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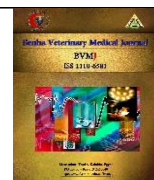

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Original Paper

Ameliorative effect of thymoquinone against acrylamide-induced hepatotoxicity in male rats

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ABSTRACT

Acrylamide (ACR) is a food toxicant detected in over-thermal cooked foods, especially carbohydrate-rich foodstuffs. ACR-induced toxicity in different organs in the body. The current study was planned to evaluate the possible ameliorative effect of Thymoquinone (TQ) against ACR-induced hepatotoxicity. Twenty-eight adult male rats were divided into four groups (seven rats/group) Control group received saline only, TQ group (20 mg/kg b.wt/day/orally), ACR group (20 mg/kg b.wt/day/orally), ACR+TQ group (administered (TQ 20 mg/kg b.wt) + (ACR 20 mg/kg b.wt) orally and once daily) for 28 days. Blood samples for serum separation and hepatic tissue specimens were taken on the last day of the experiment for determination of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol (CHO), and triacylglycerols (TAG) in addition to hepatic oxidative markers including malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and reduced glutathione (GSH). Also, histopathological and immunohistochemical examinations of the liver tissue were performed. ACR caused liver damage indicated by increasing the activity of marker enzymes, lipids profile, and reduction of the hepatic antioxidant enzymes CAT, SOD, and GPx activities and GSH content with marked elevation in MDA concentration. Also, ACR induced histopathological and immunohistochemical alterations in the hepatic tissue. TQ, however, reduced the liver tissue's oxidative stress and hepatic damage induced by ACR. Overall, the obtained findings showed that TQ's antioxidant mechanism reduced ACR-induced liver damage.

1. INTRODUCTION

Acrylamide (ACR), a water-soluble vinyl monomer, has been widely applied in multiple industries such as extraction of oil and production of paper pulp, wastewater treatment, manufacture of dyes and other monomers, biotechnology, and cosmetics (Kandemir et al., 2020). As a food toxicant, ACR can be produced during the thermal cooking of foods rich in carbohydrates over 120 °C, such as deep-frying, baking foods, and roasting (Haase et al., 2012). Many regularly consumed foods such as bread, chips, snacks, and crisps as well as breakfast cereals, biscuits, and crackers contain ACR (Tareke et al., 2002). Several interactions between amino acids, particularly asparagine, and glucose or another reducing sugar are involved in the mechanism of ACR production in food during thermal processes (Becalski et al., 2002). The rapid absorption of ACR occurred through ingestion (Dearfield et al., 1988). Toxicokinetic studies have shown that the metabolism of ACR could be occurred by Cytochrome P450 2E1 and converted to glycidamide (a more toxic epoxide derivative than ACR itself) which attacks DNA and proteins (Sumner et al., 1999). Furthermore, in people and animals, ACR exerted neurological disorders, reproductive problems, genotoxicity, and cancer as reported in several studies (Tareke et al., 2002, Parzefall 2008). He et al., (2017) suggested that ACR-induced toxicity occurred as a result of oxidative stress. Moreover, the exogenous antioxidants play a vital role in combatting ACR-induced oxidative stress by inhibiting the creation of glycidamide-DNA adducts (Yang et al., 2001, Alturfan et al., 2012).
The liver is the main organ implicated in the process of drug biotransformation. ACR-induced hepatotoxicity is closely associated with the excessive generation of ROS in cells, oxidative damage, and mitochondrial impairment (Zhao et al., 2015). Previous studies were carried out to investigate ACR-induced hepatotoxicity (Abdel-Daim et al., 2020 and Zhang et al., 2021). Consequently, considerable attention has been given to reducing ACR-induced hepatotoxicity.
Thymoquinone (TQ) is the active principle and powerful component of the volatile oil obtained from *Nigella sativa*. *Nigella Sativa* and TQ have several health-promoting effects including anti-inflammatory (Taka et al., 2015), and hepatoprotective (Noorbakhsh et al., 2018). Also, it was reported that TQ played a crucial role in lung intoxication with malathion (Abdo et al., 2021). Considering the valuable anti-toxicant, and hepatoprotective effects of TQ stated above, the present study was planned to evaluate the possible protective effect of TQ in ACR-induced hepatotoxicity in rats.

2. MATERIAL AND METHODS

2.1. Chemicals:
Acrylamide and TQ were obtained from Sigma Chemical Company (St. Louis, Mo, USA) and stored at room temperature till use. The analytical reagent used for the determination of the biochemical parameters were obtained from Bio-diagnostics Co, Giza, Egypt.
2.2. Animals:
Twenty-eight Wister Albino male rats (160±30 g) were obtained from the laboratory animal research center of the Faculty of Veterinary Medicine, Benha University, Egypt. The rats were kept at 25°C, subjected to a light/dark cycle (12:12 h), and freely

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received commercial pellets and clean drinking water supplied ad-libitum and left for seven days for adaptation before the beginning of the experiment.

2.3. Ethical approval:

All the experimental protocols were approved by the Institutional Animal Ethics Committee of the Faculty of Veterinary Medicine, Benha University, Egypt (ethical approval number BUFVTM 05-03-22).

2.4. Experimental design:

The rats were haphazardly allotted into four groups of seven rats each, as follows: The first group is the control in which the rats received saline once daily; the second is the TQ group (rats received TQ at a dose of 20 mg/kg b.wt./day, orally); Abdel-Daim et al., 2020), the third is ACR group (rats received ACR at a dose of 20 mg/kg b.wt./day, orally) (Rahangadale et al., 2012), and the fourth is ACR+TQ treated group (rats treated with ACR (20 mg/kg b. wt/day) and TQ (20 mg/kg b. wt/day) orally for 28 days.

2.5. Samplings:

Blood samples and liver tissue specimens were collected from all animals' groups at the end of the experiment (28 days).

2.5.1 Blood samples:

Blood samples for serum separation were collected from the retro-orbital venous plexus at the end of the experiment after overnight fasting in screw-capped tubes and serum was separated by centrifugation at 1200 g for 15 minutes. The separated serum was received in a samples tube, then kept in a deep freeze at -20 ^{o} C until used for subsequent biochemical analysis. All sera were analyzed for the following parameters: AST, ALT, ALP, CHO, TAG, high-density lipoprotein-cholesterol (HDL-c), and low-density lipoprotein-cholesterol (LDL-c).

2.5.2. Tissue specimen (Liver):

The liver was removed, rinsed with NaCl (0.9%), and perfused with ice-cold 50 mmol/L sodium phosphate-buffered saline containing 0.1 mmol/L EDTA. Part of the hepatic tissue specimens was stored at -80 °C till used for oxidative cascade markers (MDA, CAT, SOD, GPx, and GSH). Other parts were immediately fixed in 10% neutral buffered formalin for histopathological and immunohistochemical examination.

2.6. Analysis:

2.6.1. Biochemical analysis:

The serum AST and ALT activities were measured according to Hayashi et al, (2003). ALP according to Tietz et al, (1983). Whereas the level of total cholesterol according to Allain et al., (1974), triacylglycerol according to Fossati and Prencipe, (1982), high-density lipoprotein-cholesterol according to Lopes-Virella et al. (1977), low-density lipoprotein-cholesterol, according to Friedewald et al., (1972). Moreover, liver MDA, CAT, SOD, GPx, and GSH were determined according to the methods described by Uchiyama and Mihara (1978), Aebi (1984), Nishikimi et al. (1972), Paglia and Valentine (1967), and Beutler (1963).

2.6.2. Histopathology examinations:

The liver tissues were preserved for 24 h in 10% neutral buffered formalin. Specimens were immersed in serial dilutions of ethyl alcohol after being washed with tap water. The specimens were cut into pieces (4 m thick) and immersed in paraffin. The histological examination was performed under a light microscope after staining with hematoxylin and eosin (H&E) according to Bancroft and Cook (1994).

2.6.3. Immunohistochemical examinations:

Bax, Bcl-2, and caspase 3 Paraffin-embedded tissue sections of 3 μm thickness were rehydrated in xylene and then rehydrated by utilizing graded ethanol solutions. Slides were then inactivated with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 2 h. After that, sections were immunostained with one of the following primary antibodies; rabbit polyclonal anti-Bax antibody (GeneTex Inc., USA), and rabbit polyclonal anti-Bcl-2 antibody (GeneTex Inc., USA), rabbit polyclonal anti caspase 3 antibody (GeneTex Inc., USA) at a concentration of 1 μg/ml compromising 5% BSA in TBS and left incubated at a temperature of 4 °C overnight. The slides were rinsed by TBS, the sections were incubated with goat anti-rabbit secondary antibody. Sections were washed with TBS and incubated in a solution of diaminobenzidine (0.02%) containing 0.01% H2O2 for 10 min. Counterstaining was conducted by utilizing hematoxylin, and the slides were investigated under a light microscope.

2.7. Statistical analysis:

Data are displayed as mean±SE. Using the statistical software package SPSS for Windows (Version 21.0; SPSS Inc., Chicago, IL, USA) data were analyzed using one-way ANOVA followed by Duncan's post hoc test for multiple group comparisons. Differences were considered statistically significant at $P < 0.05$. The multivariate principal component analysis (PCA) was performed using 'Factoextra', and the 'FactoMineR' packages were built in Rstudio under R version 4.0.2. Moreover, variable importance in projection (VIP) score, hierarchical clustering heatmap between variables, and different treatments were generated using the Metabo Analyst software.

3. RESULTS

3.1. Effect of TQ administration on serum liver marker enzymes and lipids profile in ACR treatment rats:

As presented in Figure 1 of the dot plot the ACR group showed a significant increase in the activity of AST, ALT, and ALP when compared to the control group. Marked improvement was observed in the ACR plus TQ group in the activity of AST, ALT, and ALP (Figure 1 A-C). Furthermore, the levels of CHO, TAG, and LDL-c were significantly increased compared to the normal control rats (Figure 1 D-F). While the level of HDL-c was decreased in the ACR group compared to the control group (Figure 1 G). Administration of TQ showed marked improvement in the lipid profile results.

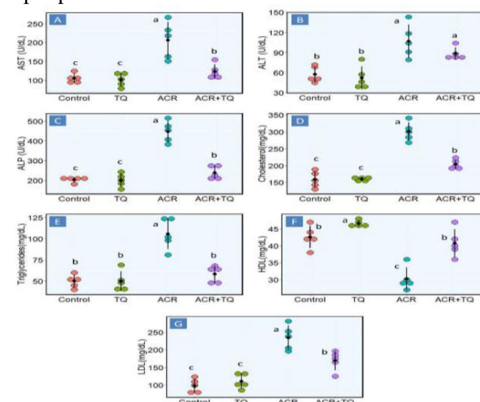

Figure (1): Effect of TQ treatment on serum liver marker enzymes and lipids profile concentrations of ACR- induced hepatotoxicity in rats (n = 5): Dot plot panel with mean (black dot) of AST (A), ALT (B), ALP (C), Cholesterol (D), Triglycerides (E), HDL (F), and LDL (G). Data are presented as Mean ± SD. Mean values with different superscript letters in the same row are significantly different at ($P \leq 0.05$).

3.2. Effect of TQ administration on liver oxidative stress and antioxidant markers in ACR-treated rats:

As presented in the dot plot panel (Figure 2), the ACR group showed a significant increase in MDA level (Figure 2 A), a significant increase in the enzymatic activity of CAT, SOD, and GPx (Figure 2 B-D), and the level of GSH (Figure 2 E) compared to the control group. Whereas the treatment with TQ ameliorates the toxic effect of ACR on the antioxidant status.

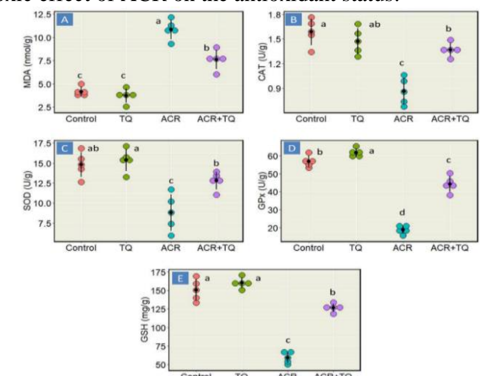

Figure (2): Effect of TQ treatment on liver oxidative stress biomarkers of ACR- induced hepatotoxicity in rats (n=5): Dot plot panel with the mean (black dots) of MDA (A), CAT (B), SOD (C), GPx (D), and GSH (F). Data are presented as Mean ± SD. Mean values with different superscript letters in the same row are significantly different at ($P \leq 0.05$).

3.3 .Multivariate analyses

3.3.1 .Correlation between variables and concentration values among different group averages

The correlation heatmap explicated in Figure (3), provides a 2D correlation matrix between all measured parameters, using colored cells to represent intuitive visualization of all the data sets. A strong positive correlation was observed between GSH and CAR and SOD and also between ALP and CHO. Whereas, a negative correlation was obviously seen between MDA and GSH. The clustering heatmap (Figure 4) provides an intuitive visualization of all data sets which summarizes the concentration values of all measured biochemical and oxidative parameters.

3.3.2 .Variable important project (VIP) score

The variable importance in projection indicated that CHO, HDL, ALP, TG, MDA, and GSH were the most significant variables in the hepatotoxicity of ACR and TQ ameliorative effect (Figure 5) .

3.3.3 .Principal component analysis (PCA) and dendrogram

As exposed in Figures 6-7, the findings showed that the majority of the investigated factors were presented in component 1 of the 3D PCA (76%) (Figure 6 A). The PCA score plot recorded that the ACR and ACR+TQ groups were segregated from other groups (Figure 6 B). Moreover, the current dendrogram (Figure 7) represented that ACR and ACR+TQ exposed animals descended from a separate branch, discriminating them from other treated animals. However, CTR and TQ groups were gathered under the other branch of the dendrogram tree.

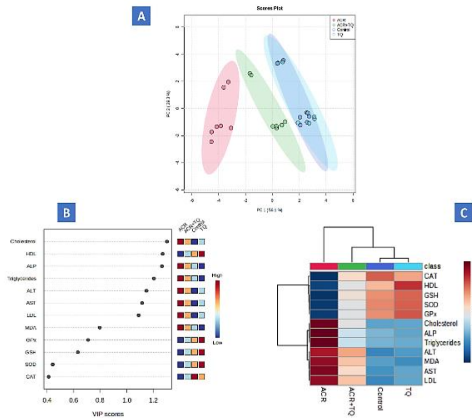

Figure (3): Principal component analysis (PCA) and data clustering analysis of the effect of ACR and/or TQ on the liver of male albino rats. A: PCA for 28 treated rats, each group represented by 7 rats. B: variable importance in projection (VIP) the colored boxes on the right display the relative concentrations of the relevant measured parameters in each study group, while, the contribution intensity is indicated by a color scale spanning from the highest (red) to the lowest (blue). C: clustering heatmap, each color cell denotes the concentration values, with the variable average in rows and different treatment sets in columns, dark red is the highest value while blue is the lowest value.

3.4. Histopathological findings:

The photomicrograph of the hepatic sections in control and TQ groups stained with hematoxylin and eosin (H&E) exhibited normal histological patterns of the hepatocytes (H) which are radially arranged in cords around the central vein (Figure 4-A and B). Meanwhile, the ACR-treated rats showed focally extensive coagulative necrosis of the hepatic cells with mononuclear cell infiltration (arrow) (Figure 4C). Moreover, the treatment with TQ reversed the hepatic lesions caused by ACR to a large extent as it showed normal histology in which the hepatocytes (H) are radially arranged in cords around a central vein (CV) (Figure 4D).

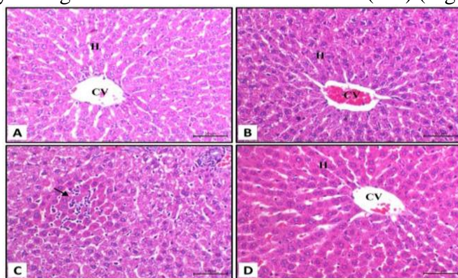

Figure 4 Photomicrograph of liver stained with hematoxylin and eosin (H&E) of the control group (Figure 4 A), showing normal histology in which, the hepatocytes (H) are radially arranged in cords around a central vein (CV). TQ group (Figure 4 B) in which the hepatocytes (H) are radially arranged in cords around a central vein (CV). ACR group (Figure 4 C) showed showing focally extensive coagulative necrosis of the hepatic cells with mononuclear cells infiltration (arrow). ACR+TQ group (Figure 4 D) showed normal histology in which the hepatocytes (H) are radially arranged in cords around a central vein (CV).

3.5 .Immunohistochemical findings

The liver of the control and TQ groups (Figure 5 A-B) showed mild immunostaining of Bax antibody within the hepatocytes. ACR group (Figure 5 C) showed marked cytoplasmic and nuclear expression of Bax antibody within the hepatocytes. ACR+TQ group (Figure 5 D) showed a decrease in the expression of Bax antibody within the hepatocytes. The Bcl2 immunostaining of the control and TQ groups (Figure 6 A-B) showed marked cytoplasmic expression of the Bcl2 antibody within the cytoplasm of hepatocytes. ACR group (Figure 6 C) showed a marked decrease in the expression of Bcl2 antibody within the hepatocytes. ACR+TQ group (Figure 6 D) showed marked increased expression of Bcl2 within the hepatocytes. The caspase 3 immunostaining of the control and TQ groups (Figure 7 A-B) showed scanty expression of caspase 3 antibody within the hepatocytes. ACR group (Figure 7 C) showed marked expression of caspase 3 (cytoplasmic and nuclear) within the hepatocytes. ACR+TQ group (Figure 7 D) showed a marked decrease in caspase 3 expression within the hepatocytes.

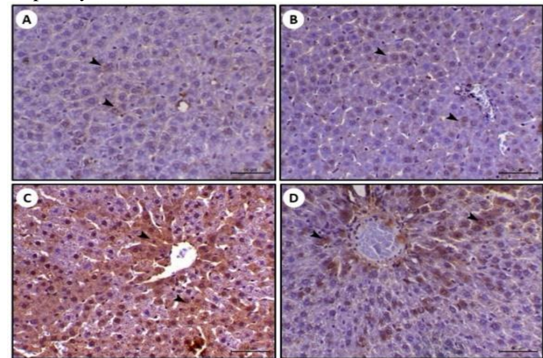

Figure (5): Effect of ACR and/or TQ on the Bax expression in the liver. Liver of the control group (Figure 3 A) showed mild immunostaining of Bax antibody within the hepatocytes (arrowheads). TQ group (Figure 3 B) showed a low level of Bax expression within the hepatocytes (arrowheads). ACR group (Figure 3 C) showed marked cytoplasmic and nuclear expression of Bax antibody within the hepatocytes (arrowheads). ACR+TQ group (Figure 3 D) showed a marked decrease in the Bax expression within the hepatocytes (arrowheads), bars = 50 µm.

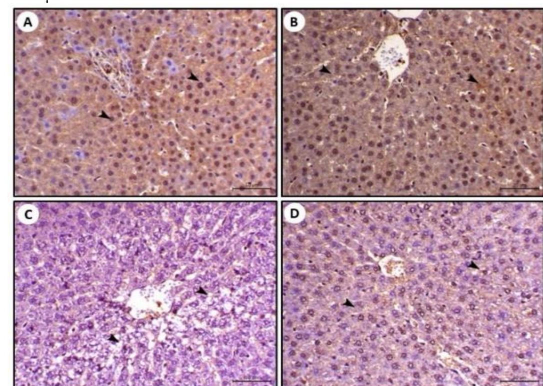

Figure (6): Liver of control animal showing marked cytoplasmic expression of Bcl2 antibody within the hepatocytes (arrowheads), Bcl2 IHC, X200, bar= 40 µm. TQ group showed marked immunostaining of Bcl2 within the cytoplasm of hepatocytes (arrowheads), Bcl2 IHC, X200, bar= 40 µm. ACR group showed a marked decrease in the expression of Bcl2 within the hepatocytes (arrowheads), Bcl2 IHC, X200, bar= 40 µm. ACR+TQ group showed an increase in the expression of Bcl2 within the hepatocytes (arrowheads), bars = 50 µm.

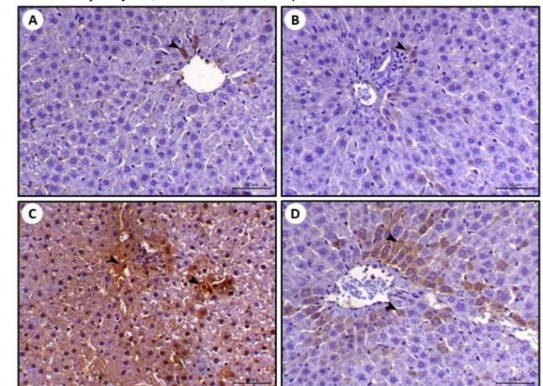

Figure (7): Liver of control animal showing mild immunostaining of caspase 3 antibody within the hepatocytes (arrowhead). TQ group showed mild expression of caspase 3 within the hepatocytes (arrowheads). ACR group showed marked cytoplasmic and nuclear expression of caspase 3 antibody within the hepatocytes (arrowheads). ACR+TQ group showed a decrease in the expression of caspase 3 antibody within the hepatocytes (arrowheads), bars = 50 µm.

4. DISCUSSION

The current study estimated the effects of TQ treatment on liver injury resulting from ACR intoxication. The action of ACR in our study resulted in increased activity of the liver enzyme markers, AST, ALT, and ALP. Hepato-toxicity of ACR resulted in damage to the lipid and protein membranes and thus the liver enzymes (indicators of hepatic damage) leak into the bloodstream (Nkosi et al., 2005). These results are inconsistent with previous data (Abdel-Daim et al., 2020). In addition, the serum levels of CHO and TG in the ACR-treated rats showed a notable increase and this may be attributed to the increase in the plasma lipoproteins synthesis and increased lipids mobilization from the liver. Our findings are in the same line with Uthra et al. (2017). Furthermore, ACR in our findings is closely related to the increased content of LDL-C. Conversely, HDL-C content was reduced in rats administrated ACR. These results are in the same line as the report performed by Cheang et al. (2021). Thymoquinone is one of the important natural antioxidants used in folk medicine and scientific research all over the world. In the current experiment, we proposed that TQ could be considered as an alternative method in the modulation of ACR liver damage. TQ showed a significant ameliorative effect in AST, ALT, and ALP activities and in the lipid profile in the damaged liver tissues. These results are attributed to the antioxidant activity of TQ which is greater than that of ascorbic acid (Staniek and Gille 2010).

The present study exposed the occurrence of significant oxidative impairment as shown by an increase in the enzymatic antioxidants (CAT, SOD, and Gpx). These findings are in harmony with (Abdel-Daim et al., 2020). Moreover, ACR which can bind with glutathione, clearly reported in our experiment, is responsible for eliminating oxygen-free radicals (Pizzino et al., 2017). Furthermore, the lipid peroxidation marker, MDA, was dramatically increased in ACR-treated rats. Reactive Oxygen Species accumulation is implicated in the lipid peroxidation process and damage of membrane lipid content and resulting in elevated MDA content (Farid et al., 2019). In addition to the lipid membrane damage, protein oxidative damage existed in a previous investigation by Ghorbel et al., (2015). Moreover, the co-administration of TQ and ACR to rats markedly decreased the content of MDA and increased the activity of CAT, SOD, and GPx, and the level of GSH. In the presence of cellular reductases, TQ, as a quinone, undergoes an electron reduction of one or two electrons (El-Najjar et al. 2010). By quinone reductase, the one-electron reduction produced semiquinones, which are then converted to ROS in the presence of molecular oxygen (Monks and Jones 2002), while the two-electron reductions produced hydroquinones, which act as antioxidants (Nagi and Almakki 2009). Guo et al. (2008) concluded that the cellular antioxidant mechanism occurred as a result of the competition of the two-electron reduction with one electron.

In this study, ACR upregulated Bax and caspase 3 and downregulated Bcl-2 expression. This suggests that ACR treatment can cause inflammation and apoptosis in renal tissues, followed by necrosis. According to different studies, the induction of apoptosis in the liver tissues can be mentioned as another mechanism that is involved in ACR-induced toxicity (Foroutanfar et al., 2020). Meanwhile, it was previously revealed that the administration of ACR led to a significant elevation in the level of caspase-3 in the liver tissue (Seydi et al., 2015). Interestingly, our data exhibited that TQ was able to reverse these alterations. TQ reduced the Bax/Bcl2 ratio and consequently inhibited caspase-3 activation in hepatic tissue. The proteins Bax and Bcl2 play important roles, with Bax acting as a pro-apoptotic factor and Bcl-2 acting as an anti-apoptotic factor (Foroutanfar et al., 2020).

In the present work, there were histological changes in the hepatic tissues of rats that administrated ACR. These changes are compatible with the findings of Uthra et al. (2017) who reported hepatocellular injury with cytoplasmic vacuolization and inflammatory cell infiltration, central vein congestion, and disruption in sinusoidal spaces and hepatic cords after acute exposure to ACR. Whereas Liu et al. (2020) demonstrated no pathological lesions in the hepatic tissue of rats treated with a chronic low dosage of ACR. These histopathological findings are attributed to the oxidative stress that occurred in the hepatic tissue and the damage to the liver membrane due to the lipid peroxidation induced by ACR. The positive effects of TQ were proved by restoring the histopathological alterations of hepatic tissue induced by ACR. In a previous study by. Additionally, it downregulates the expression of some enzymatic activity like CAT and GST (Ismail et al. 2010). Furthermore, Thymohydroquinone, the reduced form of TQ acts as hydroxyl and superoxide radical's electron donner which attacks the cell membrane polyunsaturated fatty acids. This clarifies the powerful ROS scavenging capacity of TQ (Khither et al. 2018). Additionally, El-Mahmoudy et al. (2002) elucidated the ability of TQ to inhibit the formation of NO by downregulation of the inducible nitric oxide synthase expression. On the other side, TQ could decrease inflammatory biomarkers and their expression levels (Abdel-Daim et al. 2020).

5. CONCLUSION

According to the study, oxidative stress from both enzymatic and non-enzymatic sources contributed to the hepatotoxic effects of ACR. TQ treatment may increase the hepatoprotective benefits by acting as an antioxidant against ACR hepatotoxicity. These results are significant, particularly in the assessment of the clinical importance of liver diseases.

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