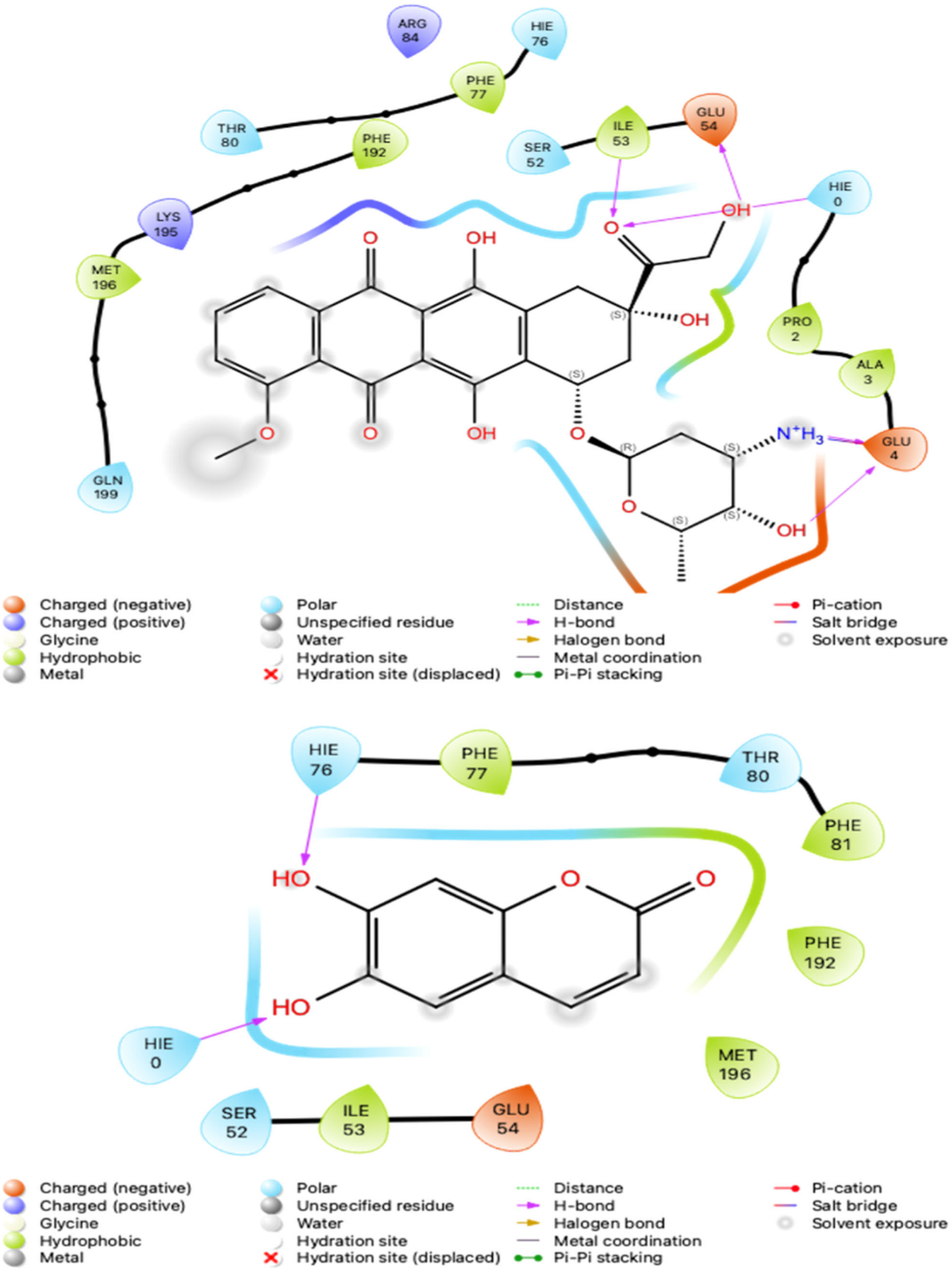

FIGURE 4 The two-dimensional docking positions of DOX, displayed at the top, and esculetin, shown at the bottom, were observed with the key amino acids located within the binding site of SOD, which PDB ID 4DVH identifies. To maintain clarity, only the amino acids that interacted were depicted.

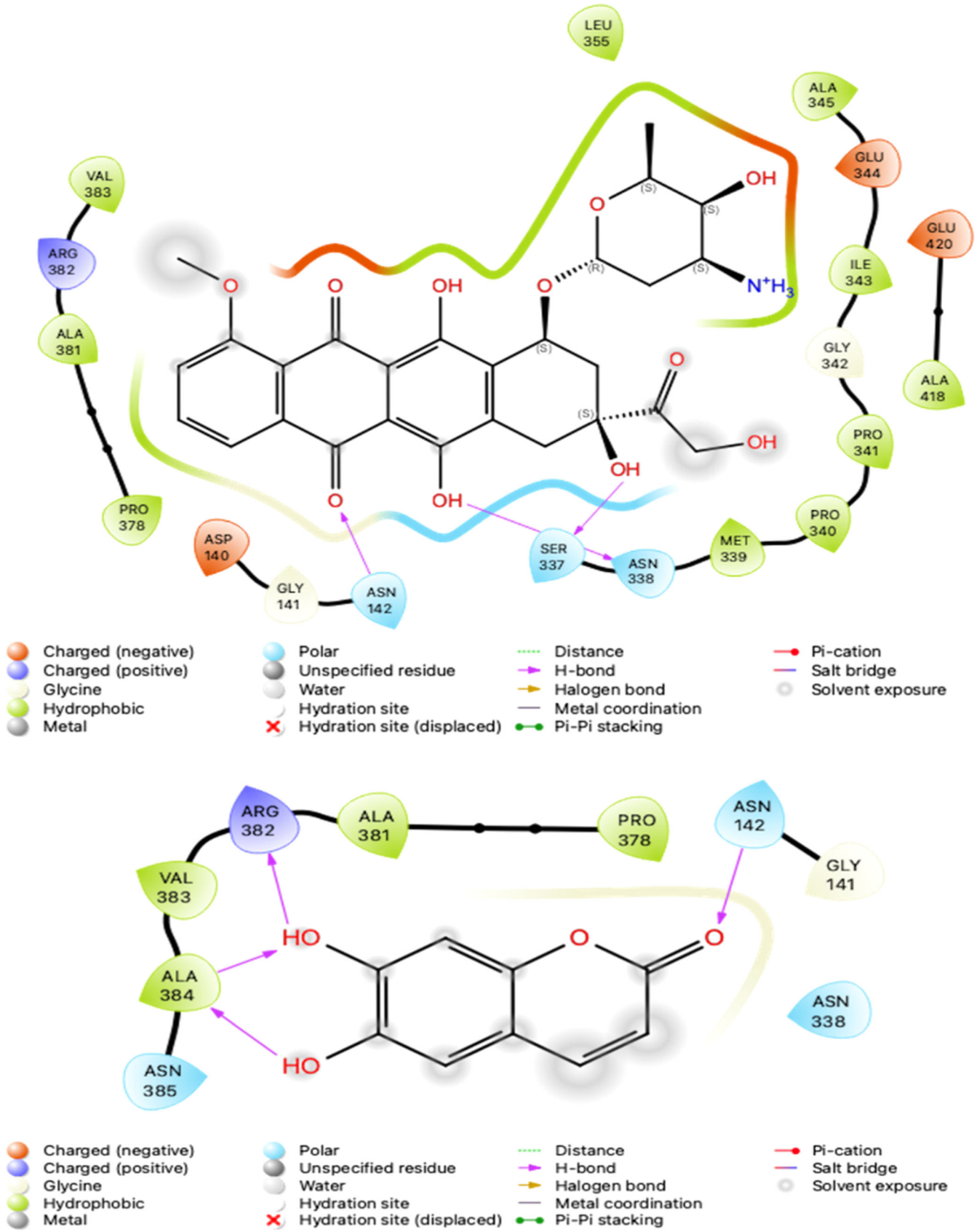

FIGURE 5 The two-dimensional docking positions of DOX, displayed at the top, and esculetin, shown at the bottom, were observed with the key amino acids located within the binding site of CAT, which PDB ID 1DGF identifies. To maintain clarity, only the amino acids that interacted were depicted.

the docking scores of DOX are comparatively lower. These lower values indicate a favorable compatibility of the agent within the binding pocket of the target receptor, resulting in the formation of a stable ligand-receptor complex.

4 | CONCLUSION

In conclusion, the results of the current study indicate that esculetin may play a role in protecting against the hepatotoxicity of DOX by improving DOX-induced changes in the antioxidant defense system in rat liver. However, further studies, safety tests and clinical studies are needed to confirm the protective properties of esculetin against DOX-induced hepatotoxicity and to precisely determine its mechanisms of action.

AUTHOR CONTRIBUTIONS

Zeynep Köroğlu: Investigation; Validation; Formal analysis. **Duygu Kizir**: Investigation; Validation; Methodology; Formal analysis; Data curation. **Melike Karaman**: Investigation; Validation; Writing - review & editing; Data curation; Formal analysis. **Yeliz Demir**: Writing - original draft; Writing - review & editing; Investigation; Formal analysis; Resources. **Cüneyt Türkeş**: Investigation; Methodology; Validation. **Şükrü Beydemir**: Software; Methodology.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All experimental procedures were performed in accordance with a protocol approved by Atatürk University Local Ethics Council for Animal Experiments (Protocol No: 2021/4–123).

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