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Quercetin protects against thioacetamide induced hepatotoxicity in rats through decreased oxidative stress biomarkers, the inflammatory cytokines; (TNF-α), (NF-κ B) and DNA fragmentation

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ABSTRACT

The present study aimed to investigate the hepatoprotective mechanisms of quercetin and silymarin in experimentally induced hepatic toxicity in rats. Mature Albino Wister rats were orally daily treated with quercetin (50 and 100 mg/kg), silymarin (50 mg/kg) for 21consecutive days, then were injected intraperitoneal with TAA (300 mg/Kg, i.p.) twice with 24 hours interval in the last two days of the experiment to induce hepatotoxicity. Afterwards, blood samples were withdrawn from all rats and liver tissue were isolated. Sera separated for determination of serum liver function tests. Liver homogenates were used for assessment of oxidative stress biomarkers, inflammatory cytokines, hepatocellular apoptosis and histopathological examinations. Moreover, hepatic DNA fragmentation levels were examined calorimetrically by diphenylamine (DPA) and electrophoreticaly by agarose gel electrophoresis. Results of the present study revealed that oral administration of quercetin (50 and 100 mg/kg) for 21 days significantly improved the elevated liver enzymes (AST and ALT), alkaline phosphatase and total bilirubin with significant decreased oxidative stress biomarkers measured as malondialdehyde (MDA) , nitric oxide (NO) and reduced glutathione (GSH) contents in liver homogenate. In addition, quercetin decrease the elevated inflammatory cytokine; tumor necrosis factor alpha (TNF-α), nuclear factor kappa beta (NF-κ B) and DNA fragmentation in a dose dependant manner. Finally, it can be concluded that quercetin displayed a protective effect against TAA-induced hepatic toxicity via mechanisms involving the alleviation of oxidative stress, antioxidant potential, and attenuation of inflammation and inhibition of hepatocellular apoptosis.

Keywords: hepatotoxicity, quercetin, silymarin, thioacetamide (TAA), rats

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INTRODUCTION

Understanding hepatotoxic injury mechanisms has arisen from cellular, functional and molecular studies in animals. The pathological lesions induced by hepatotoxic agents may be similar to those of liver diseases **(1).** Thioacetamide (TAA) is an organosulfur compound with chemical formula C2H5NS which is classified as a human carcinogen **(2)**. Its uses include textile and paper industries, leather processing, laboratories and as a stabilizer of motor fuels **(3)**. Flavin-containing monooxygenase (FMOs) systems and cytochrome P450 (Cytp450**)** metabolized TAA **(3, 4)**. In the liver microsomes, enzymes of Cyt-p450 converts TAA to thioacetamide S-oxide (TAASO) and then to thioacetamide S,S-dioxide (TAASO2) and due to oxidation process it causes oxidative stress that finally provokes centrilobular necrosis and liver injury **(5)**. TAASO is accountable for the enlargement of nucleoli, showed rise in intracellular concentration of Ca, change in cell permeability, and suppressed mitochondrial activity (6) TAASO2 enhance nitric oxide synthase (iNOS) release and nuclear factor-κB (NF-κB), leading to centrilobular necrosis **(7)**.TAASO2 denatures proteins through modification of their charge. Free radicals generated by TAA, as a result of

lipid peroxidation, may injure different cells **(8)**. Furthermore, it damages the urea cycle **(9)** and ornithine aminotransferase activity directing to serious changes in the profile of serum amino acid **(10)**.

Herbal medicine becomes a promising approach for healthcare professions. Plants contain bioactive components having desirable benefits and are usually known to be preventive of various diseases. Quercetin is a flavonoid (polyphenolic compound) bearing a catechol unit on the B-ring. It is widely distributed in some fruits and vegetables as apple, berries, bulbs, citrus fruits, onions, cereal grains, legumes, tea and cacao. Quercetin is known to have several pharmacological actions, including anti-inflammatory, antioxidant and hepatoprotective effects **(11, 12)**. Quercetin also has the ability to decrease hepatic lipids and plasma cholesterol, prevent diabetes-induced oxidative stress and save the integrity of pancreatic beta cell **(13)**.

Silymarin is extracted from "milk thistle" Silybum marianum **(14)**. It consists of mixture of flavonoid isomers. Silymarin is used mainly to cure liver dysfunctions like hepatitis, alcoholic cirrhosis (due to drug induced or viral infections), and hepatic problems associated with diabetes **(15)**. Silymarin has powerful free radical scavenging properties and the ability to enhance anti-oxidant defense systems in vivo **(16)**. Silymarin showed anti-inflammatory and anti-metastatic activities; it also has protective effect against toxicity of chemotherapy and radiotherapy**(17) .**

The aim of present work is to investigate the possible quercetin hepatoprotective mechanisms against hepatotoxicity induced by thioacetamide through estimation of its effects on oxidative stress, inflammatory response and apoptosis.

MATERIALS AND METHODS

Chemicals

TAA and quercetin were obtained from Sigma-Aldrich, USA. Silymarin was obtained from MEPACO, Egypt; all other chemicals, used throughout the experiment, were of the highest analytical grade available. Kits used for measurement of serum aspartate transaminase (AST), alanine transaminase (ALT); alkaline phosphatase (18); total bilirubin levels; hepatic malondialdehyde (MDA); nitric oxide (NO) and hepatic reduced glutathione (GSH) were purchased from(Biodiagnostic, Egypt). Kit used for measurement of hepatic Tumor necrosis factor-alpha (TNF-α) was purchased from (Raybiotech, USA). Hepatic nuclear factor-kappa beta (NF-κ B) was measured using kit from (EIAab, China).

Animals

Adult male albino Wister rats weighing 150-200 gm were obtained from the animal house at the National Research Center (Giza, Egypt), and fed a standard laboratory diet and tap water ad libitum. Experimental animals were housed in an air-conditioned room at 22–25 °C with a 12-h light/dark cycle. All animals received humane care and the study protocols were carried out according to the ethical guidelines for care and use of experimental animals approved by the Ethical Committee of the National Research Centre.

Experimental design After an acclimatization period of one week, fourty healthy male albino Wister rats were randomly assigned to five groups of eight rats per group and they were treated as follows:

Group 1: received water containing 0.1% Tween 80 orally for 21 days, (Normal control).
Group 2: received thioacetamide (300 mg/kg, i.p.) twice with 24 hours interval (positive control) according to **(1)**.
Group 3: received silymarin (50 mg/kg, p.o.) according to **(19)** for 21 days and thioacetamide (300 mg/kg, i.p.) twice with 24 hours interval in the last two days of injection.
Group 4&5: received quercetin (50& 100 mg/kg, p.o.) according to **(20)** for 21 days and thioacetamide (300 mg/kg, i.p.) twice with 24 hours interval in the last two days of injection.

Collection of blood samples

At the end of the experimental period; blood samples were withdrawn from the retro-orbital vein of each animal, under light anesthesia by diethyl ether, according to the method described by **(21)**. Blood was allowed to coagulate and then centrifuged at 3000 rpm for 15 min. The obtained serum was used to estimate the levels of AST; ALT activities according to the method of **(22)**; ALP was determined according to the method of **(23)** and total bilirubin was analyzed by the method of **(24).**

Preparation of liver homogenate

Immediately after blood sampling, animals were sacrificed by cervical dislocation under ether anesthesia and livers were collected for biochemical and histopathological examinations. Liver tissues were rapidly removed, washed in ice-cooled saline. A weighed part of each liver was homogenized, using a homogenizer (Medical instruments, MPW-120, Poland), with ice-cooled saline (0.9% NaCl) to prepare 20% w/v homogenate. The homogenate was then

centrifuged at 4000 rpm for 5 min. at 4°C using a cooling centrifuge to remove cell debris (Laborzentrifugen, 2k15, Sigma, Germany). The aliquot was divided into six parts; the 1st part was used for the assessment of lipid peroxidation (LPO) as MDA according to the methods described by Ohkawa et al.; the 2nd part was used for the determination of the level of NO by the method of Montgomery and Dymock; the 3rd part was used to estimate GSH by the method of Beutler et al.; the 4th part was used to estimate Tumor necrosis factor-alpha (TNF-α) according to the instructions of the manufacturer, using (Raybiotech, USA) ELISA kit the 5th part was used to estimate (NF-κ B) using (EIAab, China) ELISA kit; and the 6th part was used to estimate DNA fragmentation coloremetrically by diphenylamine (DPA) according to **(25)** and electrophoreticaly by agarose gel electrophoresis.

Histopathological examinations

The specimens from the liver were taken and fixed immediately in 10% neutral buffered formalin, processed for light microscopy to get (5µm) paraffin sections and stained with: Hematoxylin & Eosin (H & E) to verify histological details.

Statistical analysis

The degree in variability of results was expressed as means ± standard error of means (SEM). Data were evaluated by one-way analysis of variance followed by Tuke's multiple comparisons test. The level of significance was accepted at $P < 0.05$.

RESULTS

Effects on serum hepatic functions biomarkers.

Injection of TAA resulted in a considerable hepatic injury as assessed by a significant elevations in serum AST, ALT, ALP and total bilirubin by 78%, 98%, 17% and 70% respectively as compared to normal control values. Treatment with silymarin exerted a significant decrease in serum AST, ALT, ALP, and total bilirubin levels by 16%, 27%, 12%, and 38%, respectively as compared to TAA control group. Administration of quercetin significantly reduced TAA-induced elevations by 15%, and 17% for AST; 12% and 14% for ALT; 11%, and 14% for ALP; and 22%, and 22% for total bilirubin, respectively in dose-dependent manner, compared to TAA control group (Table 1).

Effects on hepatic oxidative stress biomarkers.

TAA injection caused a significant elevation in MDA and NO values as well as significant depletion in GSH value. Injection of TAA significantly elevated liver MDA and NO contents by 135% and 259% respectively, as compared to normal control values. Treatment with silymarin significantly decreased liver MDA and NO content by 41% and 49% respectively, compared to TAA control group. Treatment with quercetin showed a significant decrease in elevated liver MDA by 21%, and 49% (Figure 1A), and NO by 44% and 51% (Figure 1B) As compared to TAA control group, respectively. Results showed that TAA significantly decreased liver GSH content by 68.5 %, as compared to normal control group. Treatment with silymarin significantly increased the hepatic concentration of GSH by 100% compared to TAA control group. Moreover, treatment with quercetin caused a significant increase in GSH level by 74% and 152% respectively (Figure 1C) as compared to TAA control group.

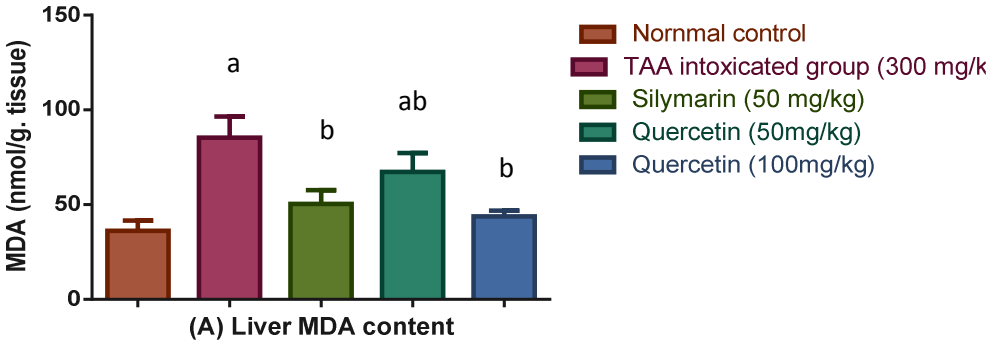
Effects on hepatic tumor necrosis factor-alpha (TNF-α) and nuclear factor-κB (NF-κ B) levels

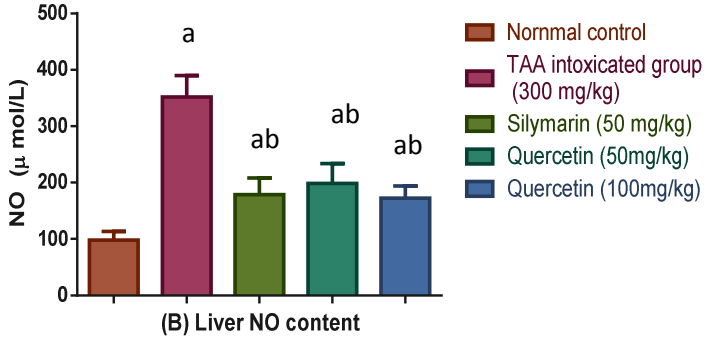
TAA injection caused a significant elevation in hepatic TNF-α and NF-κ B content by 585% and 866% respectively, as compared to normal control values. Treatment with silymarin significantly decreased liver TNF-α and NF-κ B content by 54% and 48% respectively, compared to TAA control group. Treatment with quercetin showed a significant decrease in elevated liver TNF-α by 42%, and 67% (Figure 1E), and NF-κ B by 45% and 70% (Figure 1D) As compared to TAA control group, respectively.

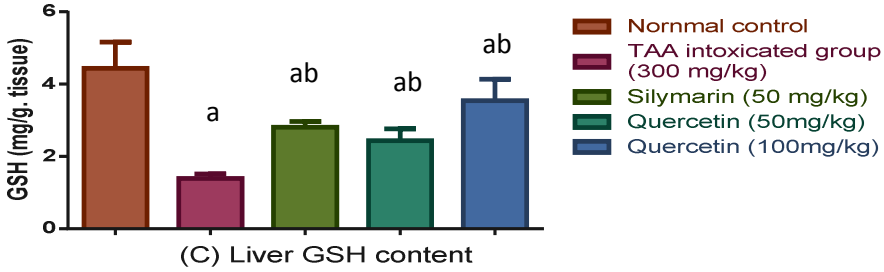
Table 1: Effect of quercetin (50 and 100 mg/kg) and/or silymarin (50 mg/kg) on serum liver function tests in TAA -induced hepatotoxicity in rats

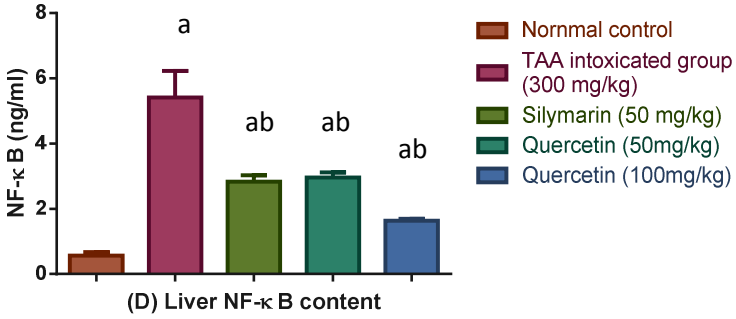
Groups/ Dose	ALT (U / ml)	AST (U / ml)	ALP (IU / L)	Total bilirubin (mg / dl)
Control	101.3 ± 2.632	99.5 ± 2.502	338.8 ± 4.809	1.963 ± 0.116
TAA (300 mg/kg b.wt)	200.8 ± 1.437 [a]	177.1 ± 3.541[a]	393.5 ± 1.212 [a]	3.338 ± 0.1973 [a]
Silymarin (50 mg/kg b.wt)	146.2 ± 5.137 [ab]	149.1 ± 3.247 [ab]	348.8 ± 5.652 [b]	2.083 ± 0.0569 [b]
Quercetin (50 mg/kg b.wt)	175.9 ± 3.742 [ab]	149.9 ± 2.983 [ab]	352.5 ± 4.677 [b]	2.593 ± 0.0368 [ab]
Quercetin (100 mg/kg b.wt)	171.9 ± 3.742 [ab]	146.9 ± 2.811 [ab]	340 ± 4.677 [b]	2.596 ± 0.0773 [ab]

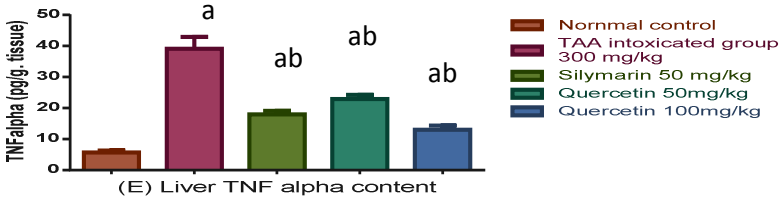
Data are presented as the mean ±S.E. of (n=8) for each group. Statistical analysis was carried out by one way ANOVA followed by "Tukey's Multiple Comparison Test". [a] Statistically significant from control group at P <0.05 . [b] Statistically significant from TAA–intoxicated group at P <0.05 .


MDA (nmol/g. tissue)
150
100
50
0
a
b
ab
b
(A) Liver MDA content
Nornmal control
TAA intoxicated group (300 mg/k
Silymarin (50 mg/kg)
Quercetin (50mg/kg)
Quercetin (100mg/kg)


NO (μ mol/L)
500
400
300
200
100
0
a
ab
ab
ab
(B) Liver NO content
Nornmal control
TAA intoxicated group (300 mg/kg)
Silymarin (50 mg/kg)
Quercetin (50mg/kg)
Quercetin (100mg/kg)


GSH (mg/g. tissue)
6
4
2
0
a
ab
ab
ab
(C) Liver GSH content
Nornmal control
TAA intoxicated group (300 mg/kg)
Silymarin (50 mg/kg)
Quercetin (50mg/kg)
Quercetin (100mg/kg)


NF-κ B (ng/ml)
8
6
4
2
0
a
ab
ab
ab
(D) Liver NF-κ B content
Nornmal control
TAA intoxicated group (300 mg/kg)
Silymarin (50 mg/kg)
Quercetin (50mg/kg)
Quercetin (100mg/kg)


TNFalpha (pg/g. tissue)
50
40
30
20
10
0
a
ab
ab
ab
(E) Liver TNF alpha content
Nornmal control
TAA intoxicated group 300 mg/kg
Silymarin 50 mg/kg
Quercetin 50mg/kg
Quercetin 100mg/kg

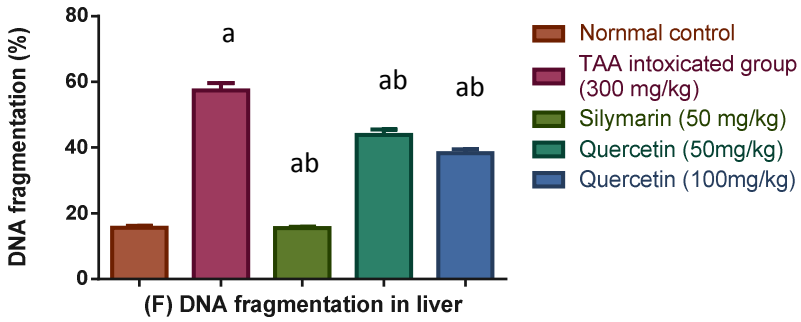


Figure 1: Effect of Quercetin (50 and 100 mg/kg) and/or silymarin (50 mg/kg) on hepatic (A) MDA, (B) NO, (C) GSH, (D) NFκ-B, (E) TNF-α levels and (F) DNA fragmentation

Data are presented as the mean ±S.E. of (n=8) for each group. Statistical analysis was carried out by one way ANOVA followed by "Tukey's Multiple Comparison Test". [a] Statistically significant from control group at P <0.05 . [b] Statistically significant from TAA–intoxicated group at P <0.05 .

Effects on hepatic DNA fragmentation levels

Injection of TAA caused a significant elevation in hepatic DNA fragmentation level by 271% compared to normal control group. Treatment with silymarin significantly decreased liver DNA fragmentation level by 20% compared to TAA control group. Quercetin showed a significant decrease in elevated liver fragmentation level by 24% and 34% respectively, compared to TAA control group (Figure 1F).

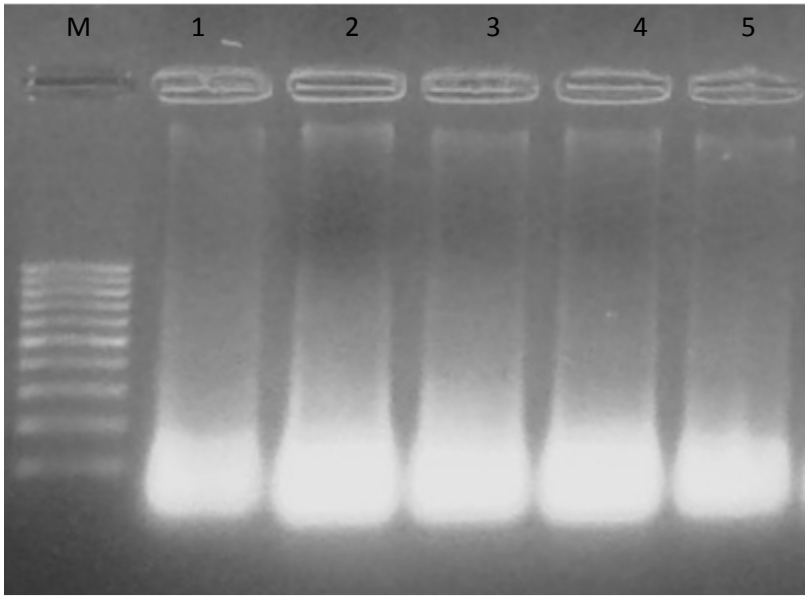


Figure 2: Electrophoretic analysis of DNA on 2% agarose gel

Lane 1: control; Lane 2: Sil ; Lane 3: toxic agent; Lane 4: Q50; Lane5: Q100; M: 100bpDNA ladder (Vivantis).

Effects on the hepatic histopathological examination

Liver of control rats showed normal hepatic parenchyma with normally arranged hepatic cords (Figure 3a). Whereas, liver of thioacetamide treated rats showed Complete loss of architectural organization with bridging centrilobular necrosis, which extend to involve most parts of hepatic lobules. Disorganization of hepatic cords and dissociation of hepatocytes that appeared atrophied with intensely eosinophilic cytoplasm and pyknotic nuclei associated with sinusoidal congestion and hemorrhage (Figure 3b) were a characteristic lesions demonstrated in this group. Abundant apoptosis (Figure 3c) and focal sinusoidal dilatation with kupffer cell activation were also demonstrated. Portal area showed congestion of portal blood vessels, biliary hyperplasia and intense portal mononuclear cell infiltration as well as sporadic necrosis of the periportal hepatocytes. On the other hand, low dose quercetin pre-treated rats showed marked improvement. No bridging necrosis was demonstrated in this group and lesions were restricted to centrilobular coagulative necrosis of hepatocytes which extends to involve few layers around central vein (Figure 3d) in addition to activation of kupffer cells with sinusoidal dilatation with mild leukocytosis and few apoptosis. The histopathological lesions were markedly alleviated in high dose quercetin pre-treated rats manifested by marked restoration of the hepatic parenchyma with centrilobular necrosis only restricted to first two rows of hepatocytes surrounding the central vein (Figure 3e) with minimal apoptosis. Regression and

mild improvement of the histopatholgical alterations were recorded in Silymarin pretreated rats as the hepatocellular necrosis was limited to the centrilobular area associated with numerous apoptosis (Figure 3f) and sinusoidal congestion.

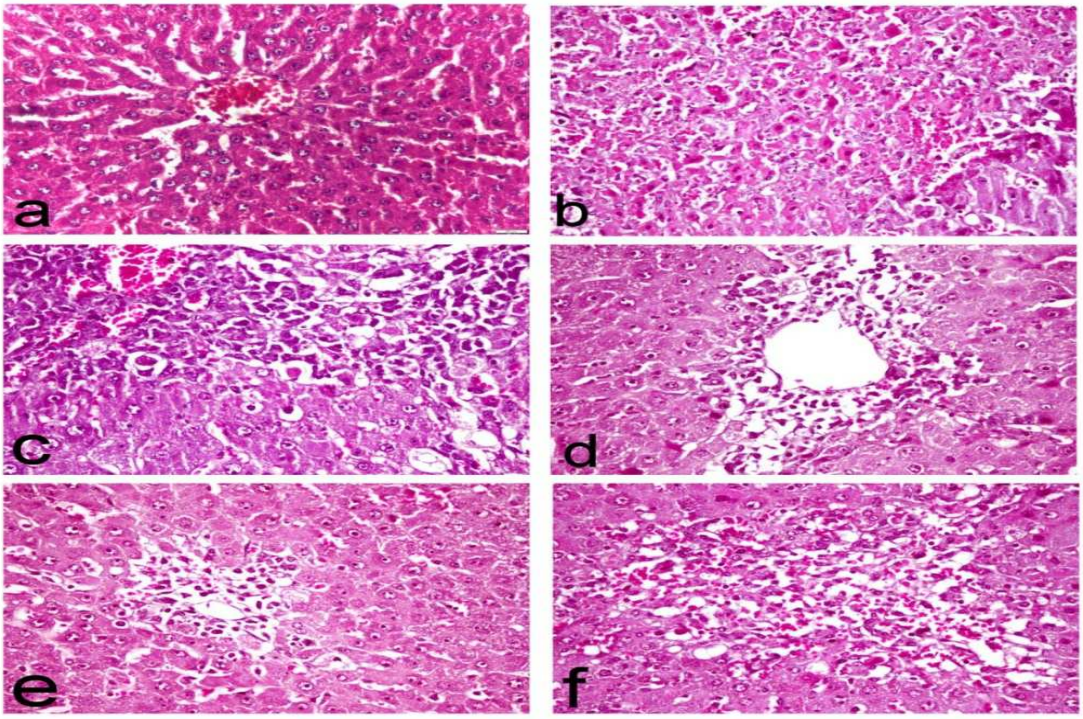


Figure 3: liver of (a) control rats showing normal hepatic parenchyma with normally arranged hepatic cords, (b) TAA treated rats showing disorganization of hepatic cords and dissociation of hepatocytes that appeared atrophied with intensely eosinophilic cytoplasm and pyknotic nuclei associated with sinusoidal congestion and hemorrhage and (c) abundant apoptosis, (d) low dose querticin pre-treated rats showing centrilobular coagulative necrosis of hepatocytes which extends to involve few layers around central vein, (e) high dose querticin pre-treated rats showing marked restoration of the hepatic parenchyma with centrilobular necrosis only restricted to first two rows of hepatocytes surrounding the central vein and (f) Silymarine pretreated rats showing hepatocellular necrosis was limited to the centrilobular area associated with numerous apoptosis (H&E, X40)

DISCUSSION

TAA is close to experimental partial hepatectomy. Metabolism of TAA in rat liver by Cyt-P450 system to thioacetamide-*S*-oxide inducing change in cell permeability, enlargement of nucleoli, increases in intracellular Ca^{++} concentration and inhibition of mitochondrial activity that provokes cell death **(18)**. TAA also decreases the protein contents in the liver through inhibition of amino acids incorporation into liver protein **(26)**.

In the present study, TAA showed a marked rise in the levels of serum AST, ALT, ALP and total bilirubin compared to the normal rats, which indicating that TAA could cause a liver damage in the rats. Histopathological study supported these results that showed complete loss of architectural organization with bridging centrilobular necrosis, which extend to involve most parts of hepatic lobules. Our results are in agreement with Yogalakshmi et al. **(1)** who observed increase in the activities of AST, ALT, ALP and total bilirubin following TAA injection in rats via substantial liver damage. The elevation of serum AST and ALT levels might be attributed to release of these enzymes into the blood circulation from the cytoplasm after injured liver structural integrity **(27)**. Moreover, an increased activity of ALP may be due to biliary flow pathological change. **(28)**. Furthermore, the elevation of serum total bilirubin level may be due to decrease in conjugation and secretion from the liver or blockage of bile ducts.

In our work, daily administration of quercetin or silymarin significantly decreased serum AST, ALT, ALP and total bilirubin levels compared to TAA-intoxicated group. Histopathological study supported these results that showed restoration of the hepatic parenchyma with decrease in centrilobular necrosis. Liu et al. **(29)** found that quercetin can protect mouse liver against nickel-induced liver damge.

TAA injection significantly elevated liver MDA content indicating increased lipid peroxidation and oxidative stress in liver while significantly decreased liver GSH content. This may be due to injection with TAA induce hepatic damage by generation of ROS and suppressed antioxidant defense mechanism **(30)** Meanwhile, TAA injection,

showed a significant elevation in liver NO content. This may be due to the formation of NO, which increases in liver disease due to activation of L-arginine-NO pathway **(31, 32).** These results are in line with Nada et al. **(33)**.

Administration of quercetin or silymarin significantly decreased elevated MDA and NO level in liver and increased hepatic GSH content. These results may be through quarcetin powerful antioxidant effect and its activity to scavenge free radical **(34)**. Ansari et al. **(35)** reported that quercetin prevent GSH oxidation and depletion owing to its specific structure, thereby protecting oxidative stress-induced neurotoxicity.

The present results showed a significant increase in hepatic TNF-α and NF-κB levels in TAA-intoxicated group in comparison with the healthy control group. This effect is attributed to induction of inducible nitric oxide synthase (iNOS) and NF- κB as a result of the formation of the reactive metabolite of TAA, thioacetamide-S, S-dioxide, that expresses proinflammatory molecules **(7)**. Our results are in agreement with Bahcecioglu et al. **(36)**.

Daily administration of quercetin or silymarin inhibited the elevation of tumor TNF-α level and hepatic NF-κB level as compared to TAA-intoxicated group. our results are in line with Liu et al. **(37)** who stated that quercetin can protect rat kidney against lead-induced inflammatory response through the inhibition of the ROS-mediated mitogen-activated protein kinases (MAPKs) and NF-κB pathway . In addition, ability of quercetin to inhibit TNF-a transcription was attributed to inhibition of signal transduction pathways of MAPK and the redox-sensitive transcription factors and activator protein 1 **(38)**. Quercetin, also, inactivated NF-κB and the inflammatory cytokines through the Toll-like receptor 2 (TLR2) and the Toll-like receptor 4 (TLR4) inhibition **(39)** and signal transducer and activator of transcription 1 (STAT1) inhibition, **(29)**.

The findings of DNA fragmentation using the diphenylamine (DPA) assay and gel electrophoresis revealed that; a significant increase was observed in DNA fragmentation in the hepatocytes after TAA administration this may be due to increased cell death via both apoptosis and necrosis **(40)**. These results are supported by histopathological study that showed abundant apoptosis in liver of TAA treated group.

Pretreatment with quercetin or silymarin inhibited DNA fragmentation when compared to TAA-intoxicated rats. The obtained results are in accordance with those obtained by Lui et al**. (41)** who found that quercetin is able to protect rat kidney against lead induced apoptosis and DNA damage.

CONCLUSION

Quercetin has a potent hepatoprotetive activity in TAA-induced hepatic injury in rats. This preventive effect of quercetin is due to powerful antioxidant properties, anti-inflammatory and anti-apoptotic effects.

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