International Journal of Innovative Research and Scientific Studies, 8(1) 2025, pages: 223-238

IJIRSS
International Journal of Innovative Research & Scientific Studies

ISSN: 2617-6548

URL: www.ijirss.com

The potential hepatoprotective effect of Erythropoietin against liver damage induced by Doxorubicin through modulation of PI3K/Akt/GSK3β and activation of Nrf2/HO-1 pathway

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Abstract

This study aims to evaluate the hepatoprotective effect of erythropoietin against doxorubicin-induced liver damage in a male rat model through PI3K/Akt/GSK3β axis modulation and activation of the Nrf2/HO-1 pathway. Doxorubicin, a chemotherapeutic antibiotic used to treat many types of cancer, has some considerable side effects that limit its clinical use, including hepatotoxicity. Erythropoietin is a glycoprotein hormone primarily recognized for its crucial function in promoting erythropoiesis. Recent studies have demonstrated the significant cytoprotective effects of erythropoietin in several organs. Thirty-six male Wistar rats were randomly separated into six groups: (Group 1) a negative control group; (Group 2) a doxorubicin induction group; (Groups 3, 4, and 5) three groups pre-treated with erythropoietin in three different doses (1000, 3000, and 6000 IU/kg); and (Group 6) a positive control group pre-treated with silymarin (100 mg/kg). On the 7th day, all groups (except group 1) were injected with a single dose of doxorubicin (20 mg/kg). On day 9 of the experiment, blood and liver samples were taken for subsequent analysis. The results showed that erythropoietin pre-treatment significantly reduced doxorubicin-induced hepatotoxicity by stimulating the PI3K/Akt and Nrf2/HO-1 pathways and suppressing GSK3β and caspase 3. In addition to that, erythropoietin pre-treatment also raised the levels of GSH, lowered the levels of MDA, and brought the levels of the liver enzymes ALT and AST back to normal compared to the induction group. In conclusion, via modulating PI3K/Akt/GSK3β and activating the Nrf2/HO-1 pathway, erythropoietin pre-treatment protected the liver against hepatotoxicity caused by doxorubicin in a dose-dependent manner.

Keywords: Doxorubicin, Erythropoietin, Hepatotoxicity, Nrf2/HO-1, PI3K/Akt/GSK3β, caspase 3, ALT, AST, GSH, MDA.

DOI: 10.53894/ijirss.v8i1.3656
Funding: This study received no specific financial support.
History: Received: 2 April 2024/**Revised:** 9 September 2024/**Accepted:** 4 October 2024/**Published:** 22 October 2024

Competing Interests: The authors declare that they have no competing interests.
Authors' Contributions: Data curation, formal analysis, investigation, project administration, resources, software, visualization, writing - original draft, and writing - review & editing, Z.J.S.; conceptualization, methodology, resources, supervision, validation, writing - review & editing, Y.M.K.; conceptualization, investigation, methodology, resources, supervision, validation, writing - review & editing, H.J.W. All authors have read and agreed to the published version of the manuscript.
Transparency: The authors confirm that the manuscript is an honest, accurate, and transparent account of the study; that no vital features of the study have been omitted; and that any discrepancies from the study as planned have been explained. This study followed all ethical practices during writing.
Institutional Review Board Statement: The Ethical Committee of the Scientific Committee in the Pharmacology and Toxicology Department at the College of Pharmacy, Mustansiriyah University, Iraq has granted approval for this study on 15 October 2023 (Ref. No. 19).
Publisher: Innovative Research Publishing

1. Introduction

The effectiveness of chemotherapy, a critical component in cancer treatment, often faces compromise from unintended side effects such as neurotoxicity, nephrotoxicity, and cardiotoxicity [1]. Moreover, hepatotoxicity poses a significant hindrance to the broader utilization of these life-saving agents [2].

Originally derived from a soil-based bacterium, doxorubicin (DOX) showed remarkable effectiveness against various cancers, including leukemia, lymphomas, and solid tumors. Its use expanded to include breast, ovarian, and soft tissue sarcomas, promoting remission and prolonging survival in cancer patients [3]. Despite its therapeutic benefits, DOX might potentially induce liver damage when administered in certain dosages, which limits its frequency of usage in medical treatment [4]. Treatment with DOX presents a significant clinical concern due to liver toxicity; up to 40% of patients exhibit increased liver enzymes [5]. DOX metabolism can produce reactive oxygen species (ROS) and ultimately oxidative stress, which is considered the main mechanism by which DOX can harm liver tissue [6].

Recent research revealed that DOX could cause damage and kill cells by interfering with the phosphoinositide-3 kinase (PI3K)/Akt pathway [7]. The PI3K pathway is a crucial mechanism for maintaining cellular homeostasis and responding to external stimuli. It is essential for migration, metabolism, cell growth, survival, and proliferation. It also coordinates several effectors, including Akt (also known as protein kinase B) [8]. Akt is a crucial node in cell signaling that occurs in response to growth factors, cytokines, and other stimuli. A high-affinity interaction with phosphatidylinositol (3,4,5)-trisphosphate (PIP3) recruits Akt to membranes. After activation, Akt phosphorylates a host of protein substrates in diverse subcellular locations [9]. Glycogen synthase kinase-3β (GSK3β), a redox-sensitive serine/threonine kinase, is abundantly expressed in cells and is involved in cell differentiation, proliferation, and apoptosis [10]. It is constitutively active and inactivated by Akt-induced phosphorylation at specific binding sites. GSK3β is known for promoting apoptosis by facilitating caspase-3 [11].

An important transcription factor is nuclear factor erythroid-2-related factor (Nrf2). It controls many normal and abnormal processes, especially the redox status of cells. In the cytoplasm, Nrf2 is stabilized by Kelch-like epichlorohydrin-related proteins (Keap1), which are adaptors for substrates of the E3 ubiquitin ligase complex. In the absence of electrophilic stimuli, Keap1, a redox sensor, acts as a negative regulator of Nrf2 [12]. Oxidative stress causes Keap1 to detach from Nrf2, leading to its dissociation. Nrf2 stabilizes, allowing it to translocate to the nucleus [13]. Through heterodimerization with small musculoaponeurotic fibrosarcoma (sMaf) proteins, Nrf2 binds to the antioxidant response element (ARE). This transcription process regulates gene expression, activates antioxidant enzymes, removes harmful substances, and facilitates cell protection mechanisms [14]. Among the numerous cytoprotective enzymes controlled by the Keap1/Nrf2 pathway is heme oxygenase-1 (HO-1). It helps break down heme to produce carbon monoxide (CO), biliverdin, and iron ions [15]. Prior research has demonstrated that DOX may impact this pathway, reduce the cell's resistance to oxidative damage, and result in cytotoxicity [16, 17]. The coordination of erythropoiesis, the process that produces red blood cells, depends on the unique hormone erythropoietin (EPO). It's a glycoprotein hormone that's mostly made and released by kidney-specific cells; however, the liver can also synthesize only a small amount of it [18]. Medical professionals often use it to treat anemia resulting from bone marrow, cancer treatment, or chronic renal disease [19, 20]. A number of new studies have provided evidence for EPO as a pleiotropic cytokine with diverse non-hematopoietic functions, and researchers found that EPO may have cytoprotective properties in addition to its main role in erythropoiesis [21]. Neurons, cardiomyocytes, hepatocytes, and other cells contain EPO receptors (EPORs) [22, 23]. Activating EPOR starts several biological pathways, including the PI3k/Akt, STAT5, and mitogen-activated protein kinase pathways [24, 25]. So, there is increased interest in finding out how EPO might help protect different organs, like the brain, heart, and liver, from cellular stress and damage [26].

Scientific studies have demonstrated that silymarin, a flavonoid, exhibits antioxidant properties and mitigates the toxicity of DOX in certain organs. This concern has been a motive to use silymarin as a positive control in this study [27, 28].

Research on the preventive properties of EPO, particularly its effects on liver health, is limited. There is a gap in understanding how EPO can mitigate oxidative stress, decrease apoptosis, and enhance liver cell survival in the context of chemotherapy-induced hepatotoxicity. Further studies are needed to understand how EPO interacts with oxidative stress and inflammatory pathways, as well as how it affects the PI3K/Akt/GSK3β and Nrf2/HO-1 pathways. This knowledge gap underscores the need for a more comprehensive understanding of EPO's mechanisms to reduce liver damage and develop novel treatment strategies. Therefore, this study aims to find out if EPO can protect against liver damage caused by DOX in a male rat model by modulating the PI3K/Akt/GSK3β pathway and activating the Nrf2/HO-1 pathway and comparing this effect to silymarin.

2. Materials and Methods

2.1. Materials

Adriamycin® 50 mg /vial (Doxorubicin hydrochloride HCL) was obtained from Pfizer/USA. Espogen prefilled plus inj. 4000 IU/0.4ml pre-filled syringe (PFS) (recombinant human erythropoietin Alpha) was purchased from LG Chem/Korea. Silymarin powder (pure) was obtained from Spectrum Chemical Corp./USA. Tween 80 (2%) were procured from Sigma Aldrich/ USA and were also used in the study.

2.2. Experimental Animals

Thirty-six, non-previously treated, adult male Wistar rats weighing around 200–220 g were taken in this work. Rats were obtained from the animal house of the Iraqi Center for Cancer Research and Medical Inheritance/Mustansiriyah University.

Before starting the intended study, animals were separated into six groups and preserved in plastic cages of (20x25x35 cm) dimensions, which were kept in a well-ventilated place with woodchip bedding. During the experimental period, climate-controlled conditions were maintained at an appropriate temperature and relative humidity, regulating the duration of light

exposure regulated by an artificial lighting system that followed a 12-hour light and 12-hour dark cycle. They left for 7 days for acclimation. Food and water were available *ad libitum* throughout the experiment.

2.3. Experimental Design

The animals were randomly divided into six groups (n = 6 per group).

Group 1: Negative control: rats were given distilled water (1 mL/kg/day) intraperitoneally (IP) for 7 days.

Group 2: The induction rats in this group were injected with distilled water (1 ml/kg/day) IP for 6 days. Each animal received a single IP injection of DOX at a dose of 20 mg/kg on day 7 of the experiment.

Group 3: Pre-treatment group: Rats in this group were injected with 1000 IU/kg/day of EPO IP for 6 days. Each animal received a single IP injection of DOX at a dose of 20 mg/kg on the seventh day.

Group 4: Pre-treatment group: Rats were injected with EPO (3000 IU/kg/day) IP for 6 days. Then, on day 7, a single IP injection of DOX at a dose of 20 mg/kg was given to each rat.

Group 5: Pre-treatment group: Rats in this group were injected with EPO 6000 IU/kg/day IP for 6 days. On day 7 of the experiment, a single IP injection of DOX at a dose of 20 mg/kg was given to each animal.

Group 6: Positive control: Rats in this group were pre-treated by an IP injection of 100 mg/kg/day of silymarin that was dissolved in distilled water and Tween 80, for 6 days. On the seventh day, a single IP injection of DOX at a dose of 20 mg/kg was given to each rat.

2.4. Drug Administration

The mean body weight for each group was taken to determine the doses of DOX, EPO, and silymarin. Each rat in groups 3, 4, 5, and 6 received calculated doses of EPO and silymarin during the first six days of the experiment, along with an equal volume of distilled water and Tween 80 for the rats in the negative control group and an equal volume of distilled water for the rats in the induction group. Silymarin was prepared by dissolving each 25 mg of silymarin pure powder in 1 ml of distilled water and 0.1 ml of Tween 80 [29]. Hepatotoxicity was induced on the 7th day through IP injection of DOX at a dosage of 20 mg/kg [30]. DOX was prepared by dissolving 50 mg of the substance in 25 ml of distilled water, in accordance with the instructions provided by the manufacturer. The injection was administered to all groups, except for group 1, which received an IP injection of distilled water. The left arm held each rat in an upright position. After wiping the abdomen with alcohol spray and gauze, the needle was gently inserted into the right lower part of the rat's abdomen. As the needle was inserted into the animal, a gentle aspiration was done to see if it had hit an internal organ. If there was no blood coming out of the aspiration, it meant that no damage had been done, and then slowly, the desired drug was injected IP [31].

2.5. Blood and Tissue Sampling

On the 9th day of the experiment, after 48 hours of administering DOX, the animals were anesthetized by injecting xylazine (10 mg/kg) and ketamine (100 mg/kg). About 5-7 ml of blood was obtained from every rat using an intracardiac puncture of the right ventricle. The serum was promptly collected by separating it from the blood and then kept at a temperature of -20 °C for further evaluations of GSH, MDA, ALT, and AST [32, 33]. After collecting blood samples, the rats were euthanized while under anesthesia. The liver was divided into two halves. One sample was stored in plain tubes at a temperature of -20 °C to conduct enzyme-linked immunosorbent assay (ELISA) analysis on Nrf2, HO-1, and caspase 3. The second half was placed in Eppendorf tubes containing 300 µl of Triazole reagent and kept at -40 °C for RT-PCR experiments.

2.6. Measurement of PI3K, Akt, and GSK3β in Liver Tissues using Real Time-Polymerase Chain Reaction (RT-PCR)

The housekeeping gene or endogenous control used in this study was β-actin, and the data were normalized to it. All the primers were obtained from Alpha DNA Ltd. (Canada). Table 1 displays all primer sequences utilized in this study's assays.

Table 1.
Primer sequences used in real-time polymerase chain reaction (RT-PCR).

Gene name	Temperature	Sequence of primers
Akt	56 ^{0}C	ACCTCATGCTGGACAAGGAC GTGTCCCGCAGAACGTCTT
β- actin	64 ^{0}C	CTATCGGCAATGAGCGGTTCC TGTGTTGGCATAGAGGTCTTTACG
GSK3β	56 ^{0}C	GAGGAGAGCCCAATGTTTCA GTGTAATCGGTGGCTCCAAA
PI3K	56 ^{0}C	GACCGAAAGTTCAGGGTCAA AAACTCCAGCCACACATTCC

The procedure was performed according to the instructions provided in the kits. Briefly, isolation of total ribonucleic acid (RNA) from liver tissues by using the TransZol Up Plus RNA Kit (obtained from TransGen Biotech, China). After that, genomic deoxyribonucleic acid (DNA) removal and complementary DNA synthesis were done using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit (bought from TransGen Biotech, China).

The QIAGEN Rotor Gene Q Real-time PCR System was used to perform the quantitative real-time PCR (qRT-PCR). With the TransStart® Top Green qPCR Super Mix kit (obtained from TransGen Biotech, China).

The expression levels and fold changes of the PI3K, Akt, GSK3β, and β-actin genes were evaluated, and the threshold cycle (Ct) was measured [34]. Using the Efficiency Correction method [35] the following equation was used to calculate the folds of gene expression from the values of CT: Fold = $2^{-\Delta CT}$.

Where ΔCT = CT of the target gene – CT of the housekeeping gene.

2.7. Determination of Nrf2 and HO-1 in Liver Tissue Homogenate

After allowing the liver samples to come to room temperature, 1 ml of Tris lysis solution was added to lyse them and extract the proteins. After the homogenate had been sufficiently homogenized, it was centrifuged at 5000 revolutions per minute for 10 minutes. After that, an Eppendorf tube was used to retain the liquid part, which was extracted using a micropipette [36].

A sandwich enzyme immunoassay kits (obtained from Cloud-Clone Corp./USA) was used, which was intended for the accurate detection of Nrf2 and HO-1 in rat tissue homogenates according to the kits' instructions.

2.8. Determination of Caspase 3 in Liver Tissues

A sandwich enzyme immunoassay kit (procured from Cloud-Clone Corp./USA) was used to accurately detect caspase 3 in rat tissue homogenates according to the procedure described in the kit.

2.9. Measurement of Serum Enzyme Markers of Liver Damage

The liver function was estimated by measuring serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. The kits used (from Cloud-Clone Corp./USA) were sandwich enzyme immunoassay, specifically designed for the quantitative detection of ALT and AST in rat serum in an *in vivo* setting, following the instructions provided in the kit.

2.10. Measurement of Oxidative Stress Biomarkers

For the determination of glutathione (GSH) and malondialdehyde (MDA), a competitive inhibition enzyme immunoassay technique was used for the in vivo quantitative measurement of GSH and MDA in rat serum following the manufacturer's specifications (Cloud-Clone Corp./USA).

2.11. Statistical Analysis

The statistical analysis was conducted using SPSS version 24 software. The study employed a one-way ANOVA test, followed by a post hoc Tukey's multiple comparison test, to identify any significant differences among the groups. The least significant difference (LSD) test was utilized to compare the means of the groups. A p-value of less than 0.05 was considered significant. In this study, the results were presented as the Mean±standard deviation (SD). Results with non-identical superscripts (a, b, c, d) exhibited significant differences ($p<0.05$). The results with two distinct letters showed no significant difference; (ab); no statistically significant difference with (a) or (b), (bc); no statistically significant difference with either (b) or (c).

3. Results

3.1. The Effect of EPO on PI3K, Akt, and GSK3β Expression

As demonstrated in Table 2, Figure 1, and Figure 2, the mean tissue PI3K and Akt fold of expressions were significantly [$p<0.05$] decreased in the induction group injected with DOX in comparison to the negative control group. In comparison to the induction group, pre-treatment with EPO at the three doses (1000, 3000, and 6000 IU/kg) resulted in elevating the mean tissue PI3K and Akt folds of expression in a dose-dependent manner. The study also found that the group that was pre-treated with silymarin had significantly higher levels of P13K and Akt in their tissues compared to the induction group [$p<0.05$]. However, the effect of silymarin on P13K and Akt was not significantly different from that of the group that was pre-treated with EPO 3000 IU/kg.

The mean tissue GSK3β fold of expression was significantly [$p<0.05$] higher in the induction group compared to the negative control group, as shown in Table 2 and Figure 3. Pre-treatment with 1000, 3000, and 6000 IU/kg of EPO (in Groups 3, 4, and 5) resulted in a dose-dependent significant [$p<0.05$] decrease in the mean tissue GSK3β fold of expression when compared to Group 2. Also, the group that was pre-treated with silymarin had a lower level of mean tissue GSK3β compared to the induction group [$p<0.05$]. However, the effect of silymarin on GSK3β was not significantly different from that of the groups that were pre-treated with EPO 3000 and 6000 IU/kg.

Table 2.
The effect of EPO on PI3K, Akt, and GSK3β expression.

Study group	PI3K	Akt	GSK3β
Negative control	1.00± 0.00^{d}	1.00± 0.00^{d}	1.00± 0.00^{c}
Induction (DOX)	0.25± 0.04^{e}	0.33± 0.08^{e}	5.22± 0.31^{a}
EPO (1000IU/kg)	1.94± 0.14^{c}	1.78± 0.14^{c}	2.18± 0.24^{b}
EPO (3000IU/kg)	2.37± 0.17^{b}	2.52± 0.18^{b}	1.26± 0.12^{c}
EPO (6000IU/kg)	2.77± 0.23^{a}	2.97± 0.16^{a}	1.03± 0.14^{c}
Silymarin (100mg/kg)	2.48± 0.13^{b}	2.65± 0.30^{b}	1.16± 0.23^{c}
LSD	0.16	0.2	0.28

Note: Mean ± SD is used to express the results. Distinct lowercase letters (a-e) indicate statistically significant differences ($P < 0.05$) between the groups in the same column. LSD: Least significant difference.

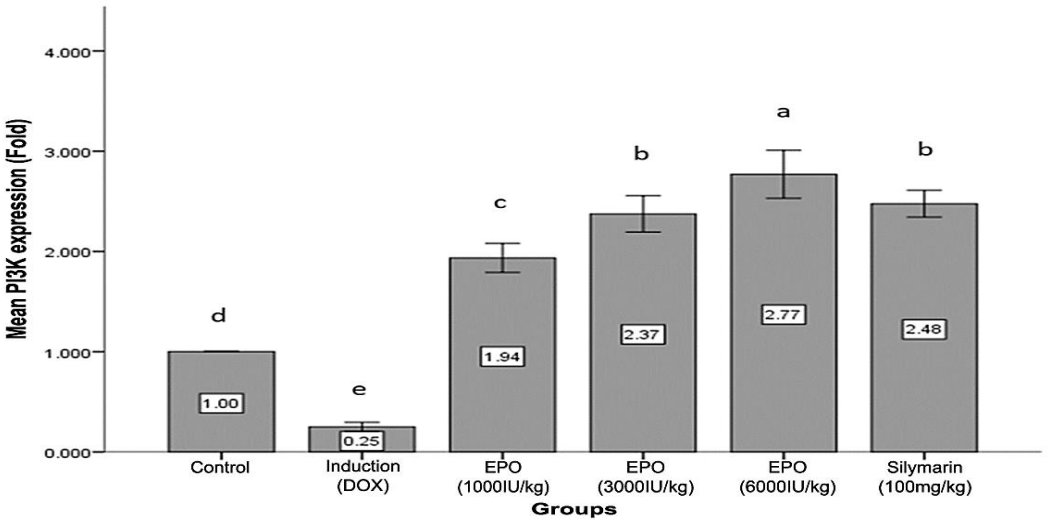

Figure 1.
The effect of EPO on PI3K expression.
Note: Mean±SD was used to express the results. Dissimilar letters (a-e) indicate a significant difference between groups (p<0.05).

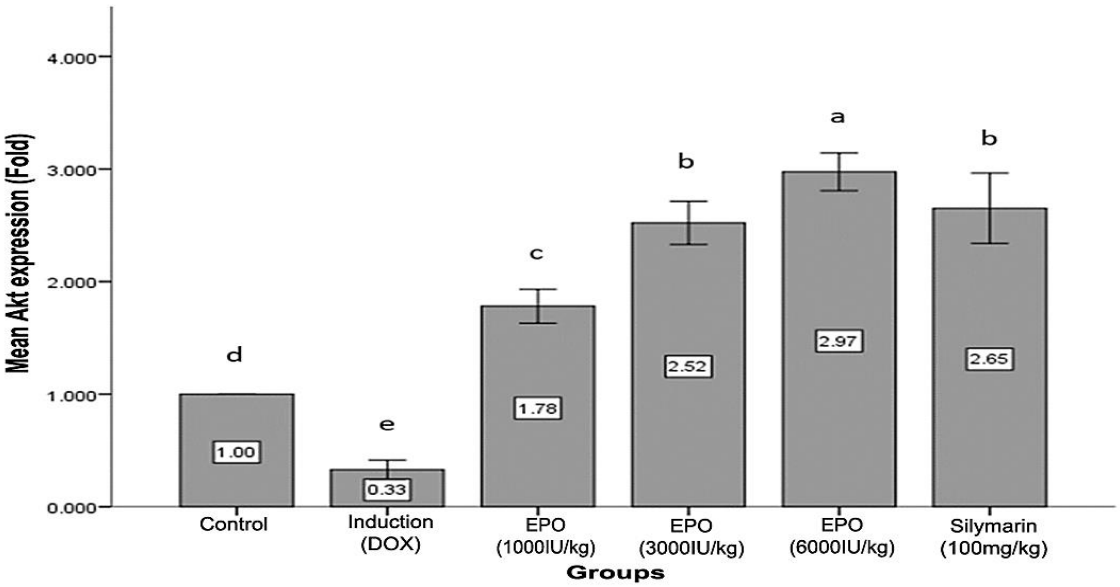

Figure 2.
The effect of EPO on Akt expression.
Note: Mean±SD was used to express the results. Different lowercase letters (a-e) above bars represent significant (P < 0.05) differences among the groups.

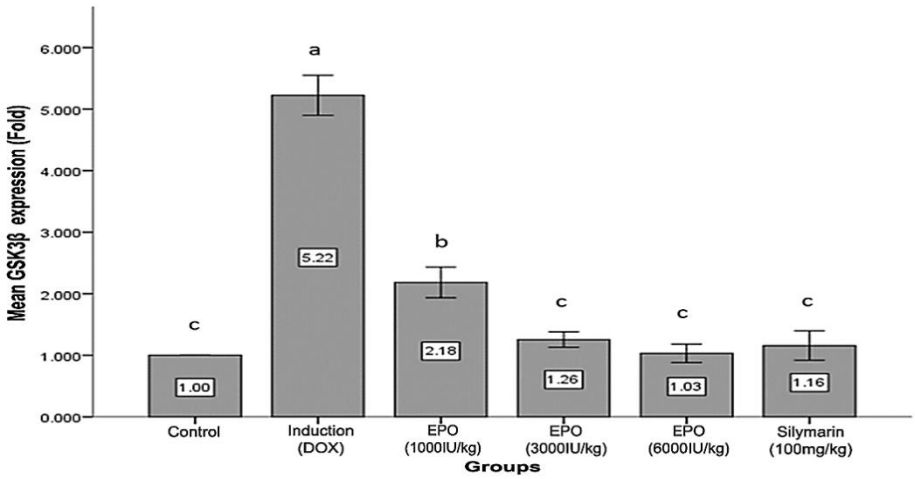

Figure 3.
The effect of EPO on GSK3β expression.
Note: Mean±SD was used to express the results. Different lowercase letters (a-c) above bars represent significant (P < 0.05) differences among the groups.

3.2. The Effect of EPO on Nrf2 and HO-1

As shown in Table 3, Figure 4, and Figure 5, the mean tissue Nrf2 and HO-1 concentrations were significantly [p<0.05] lower in the induction group injected only with DOX in comparison to the negative control group. Pre-treatment with EPO at three different doses (1000, 3000, and 6000 IU/kg) resulted in elevating the mean tissue Nrf2 and HO-1 concentrations in a dose-dependent manner compared to the induction group. Furthermore, there was no significant difference [p>0.05] between the group pre-treated with EPO 6000 IU/kg and the negative control group. In addition to that, the silymarin pre-treated group exhibited significantly [p<0.05] higher tissue Nrf2 and HO-1 concentrations in comparison to the induction group. It was also noted that the effect of silymarin on Nrf2 concentration appears to be non-significantly [p>0.05] different from the group pre-treated with EPO at the dose of 3000 IU/kg. On the other hand, the results also showed that the silymarin effect on HO-1 concentration was non-significantly [p>0.05] different from the groups pre-treated with EPO at the doses of 3000 IU/kg and 6000 IU/kg.

Table 3.
The effect of EPO on Nrf2 and HO-1.

Study group	Mean ± SD of Nrf2 (ng/mL)	Mean ± SD of HO-1 (ng/mL)
Negative control	3.13± 0.05[a]	9.46± 0.47 [a]
Induction (DOX)	1.17± 0.10[d]	3.72± 0.34[d]
EPO (1000IU/kg)	2.41± 0.07[c]	7.23± 0.23[c]
EPO (3000IU/kg)	2.88± 0.05[b]	8.57± 0.38 [b]
EPO (6000IU/kg)	3.08± 0.08[a]	9.22± 0.19 [ab]
Silymarin (100mg/kg)	2.82± 0.17[b]	8.47± 0.31 [b]
LSD	0.08	0.84

Note: Mean ± SD is used to express the results. Distinct lowercase letters (a-d) indicate statistically significant differences (P < 0.05) between the groups in the same column. Results with two different letters indicate no significant difference; (ab): No significant difference with (a&b). LSD: Least significant difference.

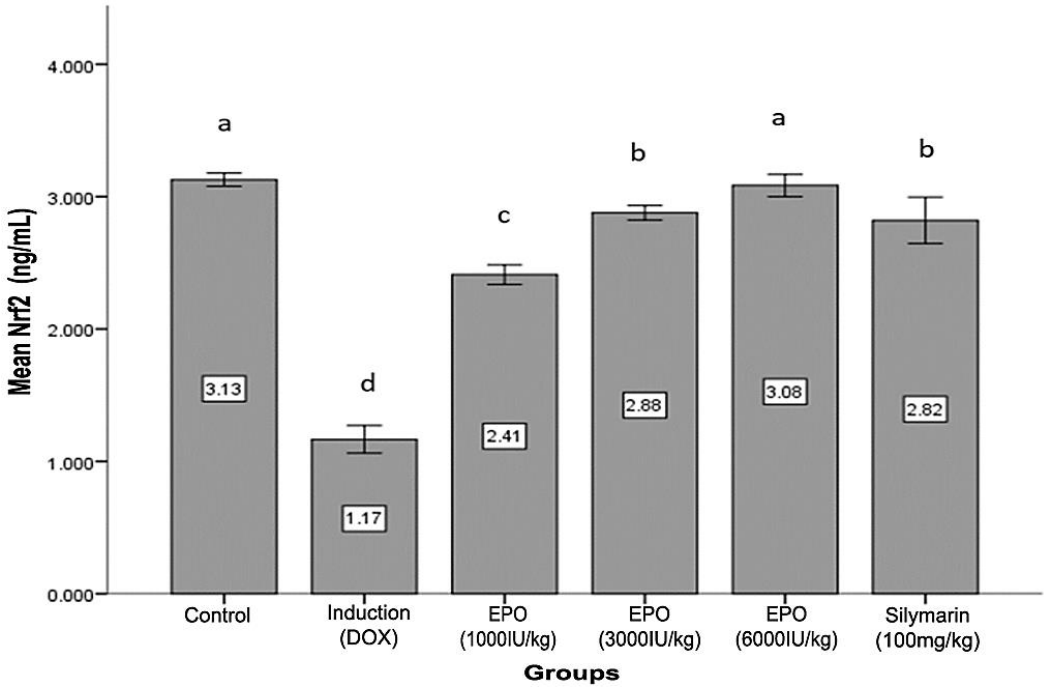

Figure 4.
The effect of EPO on tissue Nrf2.

Note: Mean±SD was used to express the results. Different lowercase letters (a-d) above bars represent significant (P < 0.05) differences among the groups.

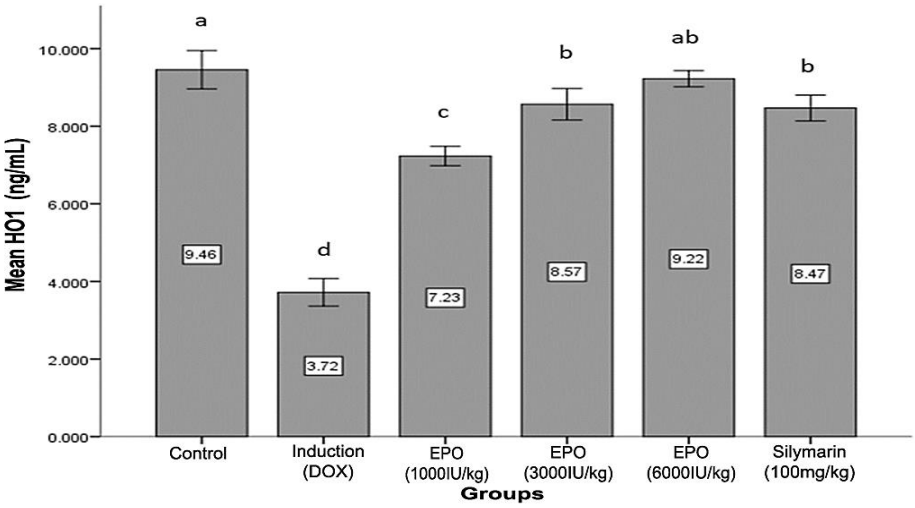

Figure 5.
The effect of EPO on tissue HO-1.
Note: Mean±SD was used to express the results. Different lowercase letters (a-d) above bars represent significant (P < 0.05) differences among the groups. Results with two different letters indicate no significant difference; (ab): No Significant difference with (a&b).

3.3. The Effect of EPO on Tissue Apoptotic Biomarker (Caspase 3)

In the induction group, DOX injection led to a significant [p<0.05] rise in the mean tissue caspase 3 concentration compared to the negative control group, as shown in Table 4 and Figure 6. While pre-treatment with EPO at all doses of 1000, 3000, and 6000 IU/kg (groups 3, 4, and 5) resulted in a dose-dependent significant [p<0.05] decrease in the mean tissue concentration of caspase 3 in comparison to the induction group. Furthermore, mean tissue caspase 3 concentration was significantly [p<0.05] decreased in the silymarin pre-treated group in comparison to the induction group, and this effect of silymarin is non-significantly [p>0.05] different from the group pre-treated with EPO 3000 and 6000 IU/kg.

Table 4.
The effect of EPO on tissue apoptotic biomarker caspase 3.

Study group	Caspase 3 (ng/mL)
Negative control	1.05± 0.08^{c}
Induction (DOX)	4.71± 0.28^{a}
EPO (1000IU/kg)	2.30± 0.12^{b}
EPO (3000IU/kg)	1.19± 0.14^{c}
EPO (6000IU/kg)	1.10± 0.11^{c}
Silymarin (100mg/kg)	1.24± 0.14^{c}
LSD	0.2

Note: Mean ± SD is used to express the results. Distinct lowercase letters (a,b,c) indicate statistically significant differences (P < 0.05) between the groups in the same column. LSD: Least significant difference.

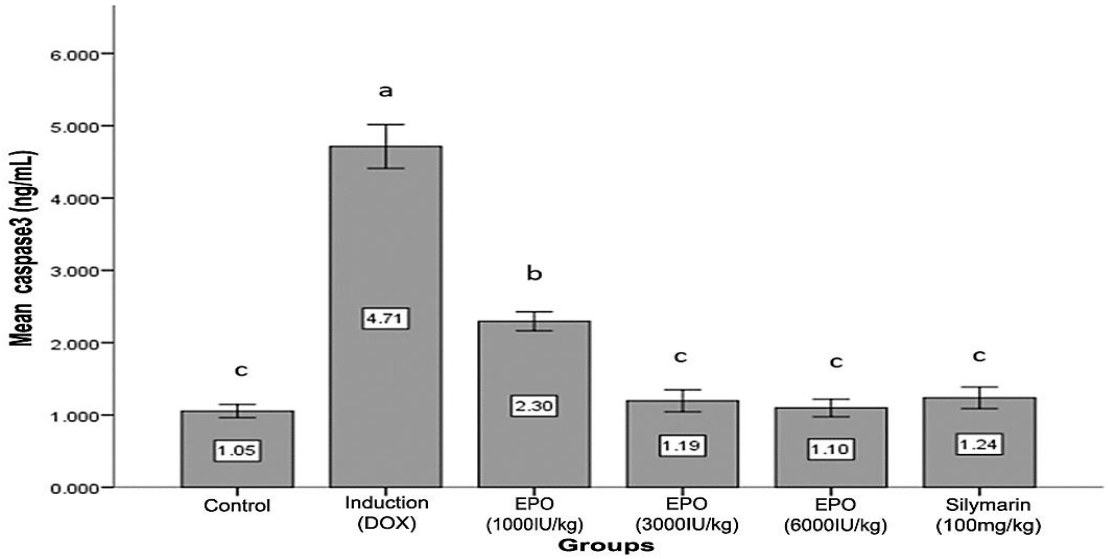

Figure 6.
The effect of EPO on tissue apoptotic biomarker caspase 3.
Note: Mean±SD was used to express the results. Different lowercase letters (a-c) above bars represent significant (P < 0.05) differences among the groups.

3.4. The Effect of EPO on Liver Function Enzymes (ALT and AST)

As shown in Table 5, Figure 7, and Figure 8, DOX injection in the induction group resulted in a significant [p<0.05] increase in the mean serum levels of the liver function enzymes ALT and AST activity in comparison to the negative control group. While pre-treatment with EPO at the doses of 1000, 3000, and 6000 IU/kg resulted in a significant [p<0.05] decrease in the mean serum levels of ALT and AST in a dose-dependent manner. In addition to that, the silymarin pre-treated group exhibited significantly [p<0.05] lower serum ALT and AST activity in comparison to the induction group. It was also noted that the effect of silymarin on ALT levels appears to be non-significantly different from the groups pre-treated with EPO at the doses of 3000 IU/kg and 6000 IU/kg. On the other hand, the results also showed that the silymarin effect on AST levels was non-significantly [p>0.05] different from the group pre-treated with EPO at the dose of 6000 IU/kg.

Table 5.
The effect of EPO on serum liver enzymes (ALT and AST).

Study group	ALT IU/L	AST IU/L
Negative control	34.55± 5.94^{c}	32.83± 4.62^{d}
Induction (DOX)	146.07± 8.54^{a}	242.67± 15.46^{a}
EPO (1000IU/kg)	56.27± 4.05^{b}	115.33± 9.66^{b}
EPO (3000IU/kg)	35.99± 4.81^{c}	58.67± 6.91^{c}
EPO (6000IU/kg)	28.68± 3.37^{c}	33.59± 4.23^{d}
Silymarin (100mg/kg)	31.42± 5.80^{c}	38.33± 4.27^{d}
LSD	8.64	10.72

Note: Mean ± SD is used to express the results. Distinct lowercase letters (a-d) indicate statistically significant differences (P < 0.05) between the groups in the same column. LSD: Least significant difference.

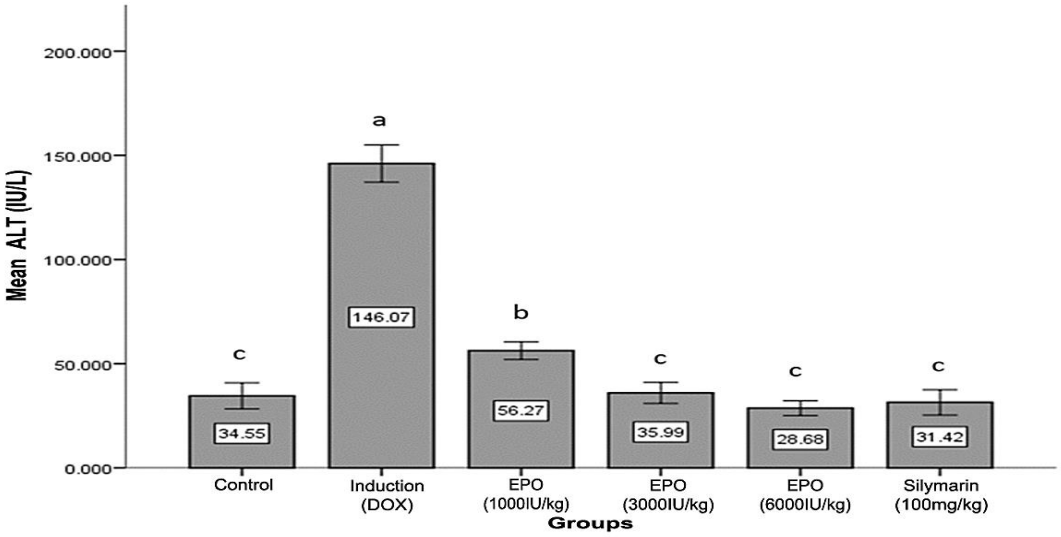

Figure 7.
The effect of EPO on serum liver enzyme ALT.
Note: Mean±SD was used to express the results. Different lowercase letters (a-c) above bars represent significant (P < 0.05) differences among the groups.

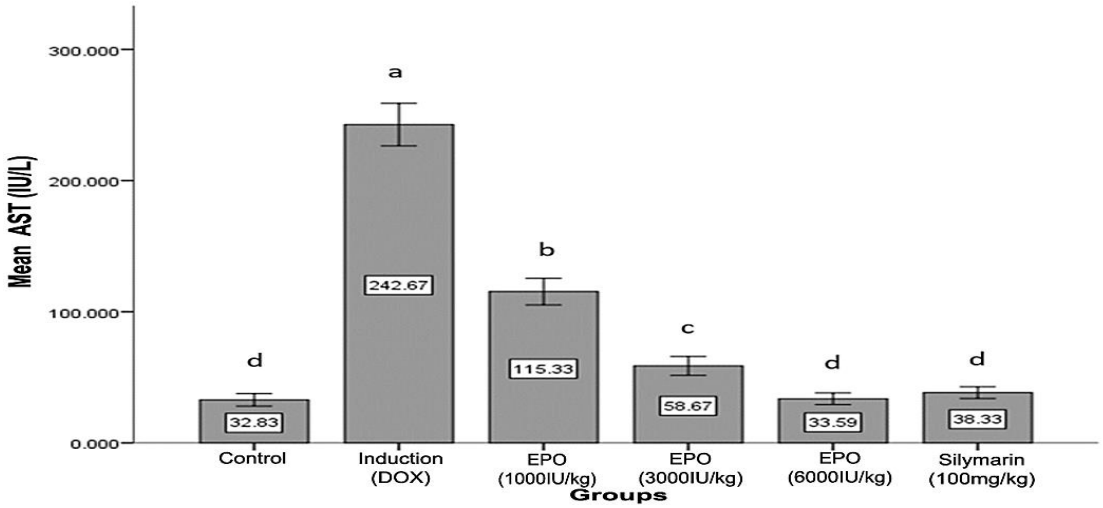

Figure 8.
The effect of EPO on serum liver enzyme AST.
Note: Mean±SD was used to express the results. Different lowercase letters (a-d) above bars represent significant (P < 0.05) differences among the groups.

3.5. The Effect of EPO on Biomarkers of Oxidative Stress (GSH and MDA)

As illustrated in Table 6 and Figure 9, the mean serum GSH levels in the induction group were significantly [p<0.05] lower than that of the negative control group. Pre-treatment with EPO at the three doses (1000, 3000, and 6000 IU/kg) led to a dose-dependent increase in serum GSH levels compared to the induction group. Moreover, the results also showed that in comparison to the induction group, serum GSH levels were significantly [p<0.05] higher for the group pre-treated with silymarin, and that silymarin's effect on GSH levels is non-significantly [p>0.05] different from the group pre-treated with EPO 1000 IU/kg.

On the other hand, Table 6 and Figure 10 show that the mean serum MDA levels were significantly [p<0.05] higher in the induction group in comparison to the negative control group. Pre-treatment with 1000, 3000, and 6000 IU/kg of EPO (in groups 3, 4, and 5) resulted in a dose-dependent significant [p<0.05] decrease in the mean serum MDA levels when compared to the induction group. Also, the group that was pre-treated with silymarin had significantly lower mean serum MDA levels compared to the induction group [p<0.05]. However, silymarin did not have a significantly different effect on MDA levels compared to the group that was pre-treated with EPO 3000 IU/kg.

Table 6.
The effect of EPO on serum oxidative stress biomarkers (GSH and MDA).

Study group	Mean ± SD of GSH (µg/mL)	Mean ± SD of MDA (nmol/mL)
Negative control	31.76± 2.75[a]	0.13± 0.01[cd]
Induction (DOX)	9.14± 1.78[c]	0.49± 0.03[a]
EPO (1000IU/kg)	24.46± 1.68[b]	0.22± 0.02[b]
EPO (3000IU/kg)	29.98± 2.76[a]	0.15± 0.02[c]
EPO (6000IU/kg)	30.96± 2.03[a]	0.11± 0.001[d]
Silymarin (100mg/kg)	25.68± 2.63[a]	0.16± 0.01[c]
LSD	2.73	0.05

Note: Mean ± SD is used to express the results. Distinct lowercase letters (a-d) indicate statistically significant differences (P < 0.05) between the groups in the same column. Results with two different letters indicate no significant difference; (cd): No significant difference with (c&d). LSD: Least significant difference.

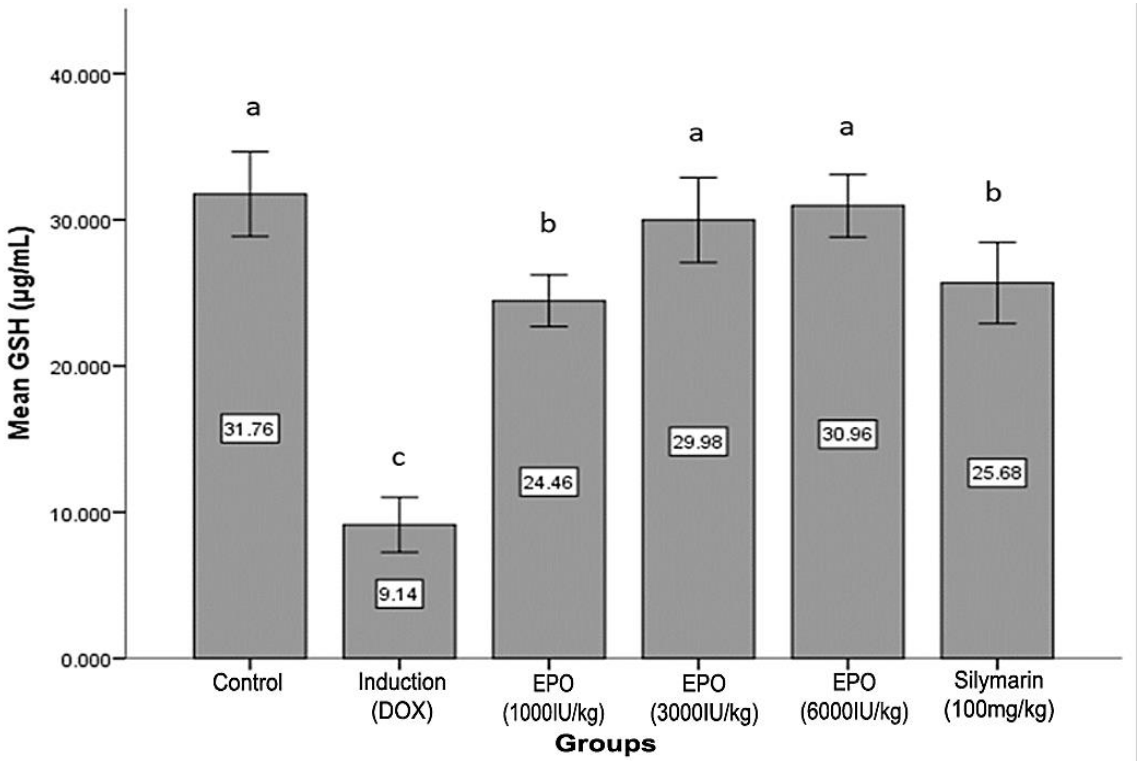

Figure 9.
The effect of EPO on serum GSH levels.
Note: Mean±SD was used to express the results. Different lowercase letters (a-c) above bars represent significant (P < 0.05) differences among the groups.

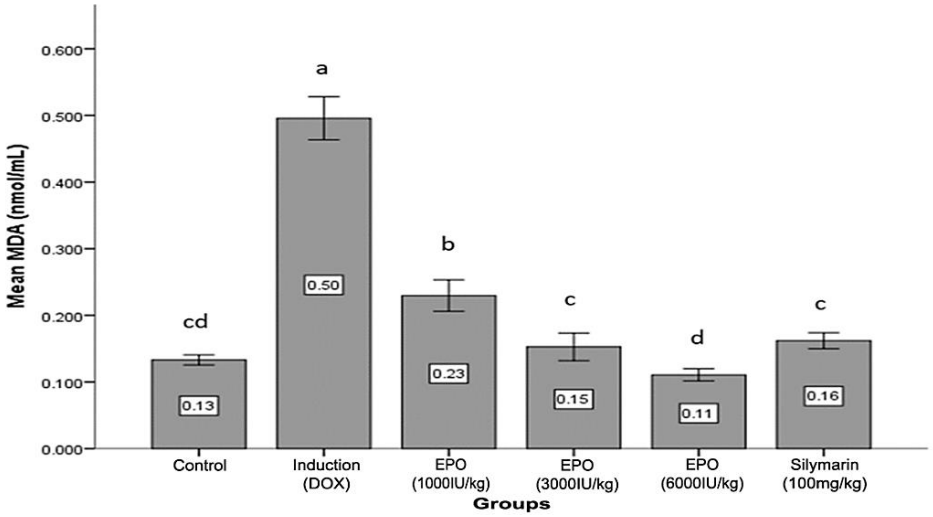

Figure 10.
The effect of EPO on serum MDA levels.
Note: Mean±SD was used to express the results. Statistically significant differences ($p<0.05$) between the findings represented by distinct lowercase letters (a, b, c & d). Results with two different letters indicate no significant difference; (cd): No significant difference with (c&d).

4. Discussion

The complex relationship between the anticancer action of DOX and the induction of hepatotoxicity has attracted a lot of attention from the medical community. Both scientists and medical professionals have worked to gain a deeper comprehension of the underlying processes of DOX-induced hepatotoxicity in order to devise methods to lessen its side effects without sacrificing its effectiveness [37, 38]. An intriguing area of research in terms of applicability is EPO, a glycoprotein well-known for its regulation of erythropoiesis [39]. Consequently, within this experiment, we postulated that EPO could potentially mitigate DOX-induced hepatotoxicity.

Compared to the negative control group (group 1), the induction group (group 2) achieved a significant reduction in tissue expression of PI3K and Akt while simultaneously elevating GSK3β levels (as presented in Table 2 and Figures 1, 2, and 3). The PI3K/Akt pathway is important for cellular survival; blocking it raises the chance of stress and cell death. GSK3β activation links to apoptosis and cell damage. Dysregulation of these processes enhances the cell damage and death caused by oxidative stress [8]. DOX exerts its effects and induces adverse effects by affecting multiple pathways and mechanisms, including the PI3K/Akt pathway. Many factors can explain why DOX inhibits PI3K/Akt. First, DOX exerts its anticancer activity by causing DNA damage in rapidly dividing cells. This process creates ROS, which cause oxidative stress. PI3K/Akt and other signaling pathways can be impaired by oxidative stress. High amount of ROS can inhibit P13K and Akt due to their redox sensitivity [40]. Second, there is intricate crosstalk and feedback regulation between different signaling pathways within a cell. DOX-induced stress and DNA damage can activate parallel signaling pathways that negatively regulate PI3K/Akt. Stress-activated kinases, namely c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK), can be triggered by DOX, resulting in the suppression of PI3K/Akt signaling. Akt phosphorylates GSK3β at serine 9, resulting in the inhibition of GSK3β activity. Under DOX, the inhibitory phosphorylation of Akt decreases. GSK3β, in its active state, has pro-apoptotic actions. It enhances apoptotic signaling by phosphorylating several substrates, including those that play a role in controlling cell survival and death [11].

Table 2 and Figures 1, 2, and 3 show that EPO was more effective than silymarin, especially at the highest dose. This suggests that PI3K, Akt, and GSK3β were significantly changed. EPO binds to its receptor and then starts downstream signaling processes. These events trigger the activation of PI3K, leading to the phosphorylation and activation of Ak. In turn, activated Akt sets off a number of cytoprotective reactions, such as the suppression of apoptotic pathways and the augmentation of cell survival mechanisms [41]. Furthermore, EPO's ability to block GSK3β adds an extra layer of defense. Akt phosphorylates GSK3β, initiating its inhibition. Blocking GSK3β with EPO results in enhanced cell viability and reduced programmed cell death [42]. EPO effectively protects the liver from DOX damage by simultaneously controlling the PI3K/Akt pathway and GSK3β.

The study results revealed a significant reduction in the levels of Nrf2 and HO-1 in liver tissue samples from the group that received DOX, compared to the negative control group (Table 3, Figure 4, and Figure 5). The findings revealed significantly higher levels of Nrf2 and HO-1 levels in the negative control group, potentially due to the regulated formation of ROS in normal cells to support various physiological processes [43]. But in healthy cells, it is anticipated that Nrf2's molecular mechanism of action in detoxification, removal of various endogenous and exogenous chemicals, and regulation of physiological oxidative stress will be highly functional [44].

Treatment with DOX results in hepatotoxicity, which is characterized by an increase in oxidative stress and the generation of ROS. An essential cellular defense mechanism against oxidative stress is the Nrf2/ARE pathway [45]. Increased cellular ROS, which can occur in normal settings or after an acute cellular injury, releases Nrf2 from its negative regulator, Keap1. Nrf2 triggers the transcription of antioxidant genes when it travels to the nucleus and binds to its ARE. This pathway is critical for redox signaling and cellular homeostasis [46]. DOX, on the other hand, can weaken this defense system by

stopping Nrf2 from activating and the production of antioxidant genes like HO-1. The drop in HO-1 levels is associated with lower cellular resilience to oxidative damage [47].

Doxorubicin disrupted the cellular antioxidant defense mechanism, as evidenced by the decrease in Nrf2 and HO-1 expression after DOX treatment. The pro-oxidative characteristics of the medication block Nrf2 activation, which reduces HO-1 expression. Another important explanation for that involves the PI3K/Akt pathway, which was shown to phosphorylate Nrf2 at serine 40, and inhibiting PI3K or Akt reduces Nrf2 activation [48]. In this experiment, it was clear that DOX administration inhibited the PI3K/Akt pathway. Thus, inhibited Akt is no longer able to phosphorylate and activate Nrf2. Inhibiting the PI3K/Akt pathway leads to an increase in the active form of GSK3β. It was found that GSK3β could phosphorylate serine 9 residues of Nrf2, promoting Keap1-independent degradation [49].

The results of this study show that EPO, particularly at the highest dose of 6000 IU/kg, demonstrated elevated levels of Nrf2 and HO-1 in comparison to the induction group. This shows that EPO strengthens the defense systems of cells against oxidative damage caused by DOX. As a positive control, silymarin possibly has some hepatoprotective and antioxidant properties [50]; nonetheless, the data show that EPO is more effective than silymarin at modifying the Nrf2 and HO-1 pathways, especially at higher doses. The findings suggest that robust Nrf2 activation and a subsequent rise in HO-1 levels, which strengthen the body's antioxidant and cell-protective responses, are responsible for EPO's capacity to prevent DOX-induced liver damage.

Both the Nrf2/HO-1 and the PI3K/Akt pathways are critical for preserving cellular homeostasis and defense against stresses. They interact at different levels, impacting one another's actions to support cell survival and protection [51]. Consequently, the interaction between these two pathways may also explain EPO's impact on Nrf2 and HO-1 levels. It has been demonstrated that the PI3K pathway's downstream effector, Akt, directly phosphorylates Nrf2, causing it to stabilize and translocate into the nucleus. Nrf2 binds to ARE in the promoters of genes, including the one encoding HO-1. Akt-mediated Nrf2 activation has a role in inducing antioxidant and cytoprotective responses [52]. Furthermore, when Akt is active, GSK3β is phosphorylated and rendered inactive, which eliminates GSK3β's ability to suppress Nrf2 and permits it to build up and trigger the antioxidant response [53]. The EPO's stimulation of both routes offers a strong cellular defense system. Akt activation supports cell survival and growth, while Nrf2/HO-1 activation helps to neutralize oxidative stress, reduce inflammation, and enhance cellular resilience. The coordination of these pathways contributes to overall cell protection [54].

The study results as shown in Table 4 and Figure 6, showed that DOX raised the levels of caspase 3 in liver tissue by a large amount compared to the negative control (group 1). DOX is well documented for its ability to damage DNA and induce oxidative stress. The cellular damages initiate a series of processes, resulting in the activation of caspase 3, which is considered a key mediator of apoptosis [17]. The activation of caspase 3 is a critical step in the apoptotic process, signifying cells' irrevocable commitment to programmed cell death [55]. DOX affects a number of apoptotic signaling cascades. By modifying the activity of kinases and phosphatases, it can shift the ratio of pro-survival to pro-apoptotic signals [56]. One possible mechanism that might explain how DOX induces apoptosis is its effect on the PI3K/Akt pathway. When GSK3β is activated and DOX-induced PI3K/Akt inhibition occurs, a series of signaling events converge to promote apoptotic pathways, which include the activation of caspase 3 [57, 58]. The breakdown of this equilibrium promotes the onset of programmed cell death, which is characteristic of DOX-induced cytotoxicity.

This research found dose-dependent, significant caspase-3 downregulation by EPO. When compared to silymarin (group 6), the higher dosages of EPO demonstrated greater efficacy (as presented in Table 4 and Figure 6), highlighting its potential as a powerful protective drug and providing support for the concept that EPO exerts its protective effects by blocking apoptotic pathways that are initiated by DOX. Complex processes underlie EPO's down regulation of caspase 3. Studies have demonstrated that EPO's antioxidant qualities reduce the production of ROS in cells [59]. Since caspase 3 activation is frequently linked to oxidative stress, lower ROS levels might lessen the effects of oxidative stress-induced apoptosis [60].

Erythropoietin impedes apoptosis by controlling several signaling pathways, including the PI3k/Akt pathway, which intersects with the process of programmed cell death [61]. One of the subsequent outcomes of EPO-mediated PI3K/Akt/GSK3β regulation is the inhibition of caspase 3 activation. One essential caspase that is in charge of executing the apoptotic process is caspase 3 [62]. Its blocking stops programmed cell death from happening, which is one way that EPO protects tissues against DOX-induced hepatotoxicity.

The hepatotoxicity caused by DOX was investigated by the assessment of liver function indicators, specifically ALT and AST. According to Table 5 and Figures 7 and 8, the results showed that, in comparison to the negative control (group 1), DOX caused a significant increase in the levels of transaminases (ALT and AST) in the blood. The results were consistent with earlier investigations on the hepatotoxic effects of DOX [63, 64].

The reported increase in ALT and AST levels following DOX administration corresponds to the well-documented hepatotoxic effects of the medication. DOX-induced oxidative stress destroys liver cells, releasing these enzymes into the circulation. This may also be associated with the suppression of the Nrf2 pathway by DOX. The inhibition of Nrf2 by DOX affect a number of cellular activities, including those related to liver function. Because Nrf2 pathway inhibition lowers antioxidant capacity, it may lead to liver damage and higher ALT and AST levels. Additionally, DOX induces the inhibition of PI3K/Akt and activates GSK3β. This action contributes to hepatocellular damage, apoptosis, and compromised cellular membrane integrity. As a result, impaired liver cells release ALT and AST.

Administering EPO at different doses resulted in a dose-dependent, significant decrease of ALT and AST levels in comparison to the induction group, as was presented in Table 5 and Figures 7 and 8. Many pathways related to EPO might link to this beneficial action. Initially, EPO has the potential to decrease the occurrence of hepatocyte cell death caused by DOX by inhibiting apoptosis. Furthermore, EPO's stimulation of Nrf2 may aid in maintaining liver function indirectly by

reducing oxidative damage. As a result, this could impede the release of ALT and AST into the bloodstream, as elevated levels of these enzymes often indicate liver injury or stress. EPO's activation of PI3K/Akt and inhibition of GSK3β collectively contribute to enhanced cell survival, reduced apoptosis, and preservation of cellular membrane integrity. The resultant decrease in hepatocellular damage leads to reduced release of ALT and AST into the bloodstream, reflecting a protective effect of EPO on liver function.

Higher doses of EPO, such as 6000 IU/kg, seem to work better in this experiment to counteract the negative effects of DOX on liver enzymes, which shows a dose-response relationship. In the same manner, silymarin administration normalized the serum ALT and AST to a level non-significantly different from the negative control group (group 1).

In this study, administration of DOX injections resulted in a significant reduction in GSH levels in the induction group as compared to the negative control group. Furthermore, Table 6 and Figures 9 and 10 demonstrate that the DOX-induced group showed significantly higher levels of MDA compared to group 1. Decreased levels of GSH indicate a higher demand for antioxidant protection, whereas elevated levels of MDA indicate oxidative damage to cell membranes caused by DOX. The results were in line with earlier studies on the hepatotoxic effects of DOX [16, 65].

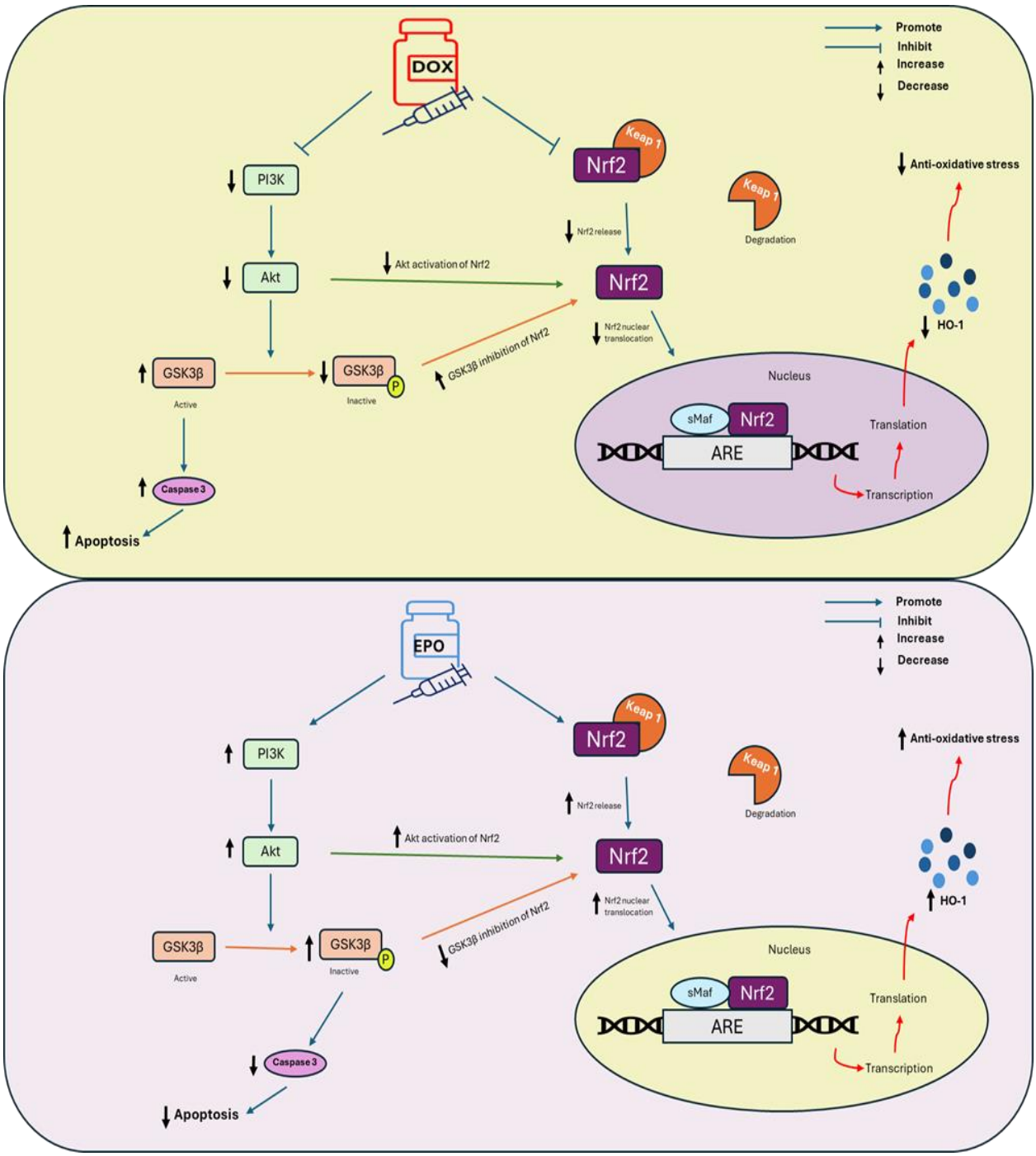

Figure 11.
An illustration of the possible molecular mechanisms that mediate the hepatoprotective effect of EPO against DOX-induced liver damage.

It is well known that DOX produces ROS, which causes oxidative stress in the liver. These ROS can overwhelm the cellular antioxidant defense systems, leading to a disruption in the balance between pro-oxidants and antioxidants [4]. GSH, a major cellular antioxidant, plays a crucial role in detoxifying ROS [66]. In the face of DOX-induced stress, there is an increased demand for GSH, resulting in its depletion. On the other hand, MDA serves as an indicator of oxidative damage to cell membranes. Elevated MDA levels are indicative of increased lipid peroxidation, a consequence of ROS attack on cell membranes [67].

It has been demonstrated that DOX inhibits the Nrf2 pathway [68]. Cells need the Nrf2 pathway to be protected against oxidative damage. A reduction in the expression of genes involved in antioxidant responses, such as those in charge of producing GSH, can result from DOX's inhibition of Nrf2. The results of the experiment show that DOX inhibited Nrf2, which decreased the levels of GSH. Simultaneously, DOX-induced Nrf2 reduction may disturb the balance between pro- and antioxidants, which results in a rise in MDA.

The current study's results demonstrate that EPO exhibits a dose-dependent effect on regulating indicators of oxidative stress, specifically GSH and MDA. Higher doses of EPO improve GSH preservation while simultaneously lowering MDA levels. These findings suggest an elevation in liver antioxidant capacity due to EPO use, thereby alleviating DOX-induced oxidative stress. At the molecular level, EPO increases the production of natural antioxidants like GSH and stops lipid peroxidation, which prevents MDA generation. This may be attributed to the regulation of the Nrf2 pathway. EPO activates the Nrf2 pathway. This increases transcription of antioxidant defense genes, including those producing GSH, and contributes to a balanced cellular redox state, thus decreasing MDA accumulation. Therefore, Nrf2's enhanced activity is crucial for cellular health. And the fact that EPO outperformed silymarin, especially at higher doses, highlights its potential as a more effective intervention against DOX-induced hepatotoxicity. Figure 11 summarizes the results of the present study.

5. Conclusions

This work highlights important information on the possible protective advantages of EPO against DOX-induced hepatotoxicity, including the following:

1. DOX-induced hepatocellular damage was caused by inhibition of PI3K/Akt (and activation of GSK3β) and Nrf2/HO-1 pathways.
2. EPO pre-treatment demonstrated significant hepatoprotective effects against DOX-induced hepatotoxicity, which occurred in a dose-dependent manner by activating the Nrf2/HO-1 antioxidant defense mechanism and modulating the PI3K/Akt/GSK3β axis.
3. The highest doses of EPO provided superior protection compared to silymarin, highlighting EPO's stronger efficacy, particularly at elevated doses, in preventing liver damage and oxidative stress.
4. EPO pre-treatment significantly normalized blood levels of aminotransferases (ALT and AST), indicating its potential to preserve liver function in the context of DOX-induced hepatotoxicity.
5. EPO also restored the balance of oxidative stress biomarkers, such as reduced GSH and MDA, underlining its potent antioxidant properties in mitigating DOX-related oxidative injury.
6. EPO pre-treatment effectively decreased the levels of tissue apoptotic biomarker caspase 3, reinforcing its anti-apoptotic and tissue-protective effects, which contribute to its overall therapeutic potential in mitigating DOX-induced liver damage.

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