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International Journal of Pharmaceutical Research & Allied Sciences, 2022, 11(3):89-97
https://doi.org/10.51847/0hqldEZBtj

Research Article

ISSN : 2277-3657
CODEN(USA) : IJPRPM

Thymoquinone Prevents Doxorubicin-induced Hepatic-injury by Mitigating the Impairment of Mitochondrial Respiration and Electron Transport

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ABSTRACT

Doxorubicin (DXR) is an anthracycline antibiotic that is commonly used in cancer treatment. The purpose of this study was to see if Thymoquinone (THY) could reduce the hepatotoxic effects of doxorubicin. All animals were divided into four groups: control (Phosphate buffer i.p. 0.5mlkg^{-1}/day), THY (10mgkg^{-1}/i.p/daily), doxorubicin (20mgkg^{-1}/i.p/single dose), THY (10mgkg^{-1}/i.p/daily) + DOX(20mgkg^{-1}/i.p/single dose on Day 7). Changes in hepatic enzymes in serum ALT, AST, and GGT, as well as tests for free radical metabolism enzymes such as Cu, Zn-SOD, CAT, and MDA were carried out in all animals that received treatments. In addition, the inflammatory markers IL-6, Nf-kB, and TNF-alpha were measured in liver tissue homogenate. Caspase, an apoptosis marker, was also measured. The liver enzymes were significantly elevated in DOX treated rats as compared to all treated groups. Thymoquinone pretreatment significantly reduced the level of hepatic enzymes as compared to DOX. Comparison of the DOX-treated group to the control group showed that SOD and catalase activities rose significantly. In the DOX-treated group, malondialdehyde levels were higher than in the control and all-treatment groups, respectively (P<0.05). The THY + DOX group had lower levels of malondialdehyde, SOD, and catalase activity than the DOX-group (P<0.05). Anti-inflammatory markers (IL-6 and TNF-alpha), as well as Caspase 3 (p<0.0001), reduced in THY-treated rats, were found to be significantly lower in THY-treated rats (P<0.0001). Biochemical findings were supported by histopathological studies. We conclude that by modulating ROS, inflammation, and apoptosis, thymoquinone significantly reduces DOX-induced hepatotoxicity in rats.

Key words: *Thymoquinone, Doxorubicin, Hepatoxicity, Mitochondrial dysregulation, Oxidative stress*

INTRODUCTION

In the present era of cancer treatment, the anthracycline family drug class is frequently used to treat various types of cancers. An example of a chemotherapeutic agent that belongs to this family is doxorubicin (DOX). It has been used to treat cancer for more than 40 years [1]. However, it has severe toxicity against vital organs of the body which can limit its use [2, 3].

In the pathophysiology of DOX-induced cytotoxicity, oxidative stress plays an essential role, which suggests that antioxidants can effectively relieve DOX-induced toxicity. Several studies have shown that DOX chemotherapy caused significant liver toxicity [4-8]. Increased biomarkers' levels for hepatotoxicity in response to DOX administration, such as hepatic transaminase enzymes ALT and AST levels, were reported in many studies [4, 5, 9]. The reason why these enzymes are elevated may be due to ROS overproduction. Furthermore, excessive ROS production can cause inflammation by activating the transcription factor (NF-kB), resulting in the massive production of cell adhesion molecules, chemokines, and pro-inflammatory cytokines, thereby increasing DOX

cytotoxicity. Research has found that the inflammation-related transcription factor NF-kB, as well as its interaction with apoptosis, can cause hepatic injury [10-12].

A variety of active herbal extracts or active components derived from herbal medicines have been shown in earlier studies to be highly effective at counteracting the adverse effects of DOX [13]. However, it has been established in the literature that Thymoquinone (THY) derived from Nigella Sativa can effectively prevent the toxicity produced by DOX. Thymoquinone is one of the most potent components extracted from *Nigella sativa*. It has been demonstrated that THY produced a variety of pharmacological actions such as anti-inflammatory, anticancer, and antioxidant [14, 15]. Numerous studies found that THY may have a protective effect against acute hepatotoxicity caused by paraquat [16], acetaminophen [17], cisplatin [18], and CCl_4 [19].

However, fewer studies have focused on its protective mechanism against hepatotoxicity when DOX is administered. A study by Akin *et al*. (2018) showed that TQ can produce a significant hepatoprotective effect in rats treated with DOX. This study concluded that TQ inhibits cell inflammation and apoptosis in the liver tissue. Also, this study showed it caused a reduction in oxidative stress output resulting from DOX administration and may enhance the antioxidant defence system [20].

Furthermore, excessive ROS production can cause inflammation by activation of NF-kB, resulting in the massive production of cell adhesion molecules, chemokines, and pro-inflammatory cytokines, thereby increasing DOX cytotoxicity. NF-kB, an inflammation-related transcription factor, and its interaction with apoptosis have been shown in studies to cause hepatic injury [21-23]. Although the mechanism of hepatotoxicity has not been fully understood, it has been linked to mitochondrial dysfunction.

Inner mitochondrial membranes contain a significant amount of Cytochrome c is a small, water-soluble hemeprotein that functions as an electron carrier. The transfer of electrons from complex III to complex IV is one of the functions cytochrome c performs to facilitate the production of cellular energy [24]. Cytochrome c-caspase cascade is responsible for mitochondrial respiration and thereby manages the energy demand of the cells which if not met leads to apoptosis. This study aimed to investigate the potential protective mechanisms like inflammatory, apoptotic, and mitochondrial dysregulation of THY against DOX-induced hepatotoxicity in rats.

MATERIALS AND METHODS

Preparation of Thymoquinone stock solution

Thymoquinone (Angene, China; AGN21-456813-1) stock solution (20mg/mL) was made by dissolving it in Dimethyl Sulfoxide (DMSO) and keeping it at 4°C, in the dark, for future use. The needed dose of thymoquinone was administered to the animals with a final DMSO blood concentration < 0.5% prior to I. P. injection with an aliquot of the stock solution that had been diluted in PBS.

Treatments and study design

Thirty-two male Wistar rats weighing 210–230 g were obtained from the Animal house facility at the Faculty of Pharmacy, King Abdulaziz University, Saudi Arabia (PAH: 2021-0005). The rats were kept in animal cages with free access to rat chow and water, animal room was climate-controlled having a 12-hour light/dark cycle, temperature of 22 ± 3°C and humidity (60±5%).

After seven days of acclimatization, the rats were divided randomly into four different groups of eight rats each. The Control group (received phosphate buffer 0.5 $mlkg^{-1}$, i.p.), Doxorubicin hydrochloride Injection (Cellcys, India; LDXRBC1001) (DOX) group (single dose 20 $mgkg^{-1}$,i.p. on Day 7), THY 10 $mgkg^{-1}$ and THY 10 $mgkg^{-1}$ + Dox (THY+DOX) group. Thymoquinone was administered intraperitoneally at 10 $mgkg^{-1}$ single daily dose for two weeks. Vehicle (0.5% Phosphate buffer) was given to the rats in the control and DOX groups. THY+DOX treated groups were injected intraperitoneally with DOX (20 $mgkg^{-1}$) in normal saline (10 $mlkg^{-1}$) one hour after THY administration on the Day 7. After the 14th day, blood was collected and the rats were sacrificed, and the liver tissue was divided into two portions: the first for biochemical analysis while the second was for histopathology.

Clinical signs

Throughout the experiment, animals were monitored for clinical signs on a daily basis.

Preparation of serum

The blood sample was collected into serum separation gel tubes and then allowed to clot for 30 minutes. The sample was then centrifuged (Hermle Z326K, Germany) for a duration of five minutes at 3500 rpm. After that, the supernatant was separated using a pipette and promptly frozen at -20° C until analyzed.

Preparation of liver tissue homogenate

The liver tissues were rinsed in ice-cold phosphate buffer Saline (PBS) pH7.4 after being kept at -80 °C for biochemical analysis. Tissues were then minced into small pieces and homogenized (Polytron homogenizer- PT 3100, Kinematica AG, Switzerland) with PBS at a concentration of 100mgml^{-1} of PBS using Brinkman instruments on ice. The resulting suspension was then put through two cycles of freezing and thawing to further disrupt the cell membrane. After that, it was centrifuged for 15 minutes at 1500g (Hermle Z326K, Germany) to collect the supernatant, which was then stored at -80 °C until assayed.

Assessment of serum hepatotoxicity markers

The serum levels of ALT (MBS269614), AST (MBS264975), LDH (MBS269777), and GGT (MBS9343646) were measured using the commercial ELISA kits according to the manufacturer's manual. All kits were manufactured by MyBioSource, Inc. (SanDiego, CA, USA).

Assessment of inflammatory markers in liver

The levels of NF-kB (MBS268833), and IL-6 (MBS726707), in liver tissues were detected according to the manufacturer's manual. All kits were manufactured by MyBioSource, Inc. (SanDiego, CA, USA).

Assessment of Lipid peroxidation and apoptosis

The tissue levels of Malondialdehyde (MBS738685), SOD (MBS036924), and Catalase (MBS726781) were assayed in the liver tissue homogenate as an indication of the lipid peroxidation while Caspase 3 (Casp3) *(MBS018987)* was assayed in the liver tissue homogenate as an indication of the apoptosis and mitochondrial dysfunction respectively. All kits were manufactured by MyBioSource, Inc. (SanDiego, CA, USA).

Histological examination

The liver tissue was fixed in formalin at a concentration of 10%, the liver tissues were then embedded in paraffin and sectioned into 5-mm sections. After being stained with hematoxylin and eosin (H&E), the sections were photographed using a light microscope (Olympus light microscope, model: BX51TF- Japan).

Statistical analysis

All results are shown as Mean ± SEM. The data were analyzed using 1-way ANOVA with Holm-Šídák's multiple-comparisons test was performed using GraphPad Prism version 9.4.0 for MacOS, GraphPad Software, CA, USA. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Clinical signs

There was no mortality in any experimental animal group during the entire study duration.

Thymoquinone's protective effect against DOX-induced hepatic injury

Insufficiency and/or injury to the liver alter their transport mechanism and modulate membrane permeability. This might cause the release of specific enzymes from hepatic cells, resulting in a low range of ALT, AST, and GGT in hepatic cells. Increased levels of these enzymes in serum, on the other hand, might be used clinically as indications of liver injury [25]. In the current study ALT, AST, and GGT serum concentrations were measured to see if THY pre-treatment prevented DOX-induced changes in hepatic functions. As shown by the rise in serum ALT, AST, and GGT concentrations following a single DOX injection, we found that a single dose of DOX was causing hepatic injury. Nonetheless, THY pre-treatment reduced the DOX-induced increase in these markers' concentrations to normal levels **(Figures 1a-c)**.

ALT is more likely to be formed when the liver is damaged since it is found in hepatocytes. In clinical settings, the modification of liver enzymes and inflammatory markers by abnormal enzymatic level is crucial for identifying liver damage [26, 27]. Thymoquinone (10 mgkg^{-1}) treatment resulted in a significant decline in enzymatic activity and hence a substantial anti-hepatotoxic effect [19]. According to our data, DOX increased the

serum hepatic enzyme function, comprising AST, ALT, and GGT levels. Similar effects were seen in various animal models of DOX-induced hepatotoxicity [28].

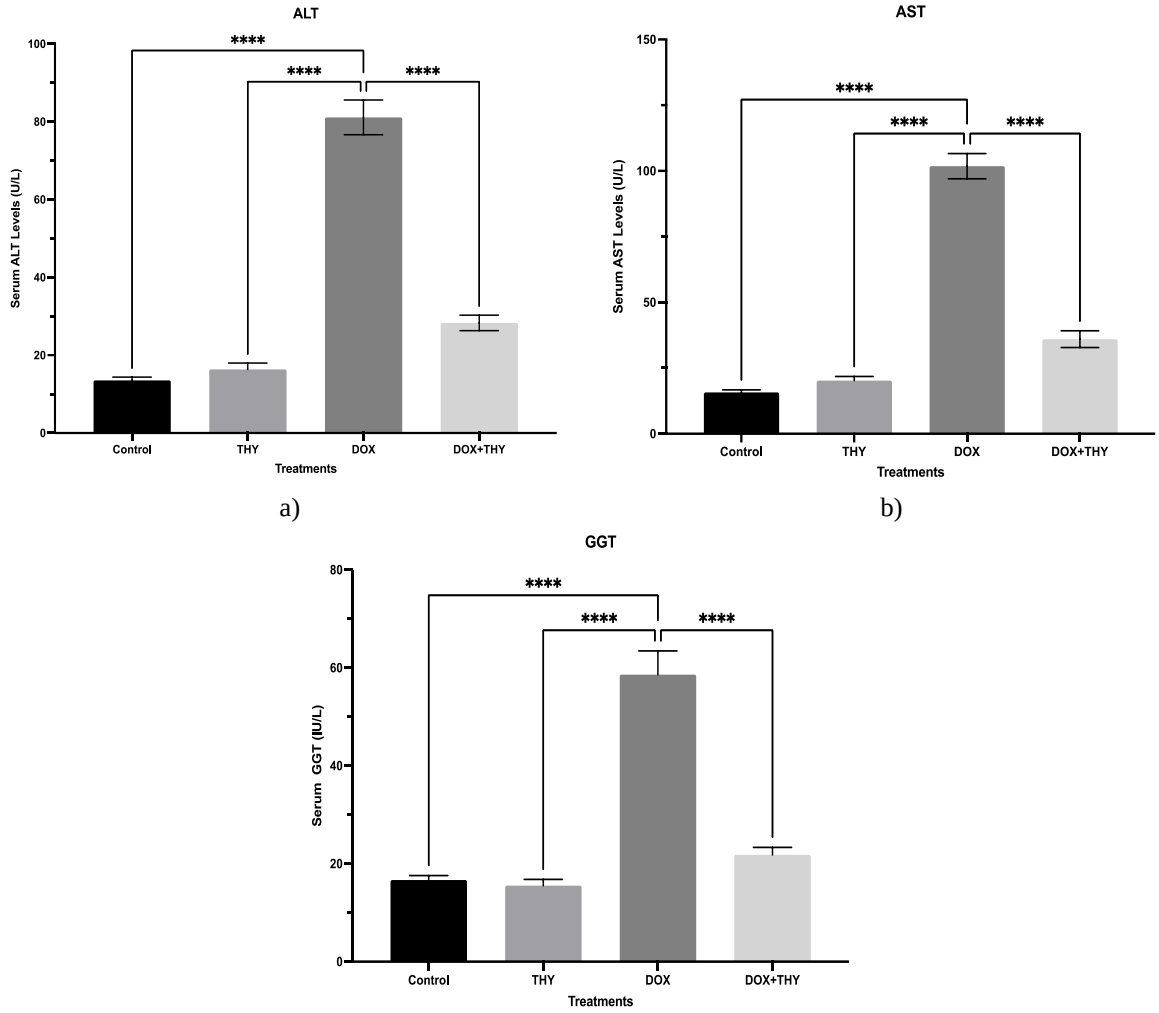


Figure 1. Effects of Thymoquinone pre-treatment on serum levels of (a) ALT, (b) AST, and (c) GGT in male Wistar rats receiving study treatments. DOX increased ALT, AST, and GGT, and Thymoquinone attenuated these enzymes. Data were analyzed using One-way ANOVA with Holm-Šídák's multiple-comparisons test. Error bars represents the mean ±SEM (n = 8), where **** $p<0.0001$, *** $p < 0.001$, ** $p < 0.01$, *$p > 0.05$ and ns.; THY ,Thymoquinone alone (10 $mgkg^{-1}$, i.p.), DOX, doxorubicin group (20 $mgkg^{-1}$, i.p.); DOX + THY, group of Doxorubicin and Thymoquinone (10 $mgkg^{-1}$, i.p.).

Thymoquinone's effects on DOX-Induced oxidative stress markers and the inactivation of enzymatic antioxidants

Natural extracts have been linked to therapeutic molecules that modulate oxidative stress-mediated toxicity, including acute liver failure [11]. Hepatic tissue MDA levels, SOD, and CAT activity were determined in hepatic cells to further investigate THY's protective effect. DOX (20 $mgkg^{-1}$) increased tissue MDA levels while decreasing CAT and SOD enzymatic activities, according to the findings. Nonetheless, at the given dose of 10$mgkg^{-1}$, THY prophylactic treatment significantly restored LPO, SOD, and CAT catalytic activity. These findings revealed that THY has a free radical scavenging protective effect **(Figures 2a-c)**.

Antioxidant-mediated enzymes are thought to constitute the first-line of defense against ROS generation in living organisms [29]. Nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) is a critical essential regulatory element that regulates hundreds of antioxidant proteins. The transcription factor Nrf2 activates many antioxidant proteins, including GPX, SOD, HO-1, and CAT. Our results showed that tissue SOD levels were lower in rats given DOX than in rats in the control group. This was in line with other studies [30, 31]. However, THY pre-treatment reversed the observed change in SOD levels. Lipid peroxidation is a well-established indication of oxidative stress [32, 33]. DOX treatment has been shown to enhance MDA content in studies [31, 34, 35].

The current study found a substantial decrease in hepatic antioxidant defense in DOX-treated rats. MDA levels were considerably higher in the livers of DOX-treated rats. This implies that DOX can generate free radicals after oxidative damage to biological components and lipid peroxidation in the membrane [36]. Furthermore, pre-treatment with THY reduced these effects which correspond to the findings of earlier research [37, 38]. Catalase (CAT) is an antioxidant enzyme that decomposes H2O2 into O2 and H2O. This response functions as a defense mechanism against ROS generation [29]. This study found a significant reduction in CAT enzymatic activity in DOX-treated rats compared to control animals, which is consistent with earlier research [39]. Nonetheless, THY administration alleviated the changes in CAT activity in the hepatic system, emphasizing thymoquinone's preventive and antioxidant capabilities.

Thymoquinone's effects on DOX-induced inflammation

The tissue levels of inflammatory markers NF-kB, IL-6, and TNF-α were found to be markedly higher in the DOX group than in the control group ($p<0.0001$) **(Figures 3a-c)**. Pre-treatment with THY and DOX markedly decrease the level of these inflammatory markers ($p<0.0001$).

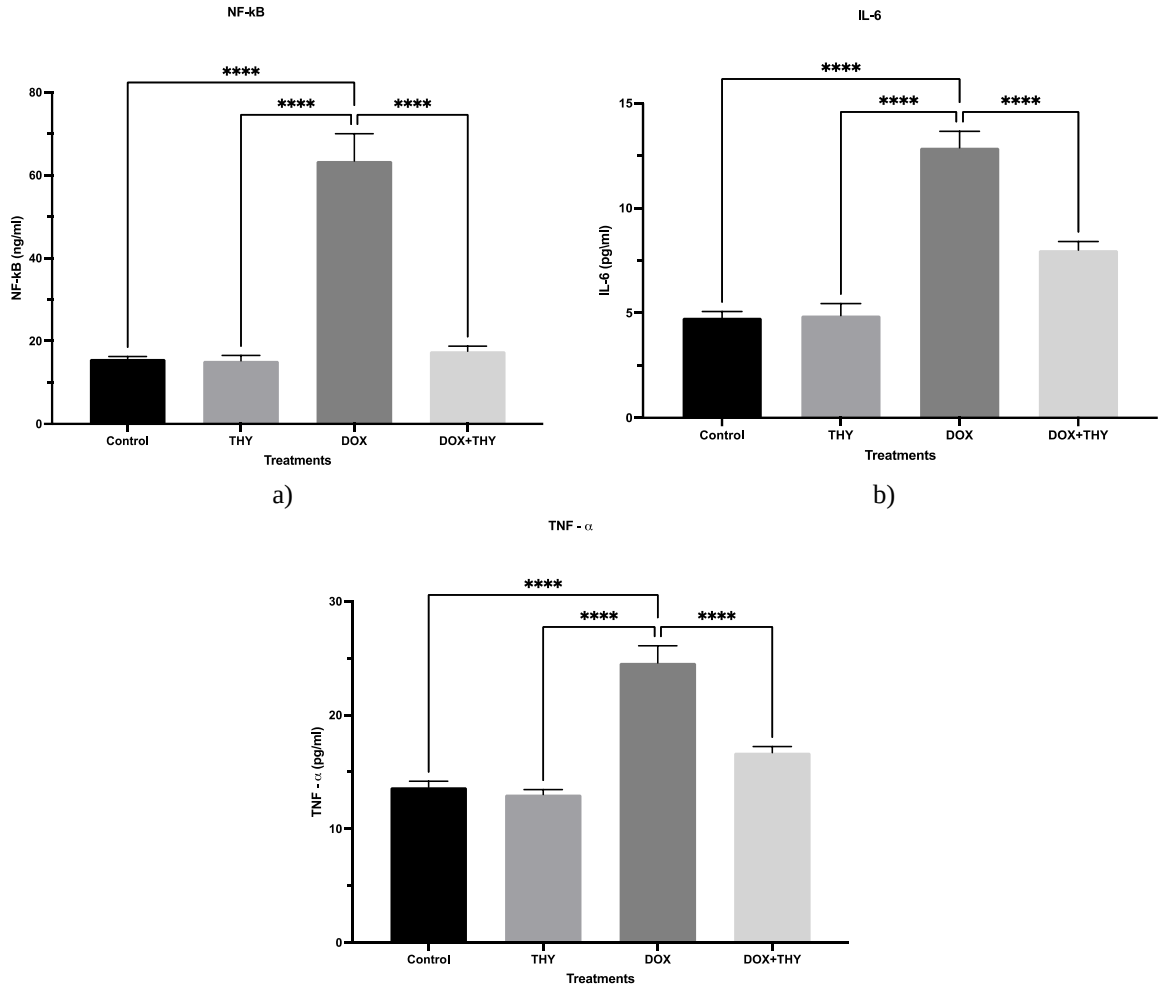


Figure 2. Effects of Thymoquinone pre-treatment on inflammatory markers NF-kB (A), IL-6 (B), and TNF-alpha (C), in male Wistar rats treated with DOX. Thymoquinone reduced the inflammation induced by DOX. Data were analyzed using one-way ANOVA with Holm-Šídák's multiple-comparisons test. Error bars represent the mean ±SEM (n = 8), where **** $p<0.0001$, *** $p < 0.001$, ** $p < 0.01$, *$p > 0.05$ and ns.; THY ,Thymoquinone alone (10 $mgkg^{-1}$, i.p.), DOX, doxorubicin group (20 $mgkg^{-1}$, i.p.); DOX + THY, group of doxorubicin and Thymoquinone (10 $mgkg^{-1}$, i.p.).

Thymoquinone's effect on DOX-induced apoptosis

Oxidative stress has been studied as a key factor in the induction of apoptotic cascades [40]. There is substantial evidence linking the mitochondrial membrane to various cases of organ dysfunction. These modifications were

shown to be the result of excessive ROS generation, which activated the intrinsic cascade in the apoptotic machinery [41-43]. It has been demonstrated that damaged mitochondrial membranes can leak cytochrome c into the cytoplasm, triggering apoptotic signaling including caspase-9 activation [44]. Other enzymes, such as caspase-3, may be activated as a result of this process [45]. In the current study, we assessed caspase 3 levels in hepatic tissue.

Doxorubicin treatment causes a significant elevation in tissue caspase-3 levels as compared to the control group. Regarding the pre-treatment with THY, there was a significant reduction in caspase-3 tissue levels compared to the DOXgroup ($p<0.0001$) **(Figure 3)**. These results are confirming the potent anti-apoptotic effect by modulating the mitochondrial cytochrome c- caspase pathway thereby influencing mitochondrial respiration and electron transport chain enzymes.

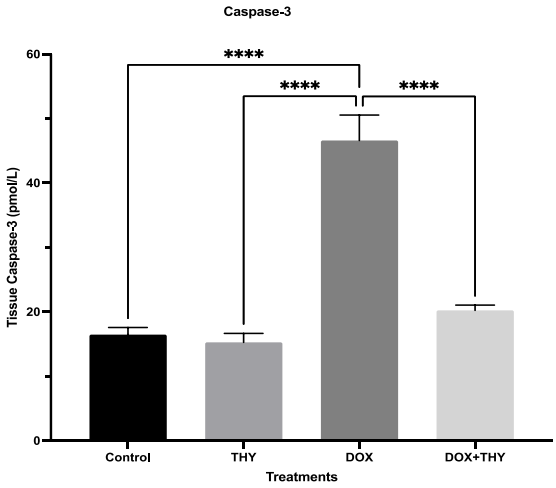
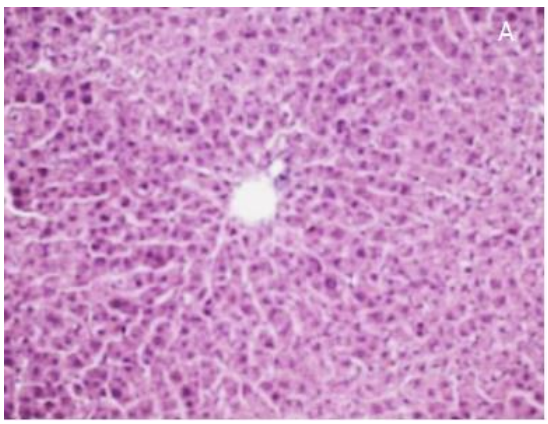


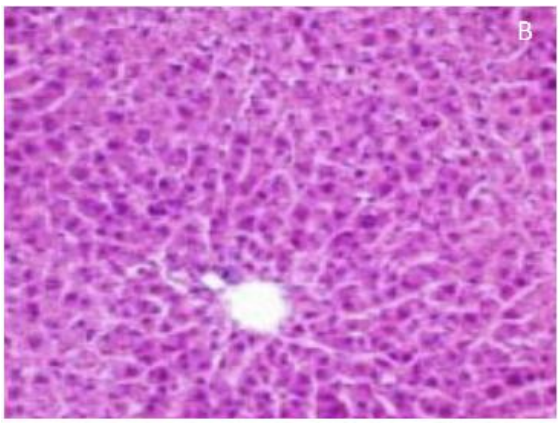
Figure 3. Effects of Thymoquinone pre-treatment on Caspase 3, in male Wistar rats treated with DOX. Thymoquinone reduced the Caspase 3 induced by DOX. Data were analyzed using one-way ANOVA with Holm-Šídák's multiple-comparisons test. Error bars represent the mean ±SEM (n = 8), where **** $p<0.0001$, *** $p < 0.001$, ** $p < 0.01$, *$p > 0.05$ and ns.; THY ,Thymoquinone alone (10 $mgkg^{-1}$, i.p.), DOX, doxorubicin group (20 $mgkg^{-1}$, i.p.); DOX + THY, group of doxorubicin and Thymoquinone (10 $mgkg^{-1}$, i.p.).

Thymoquinone's effect against Dox-induced hepatic histological changes

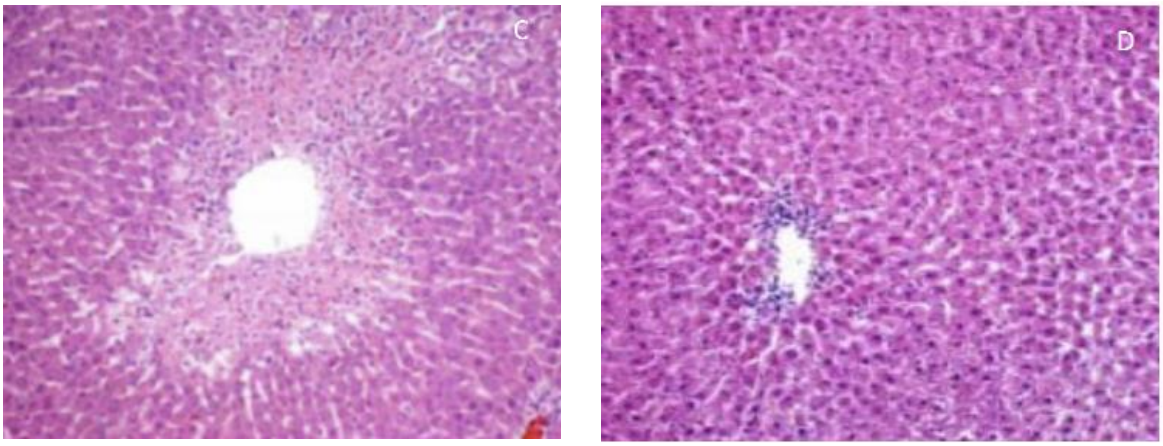
To validate the results of our biochemical tests, using the light microscope we examined the histological architecture of hepatic tissue following DOX exposure. In control and THY-treated rats, we found normal hepatic architecture with no vascular congestion, hepatic necrosis, or mononuclear inflammatory cell infiltrates **(Figures 4a and 4b)**. DOX exposure, on the other hand, induced considerable damage in hepatic cells, as evidenced by the development of sporadic hepatocyte necrosis with extensive neutrophilic infiltration and vascular congestion **(Figure 4c)**. Nonetheless, pre-treatment with THY revealed the presence of a control vein surrounded by normal hepatocytes with mild vascular congestion **(Figure 4d)**.



a)



b)



c) d)

Figure 4. Effects of THY pre-treatment on representative micrographs of the H&E stain liver tissues of 0.5 mm. (A) Control group: shows normal liver tissue artchitecure. B) THY group shows normal histology observed THY only treated rat liver (C) DOX group shows irregular architecture of liver tissues, as indicated by arrows. (D) THY pre-treatment reduced harmful effects of Dox and showed mild improvement from necrosis and inflammatory cells. All images are of 40X magnification.

CONCLUSION

According to our histopathological tests, when 10 $mgkg^{-1}$ of THY is given to liver tissue, it has a strong therapeutic effect. Also, our biochemical results confirmed the histopathological findings and showed that THY lessens the harmful effects of DOX hepatotoxicity by activating the antioxidant defense system and stopping increased inflammation and apoptosis. Based on the results of this study, we think that the antioxidant effects of THY should not be overlooked in DOX hepatotoxicity and that it could be used as an alternative therapy to attenuate the side effects of DOX chemotherapy. In the near future, it would be best to do further research, such as measuring ATP level, cytochrome c Oxidase and mitochondrial dynamics in both acute chronic doxorubicin models with THY as an adjuvant to DOX treatment.

ACKNOWLEDGMENTS : None

CONFLICT OF INTEREST : None

FINANCIAL SUPPORT : The Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah, Saudi
Arabia has funded this project, under grant no. (KEP MSc-42-140-1443).

ETHICS STATEMENT : All animal experiments were performed according to the Implementing Regulations of the Law of Ethics of Research on living Creatures in the Kingdom of Saudi Arabia and approved by Research Ethics Committee, Faculty of Pharmacy, King Abdulaziz University (Approval number # PH/1443/07).

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