The Potential Protective Effect of Quercetin on Thiamethoxam Induced Hepatotoxicity in Adult Male Albino Rats

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Abstract:

Background: Since, human exposure to thiamethoxam (TMX) is highly frequent as it is one of the most widely used pesticides nowadays and due to potential adverse health effects including hepatotoxic effects, TMX has become an issue of concern to public health.

Aim of the work: was to evaluate the possible protective role of quercetin administration on thiamethoxam induced hepatotoxicity in adult male albino rats.

Methods: Thirty adult healthy male albino rats, were included in this study. Rats were divided into five equal groups. Each group consists of six rats as follows: Group I (negative control group): Rats received only regular diet and tap water daily. Group II (positive control group): rats received one mL of normal saline 0.9% NaCl (solvent of thiamethoxam and quercetin). Group III (quercetin treated group): Each rat received quercetin at 50 mg/kg body weight (BW), dissolved in five ml of saline 0.9%. Group IV (Thiamethoxam treated group): rats received TMX at 78.15 mg/kg BW dissolved in five ml of saline 0.9%. Group IV (Quercetin +TMX treated group): With the same mentioned doses and duration, quercetin was administered one hour before TMX

Results: Administration of thiamethoxam led to a very highly significant increase ($p< 0.001$) in the mean values of serum levels of GDH, AST and ALT, significant reduction in the mean values of hepatic glutathione peroxidase and catalase and a very highly significant increase in the mean values of serum levels of MDA with histopathological changes of hepatic architecture along with increase caspase-3 immunoreaction and increase DNA damage changes in comet assay. Quercetin administration along with TMX ameliorate all studied parameters.

Conclusion: Quercetin plays an important role in protection against thiamethoxam liver toxicity which is indicated by improving the biochemical, histopathological,

immunohistochemical and comet assay results in groups treated with quercetin and thiamethoxam.

Keywords: Quercetin, Thiamethoxam, Hepatotoxicity, Albino Rats.

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Introduction:

Chemicals known as pesticides are released into the environment to improve crop yields and food grain storage by eliminating disease-transmission vectors. However, there is now significant concern about the health risks associated with exposure to these substances (1).

Farmers are exposed to pesticides through their skin, food, and respiration, which subsequently circulate through their blood and impact different organs (2).

Neonicotinoid is an acronym for "new nicotine-like insecticide." Due in part to their apparent low risk to non-target organisms and the environment, neonicotinoid insecticides were once thought to be perfect substitutes for certain insecticides (such as carbamates and organophosphates) (3).

Neonicotinoids consist of seven compounds that are divided into three generations: dinotefuran is the third generation, thiamethoxam and clothianidin are the second generation, and imidacloprid, nitenpyram, acetamiprid, and thiacloprid are the first generation (6).

A broad-spectrum second-generation neonicotinoid, thiamethoxam (TMX) is used as a contact and systemic insecticide to manage sucking and chewing pests. Applications for TMX include foliar, soil, and seed treatments for the majority of crops, fruits, vegetables, and cotton (8).

Numerous studies have demonstrated that TMX upset the equilibrium between antioxidant and reactive oxygen species (ROS), leading to oxidative stress and the lipid degradation of cell membranes, which is indicated by elevated lipid peroxidation, as well as DNA damage. This is why research has been done on the impact of oxidative stress and ROS on the neurotoxicity, hepatotoxicity, nephrotoxicity, and reproductive cytotoxicity linked to TMX (9).

One important class of polyphenolic chemicals with a wide range of medicinal and biochemical uses is flavonoids (10).

The most well-known flavonoid, quercetin (3,5,7,3',4'-pentahydroxyflavon), is a member of the flavonol subclass and is present in a variety of foods, including red apples, red grapes, broccoli, onions, red and green cabbages, red and green tea, olive oil, almonds, and more (11).

Anticarcinogenic, anti-inflammatory, antibacterial, anti-allergic, anti-proliferative, antioxidant, neuroprotective, hepatoprotective, and antiviral activities are among the advantageous properties of quercetin (12). Quercetin also has the ability to scavenge free radicals, chelate divalent cations, block certain enzymes, prevent DNA damage, and effectively mitigate the risk of a number of disorders (13).

Materials and Methods

This Sub chronic in vivo experimental study was conducted at Forensic Medicine and Clinical Toxicology, Histology & Cell Biology Departments and Animal House of Faculty of Medicine, Zagazig University. Approval for performing the experimental design was obtained from the Institutional Animal Care and Use Committee, Zagazig University (ZU-IACUC) with the approval number: ZU-IACUC/3/F/294/2022.

Materials:

Chemicals:

1. Thiamethoxam

Thiamethoxam (TMX) 98% CAS number 153719-23-4 is a creamy, odorless powder that is chemically stable at room temperature. It was acquired from the Sigma-Aldrich Co. branch in Cairo, Egypt, and produced by the Sigma-Aldrich Company, Louis St., USA.

Quercetin

A yellow powdered quercetin dihydrate extrapure, 99%, CAS number 6151-25-3, was obtained from Sisco Research Laboratories Pvt Ltd in India.

2. Reagents and commercial kits:

We purchased kits from Sigma and Bio-diagnostic chemical businesses in Cairo, Egypt, for the enzymes glutamate dehydrogenase (GDH), aspartate transaminase (AST), alanine transaminase (ALT), glutathione peroxidase, catalase, and nuclear erythroid 2-related factor (Nrf2).

3. Normal saline (0.9% Nacl solution).

It was obtained from local pharmacy. It is a vehicle for both thiamethoxam and quercetin.

Animals:

Thirty adult male albino rats were obtained from animal house of faculty of medicine, Zagazig University. All animals received a good care in compliance with the animal care guidelines of the National Institutes of Health (NIH), and the Institutional Animal Care and Use Committee, Zagazig University (ZU-IACUC).

Throughout the experiment, numerous unintentional observations about the influence of the environment have been made. According to **Romanovsky et al. (14)** in order to eliminate fallacies. The temperature in the cage and the animal housing, which both require adequate ventilation with A 12-hour light cycle, an ambient temperature range of 22 ± 2°C, and a relative humidity of 50 ± 5%. Because noise can influence animal behavior, a low volume was maintained. All of the animals underwent two weeks of passive preliminaries prior to the experiment's start in order to acclimate to their new surroundings, determine their physical health, and rule out any sick animals **(15).** Before and during the experiment, the rats were fed a well-balanced diet that was full of all the nutrients they needed to stay healthy. It includes grains and bread; water was provided in distinct, hygienic containers.

Experimental Procedure:

***Period of study*:**

According to **Khaldoun-Oularbi et al. (16)** the experimental study's duration was six weeks. Oral gavage of quercetin and thiamethoxam was administered once a day, six days a week.

***Grouping of animals*:**

This study comprised thirty adult male albino rats in good health, weighing between 150 and 200 grams, and ranging in age from eight to ten weeks. There were five equal groups of rats with six rats in each group, as follows:

Group I (negative control): rats were given merely a standard food and tap water every day. **Group II (positive control):** One ml of normal saline 0.9% NaCl (thiamethoxam and quercetin solvent) was given to each rat.

Group III (quercetin treated): rats were given 50 mg/kg body weight (BW) of quercetin (11), which was dissolved in five ml of 0.9% saline.

Group IV (thiamethoxam treated): TMX was administered to each rat at a dose of 78.15 mg/kg BW (17) in five milliliters of 0.9% saline. The study's chosen dosage of TMX is equivalent to 1/20 of the rats' oral LD50. (1,563) mg/kg/BW (18).

Group IV (Quercetin +TMX treated): The same dosages of TMX and quercetin were given and quercetin was given an hour before TMX (19).

At the end of six weeks (24 hours from the last dose), The rats were anaesthetized with pentobarbital 60 mg/kg body weight. Venous blood samples collected from animals by means of micro–capillary glass tubes from the retro-orbital plexuses (20) in accordance with the procedure described by Johnson, (21).

Blood collected in plain tube for measurement of:

- **Hepatic parameters:** glutamate dehydrogenase enzyme (GDH), the early indicators of liver damage. Aspartate transaminase (AST) and alanine transaminase (ALT).

- **Oxidative stress marker:** Serum Malondialdehyde (MDA).

Rats were slaughtered after blood samples were collected, and the liver was promptly and meticulously dissected to remove any RBCS and blood clots, liver perfused with a phosphate buffered saline (PBS), that contain 0.16 mg /mL heparin to remove any clots (22),

Parts of liver tissues were stored in liquid nitrogen at -80°C till subsequent biochemical analysis of glutathione peroxidase and catalase done.

Also parts of liver tissues were isolated over ice and then processed for alkaline single cell gel electrophoresis (comet assay).

The remaining parts of liver tissues were fixed in 10% formalin, processed and embedded in paraffin blocks that prepared for Hematoxylin and Eosin stain (H&E) and Immunohistochemical staining

Biochemical Studies:

I. Estimation of hepatic parameters:

Estimation of GDH according to the method described by **Calmels et al. (24)**. Estimation of AST and ALT according to the method described by **Murray, (25)**.

II. Oxidative stress markers:

Glutathione peroxidase, catalase and serum MDA estimation using the techniques recommended by **Iskusnykh et al. (26), Hadwan. (27)** and **Lovrić et al. (28)** respectively.

Histopathological studies: Liver tissues were fixed in 10% formalin neutral buffered formalin for 24 hours, dehydrated in ethyl alcohol, cleared in xylene and embedded in paraffin blocks and processed to create slices with a thickness of 5 u. Hematoxylin and Eosin(H&E) stains were applied to these sections using the procedure outlined by **Bancroft and Gamble (29)**.

The immunohistochemistry analysis of caspase-3 was conducted using the methodology outlined by **Mustafa et al. (30).**

Image Analysis (Morphometric study):

An Olympus microscope with a digital camera was used to view and record three to four non-overlapping fields per slide. A scale bar is shown with micrographs. The Image J (v1.50) tool was used to analyze the images in accordance with **Horai et al. (31)** as follows:

1. At X400, liver parameters (hepatocyte and Kupffer cell counts) were examined.
2. The number of Caspase-3 positive cells at X400 for the liver was used to examine Caspase-3 immunostaining.

Scoring of liver injury

The liver's histopathological alterations were inspected and evaluated. The accompanying hepatic injury assessment system was carried out in accordance with **Ishak et al. (32).** Liver damage was scored at 400x magnification on slices stained with H&E. The related liver injury is categorized into four grades using the Ishak rating method as follows:

Grade 0: no pathological changes.

Grade 1: mild congestion or the presence of injured hepatocytes.

Grade 2: moderate congestion or distorted hepatocytes.

Grade 3: manifest congestion or severely injured hepatocytes.

Grade 4: severe congestion or the presence of severely injured hepatocytes.

A) Single cell gel Electrophoresis (Comet) assay:

The Comet assay was carried out at the Ministry of Agriculture and Land Reclamation's Animal Reproductive Research Institute (ARRI) of the Agricultural Research Center **(El Haram, Giza)**; according to the method of **Singh et al. (33) & Khan et al. (34).**

Techniques for Statistical Analysis:

According to **IBM (2020)**, the Statistical Package of Social Science (SPSS) software version 27 was used to computerize and statistically analyze the gathered data. The least significant difference (LSD), t-test, and one-way analysis of variance (ANOVA or F-test) were employed.

Results

I- Biochemical Results:

Biochemical parameters of control groups:

Comparing the laboratory results of biochemical tests regarding serum biomarkers of liver functions and oxidative stress biomarkers of the negative and positive control groups, there were no statistically significant difference between them all over the period of the study ($p > 0.05$). So, negative control group was used for comparison with other groups.

Table (1): Statistical comparison between negative and positive control groups as regard mean values of liver function tests and oxidative stress parameters using student t-test test for six weeks

Groups / Parameters	Negative control	Positive control	T	P
	Mean ± SD			
Liver function tests				
GDH (ng-ml)	7.55±1.86	6.99±1.39	0.588	0.570 NS
AST (IU/L)	104.76±7.27	105.76±8.12	0.2450	0.8114 NS
ALT (IU/L)	53.83±4.66	55.27±8.95	0.350	0.733 NS
Oxidative stress parameters				
Hepatic Glutathione peroxidase (ng-ml)	222.47±13.54	227.77±10.00	0.771	0.459 NS
Hepatic Catalase (ng-ml)	10.00±1.21	9.83±1.07	0.252	0.806 NS
Serum MDA (nmol /L)	0.52±0.14	0.47±0.13	0.700	0.500 NS

Number of rats in each group=6 rats. All values are expressed as mean± SD. SD: standard deviation, NS: non-significant (P >0.05. AST: Aspartate transaminase, ALT: Alanine transaminase, GDH: Glutamate dehydrogenase

MDA: Malondialdehyde.

Liver function tests (GDH, AST and ALT):

The ANOVA test revealed a highly significant difference (p<0.001) in the mean blood GDH, AST and ALT values across all studied groups, as seen in (Table 2 and Figures 1&2).

By Least significance difference (LSD) test, when comparing the quercetin-treated group to the negative control group, the mean values of the serum levels of GDH, AST, and ALT did not differ significantly.

The results of the study revealed a highly significant increase (p<0.001) in the mean serum levels of GDH, AST, and ALT values in TMX treated group when compared to control group.

When quercetin and TMX were administered together, the mean serum levels of GDH, AST, and ALT were significantly lower (p<0.001) than in the TMX-treated group.

Table (2): Statistical comparison among different studied groups as regard mean values of serum levels of liver enzymes (GDH, AST and ALT) for six weeks using ANOVA and LSD test

Groups / Parameters	Negative control	Quercetin	Thiamethoxam	Quercetin+ thiamethoxam		P-value
	Mean ± SD					
GDH (ng-ml)	7.55±1.86	6.99±1.39	37.95±1.93 a,b	16.36±7.93a, b, c	69.823	<0.001**
AST(IU/L)	104.76±7.27	108.76±7.26	193.85±7.94 a,b	117.66±10.59 a,b,c	156.545	<0.001**

ALT(IU/L)	53.83±4.66	61.33±6.93	168.58±7.60 a,b	74.02±11.27 a,b,c	268.577	<0.001**

Number of rats in each group=6 rats. All values are expressed as mean± SD. (SD: standard deviation).

ANOVA: One Way Analysis of Variance.

LSD: least significant difference **: very highly significant (P<0.001).

AST: Aspartate transaminase ALT: Alanine transaminase.

GDH: Glutamate dehydrogenase.

a: significantly different as compared to control group (p < 0.001).

b: significantly different as compared to quercetin group (p < 0.001).

c: significantly different as compared to TMX treated group (p < 0.001).

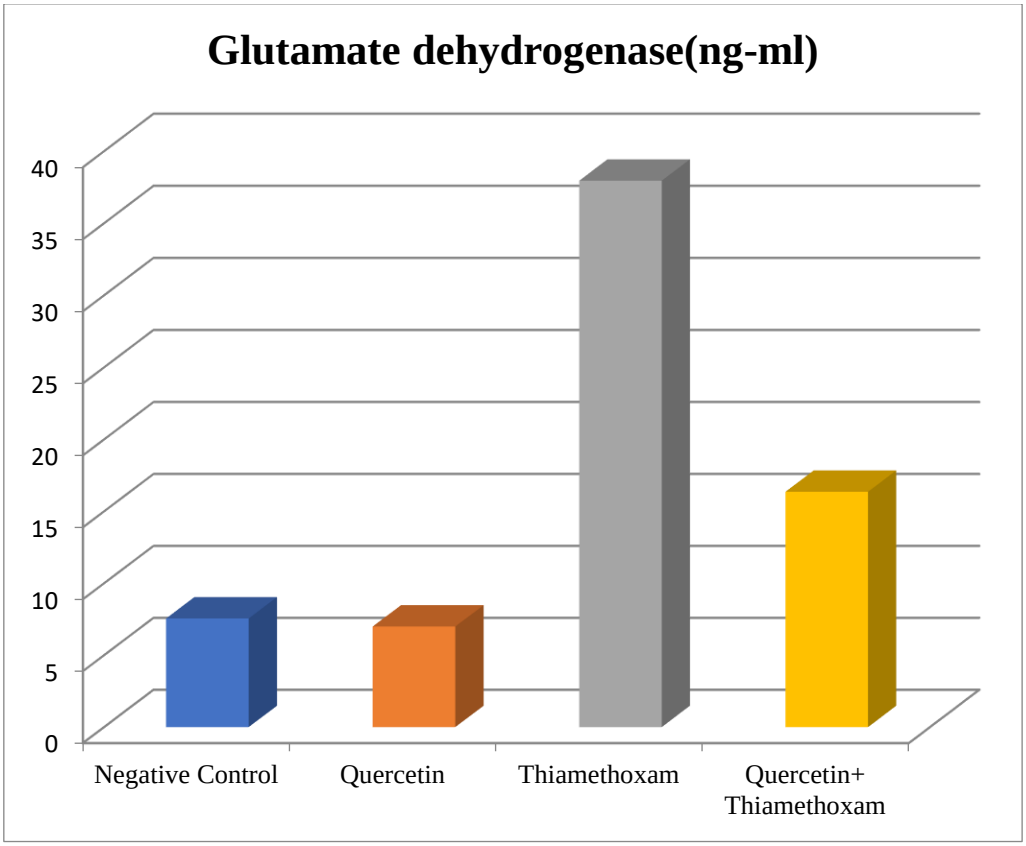

Figure (1): Bar chart showing graphical comparison among different studied groups as regard mean values of serum glutamate dehydrogenase (GDH) after six weeks.

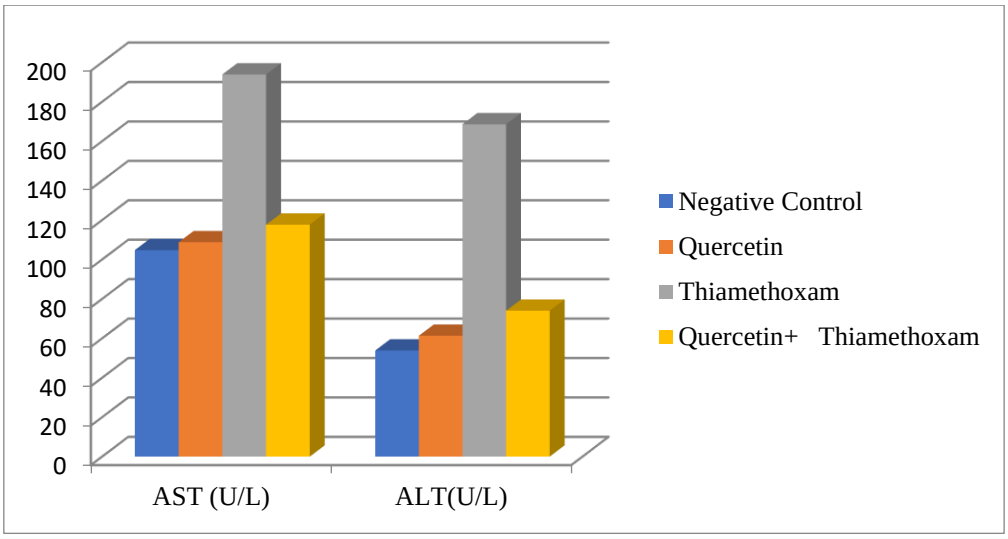

Figure (2): Bar chart showing graphical comparison among different studied groups as regard mean values of serum aspartate transaminase (AST) and alanine transaminase (ALT) after six weeks.

❖ **Oxidative Stress Biomarkers: -**

1. **Glutathione peroxidase and Catalase in liver:**

According to Table 3, the ANOVA test revealed a highly significant difference ($p<0.001$) in the mean values of hepatic glutathione peroxidase and catalase in all studied groups.

By Least significance difference (LSD) test when comparing the quercetin-treated group to the negative control group, the mean values of the hepatic glutathione peroxidase and catalase levels were not significantly different.

Administration of TMX for six weeks revealed a highly significant decrease ($p<0.001$) in the mean values of hepatic glutathione peroxidase and catalase when compared to negative control and quercetin treated groups.

When quercetin and TMX were administered together, the mean values of hepatic glutathione peroxidase and catalase increased significantly ($p<0.001$) in comparison to the TMX-treated group.

2. **Serum Malondialdehyde (MDA):**

The ANOVA test revealed a highly significant difference ($p<0.001$) in the mean serum MDA values across the several studied groups, as indicated in Table 3 and Figure 3.

By Least significance difference (LSD) test, the quercetin-treated group's mean serum MDA levels did not differ significantly from those of the negative control group.

Administration of TMX for six weeks revealed a highly significant increase ($p<0.001$) in the mean values of serum MDA when compared to negative control and quercetin treated groups.

When quercetin and TMX were administered together, the mean serum level values of MDA increased significantly ($p<0.001$) in comparison to the TMX-treated group.

Table (3): Statistical comparison among different studied groups as regard mean values of oxidative stress parameters for six weeks using ANOVA and LSD tests

Groups / Parameters	Negative control	Quercetin	Thiamethoxam	Quercetin+ thiamethoxam	F	P-value
	Mean ± SD					
Hepatic Glutathione peroxidase (ng-ml)	222.47±13.54	216.48±14.71	34.09±10.13 a,b	93.79±7.34 a,b,c	373.535	<0.001**
Hepatic Catalase (ng-ml)	10.00±1.21	9.21±1.04	0.58±0.02 a,b	2.48±0.68 a,b,c	176.881	<0.001**
Serum MDA (nmol-ml)	0.52±0.14	0.48±0.13	5.81±0.88 a,b	0.91±0.12 a,b,c	190.418	<0.001**

Number of rats in each group = 6rats. All values are expressed as mean ± SD. (SD: standard deviation). ANOVA: One Way Analysis of Variance, **: very highly significant (P<0.001).

MDA: Malondialdehyde,

a: significantly different as compared to control group ($p < 0.001$).

b: significantly different as compared to quercetin group ($p < 0.001$).

c: significantly different as compared to TMX treated group ($p < 0.001$)).

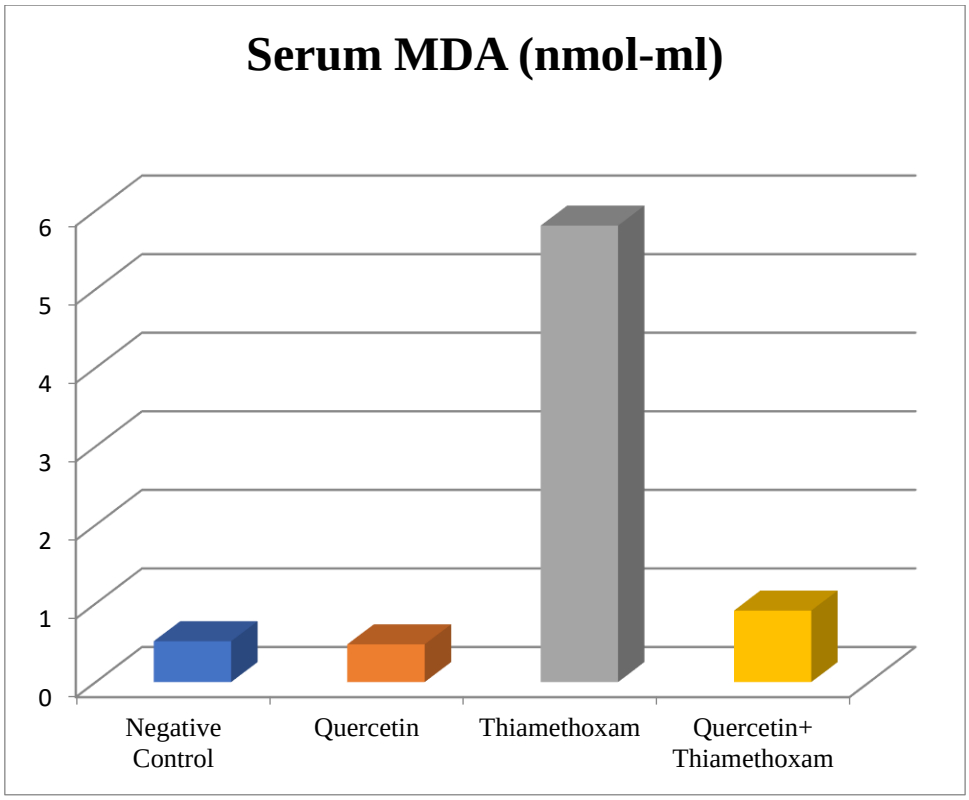

Figure (3): Bar chart showing graphical comparison among different studied groups as regard mean values of serum malondialdehyde (MDA) after six weeks.

Histopathological results:

1. Macroscopic features:

Cut sections of the liver were normal in all studied groups, and there were no aberrant tumours or cystic alterations to be seen.

2. Microscopic characteristics:

A. Control and quercetin-treated groups:

The histopathological analysis of H&E-stained liver sections from adult male albino rats in the negative control, positive control, and quercetin-treated groups revealed normal hepatic lobular architecture; the connective tissue stroma contained branches of the portal vein, hepatic artery, and bile duct within hexagonal or pentagonal lobules with central veins and peripheral hepatic triads (portal areas). Cords of hepatocytes radiate outward from the central vein. They featured big, pale vesicular nuclei and acidophilic cytoplasm that looked stippled. Endothelial cells bordered the sinusoidal gaps that separated these cords (figure 4A&B).

B. Thiamethoxam -treated group:

Liver tissues of TMX treated rats showed deformation of the liver, including lymphocyte infiltration (in the portal region) and a loss of the normal hepatic cord pattern along with cytoplasmic density. Additionally, sinusoidal congestion was noted. Lymphocyte-induced sinusoidal constriction was observed. Most hepatocytes had little, dark, disintegrated cytoplasm in

their nuclei. Higher numbers and hyperactivation of hepatic Kupffer cells were observed (Figure 4 C1&C2).

C. Quercetin + Thiamethoxam group:

Normal central vein, limited blood sinusoidal congestion, and minimum cellular infiltration all demonstrated a significant improvement in the hepatic architecture following quercetin and TMX administration. Few hepatocytes contained tiny, black nuclei, but the majority of liver nuclei were vesicular and brilliant. A small number of Kupffer cells were also observed (Figure 4D).

Figure (4): Photomicrographs of liver sections from different study groups. **(A&B)** represent the normal control and quercetin treated groups, correspondingly, show normal hepatic histological structure. Hepatocytes (arrow) are organized in hepatic cords radiating from the central vein (C). Blood sinusoids run between neighbouring hepatocytes (S) and containing Kupffer cells (curved arrow). **(C1 &C2)** TMX-treated rats exhibit increased sinusoidal congestion (S) and marked lymphocytic infiltration (double arrow) mainly in the portal area. Most of hepatocytes have small dark nuclei (arrow) with fragmented cytoplasm whereas, some hepatocytes had bright vesicular nuclei (curved arrow) with increased number of Kupffer cells (arrowhead). **(D)** Quercetin +TMX livers rats exhibit increased sinusoidal congestion (S) and marked lymphocytic infiltration (double arrow) mainly in the portal area. Most of hepatocytes have small dark nuclei (arrow) with fragmented cytoplasm whereas, some hepatocytes had bright vesicular nuclei (curved arrow). Increased number of Kupffer cells (arrowhead) is noticed. **(H & E, X400).**

Immunohistochemical results:

1) ***Control and quercetin treated groups:***

The cytoplasm of hepatocytes in the control and quercetin-treated groups' liver slices displayed negative caspase-3 immunoreactivity, according to immunohistochemical analysis (figure 5 A&B).

2) **Thiamethoxam treated group:**

After six weeks, as compared to the negative control group, immunohistochemical analysis of the liver sections of the TMX treated group revealed high positive caspase-3 immunoreactivity in the cytoplasm of hepatocytes (figure 5c).

3) **Quercetin + TMX group:**

After six weeks, the light microscopic analysis of immunohistochemically stained liver sections revealed weak positive caspase-3 immunoreactivity in the hepatocyte cytoplasm in comparison to the TMX group (figure 5D).

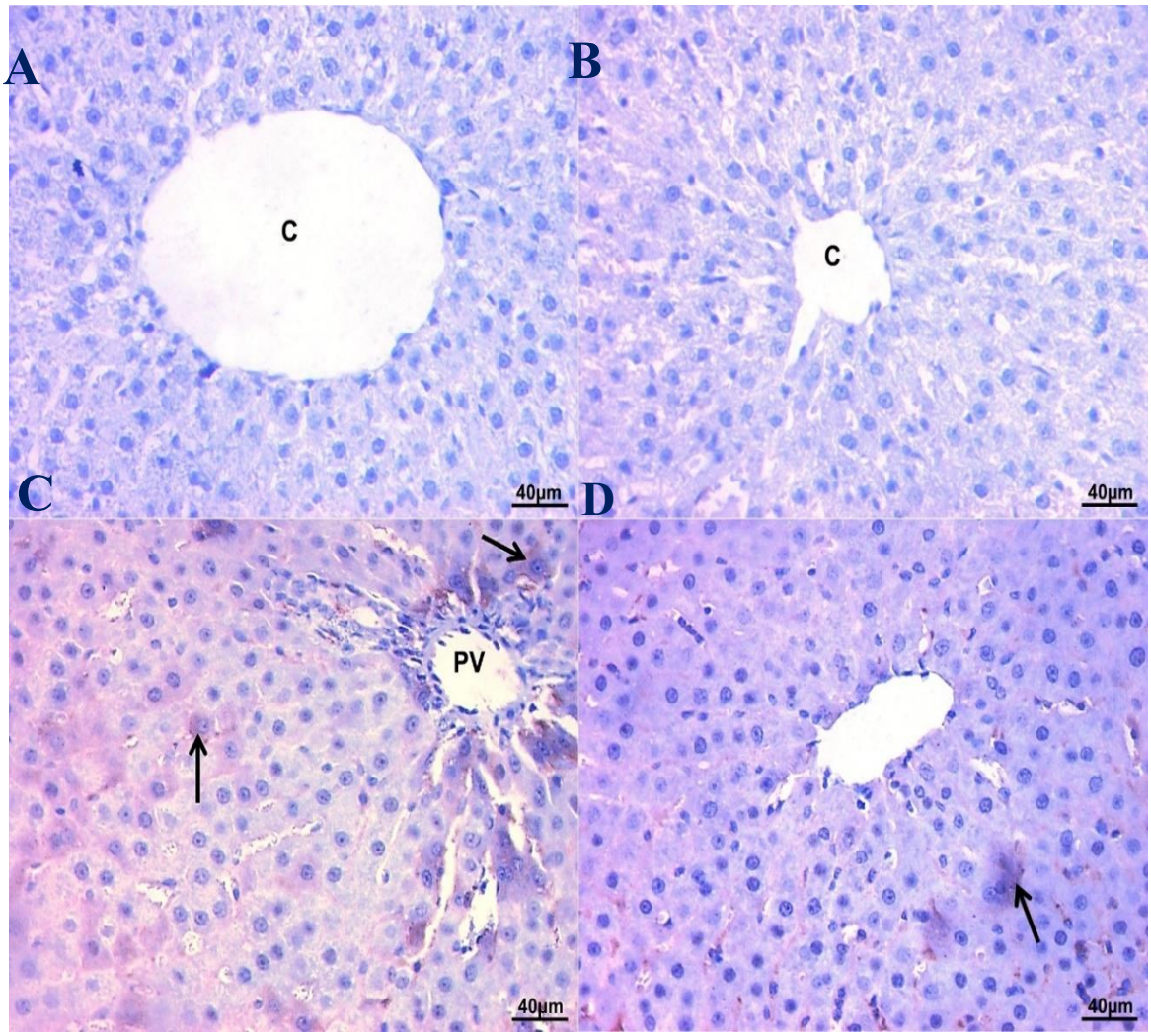

Figure (5): Photomicrographs of Caspase-3 immunohistochemical reaction in different experimental groups. (**A & B**), represent normal and quercetin treated groups respectively, show negative Caspase-3 immunoreaction. (C) TMX treated rats showed a strong reaction to caspase-3 (arrow) around the portal vein (PV). (D) Quercetin + TMX ingested rats demonstrate a weak cytoplasmic immunoreaction to Caspase-3 (brown colour indicates a positive Caspase-3 immunoreaction (X400).

Morphometric study of histopathological result:

A) Number of hepatocytes, Kupffer cells, Caspase- 3 positive cells in liver:

The mean numbers of hepatocytes, Kupffer cells, and Caspase-3positive cells in the hepatic tissues in the negative and positive control groups were within normal ranges. Over the course of the trial, there was no statistically significant difference between them ($p>0.05$). In order to compare other groups, the negative control group served as a standard reference. **(table 4).**

Table (4): Statistical comparison between negative and positive control groups as regard mean values of number of hepatocytes, number of Kupffer cells, Caspase-3 positive cells in hepatic tissues of different studied groups for six weeks using student t-test test

Groups / Parameters	Negative control	Positive control	T	P
	Mean ± SD			
No of hepatocytes	330.83 ±15.35	334.33 ±12.09	0.439	0.670 NS
No of Kupffer cells	56.33 ±1.96	52.00 ±5.21	1.904	0.086 NS
Caspase-3positive hepatocytes	0.0 ±0.0	0.0±0.0	—	—

Number of rats in each group = 6 rats. All values are expressed as mean± SD. (SD: standard deviation), NS: non-significant (P >0.05).

The ANOVA test revealed a highly significant difference ($p<0.001$) in the mean values of the number of hepatocytes, Kupffer cells, and caspase-3 positive cells in hepatic tissues in all studied groups (Table 5).

By Least significance difference (LSD) test, when comparing the quercetin-treated group to the negative control group, there was no discernible difference in the mean numbers of hepatocytes, Kupffer cells, and caspase-3 positive cells in the hepatic tissues.

Comparing the hepatic tissues of the TMX-treated and quercetin + TMX groups to the negative control and quercetin-treated groups, the results revealed a highly significant increase in Kupffer cells and Caspase-3 positive cells and a very significant decrease ($p<0.001$) in the mean values of hepatocytes.

When compared to the TMX-treated group, the administration of quercetin in addition to TMX resulted in a very significant decrease in the mean values of Kupffer cells and Caspase-3 positive cells in hepatic tissues and a very significant increase ($p<0.001$) in the mean values of hepatocytes.

B) Score of hepatic injury:

According to Table 5, the ANOVA test revealed a highly significant difference ($p<0.001$) in the mean values of the hepatic tissue injury score among different studied groups.

When comparing the quercetin-treated group to the negative control group, there was no discernible difference in the hepatic tissue injury score.

Comparing the TMX-treated and quercetin + TMX groups to the negative control, there were a very substantial increase ($p<0.001$) in the mean values of the hepatic tissue injury score.

When quercetin and TMX were administered together, the mean values of the hepatic tissue injury score decreased significantly (p<0.001) in comparison to the TMX-treated group.

Table (5): Statistical comparison among different studied groups as regard mean values of number of hepatocytes, Kupffer cells, Caspase-3 positive cells and hepatic injury score in hepatic tissues of different studied groups for six weeks using ANOVA and LSD tests

Groups / **Parameters**	Negative control	Quercetin	Thiamethoxam	Quercetin+ thiamethoxam	F	P-value
	Mean ± SD					
No of hepatocytes	330.83 ±15.35	350.50±41.47	234.66±2.58a,b	290.16±8.20a,b,c	30.96	<0.001**
No of Kupffer cells	56.33 ±1.96	59.33±6.62	89.16±6.17a,b	74.83±8.61a,b,c	34.54	<0.001**
Caspase-3positive hepatocytes	0.0 ±0.0	0.0±0.0	26.83±2.13 a,b	5.83±1.60a,b,c	546.48	<0.001**
Liver injury score	0.00±0.00	0.00±0.00	3.36±0.45 a,b	1.86±0.56a,b,c	121.763	<0.001**

Number of rats in each group=6 rats. All values are expressed as mean± SD. (SD: standard deviation), **: very highly significant (P<0.001).

a: significantly different as compared to control group (p < 0.001).

b: significantly different as compared to quercetin group (p < 0.001).

c: significantly different as compared to TMX treated group (p < 0.001)).

Comet assay results:

In terms of mean values of the percentage of tailed nuclei, the percentage of untailed nuclei, the length of the tail, the percentage of tail DNA, and tail moment unit in the liver after six weeks, the results of this study revealed no significant difference (P>0.05) between the negative control group and the vehicle positive control group, so negative control group is used as standard for comparison with other studied groups (Table 7).

Table (6): Statistical comparison between negative and positive control groups as regard mean values of Comet parameters in hepatic tissue for six weeks using student t-test test.

Groups / Parameters	Negative control	Positive control	T	P
	Mean ± SD			
Tailed nuclei %	3.66±0.81	3.16±0.75	1.103	0.296 NS
Untailed nuclei %	96.33±0.81	96.83±0.75	1.103	0.296 NS
Tail length	1.26±0.05	1.25±0.04	0.634	0.540 NS
Tail DNA%	1.12±0.07	1.09±0.06	0.669	0.519 NS
Tail moment	1.40±0.04	1.33±0.08	1.740	0.112 NS

N.B All values are expressed as mean± SD. (SD: standard deviation). Number of rats in each group= 6 rats. NS: non-significant (P >0.05). DNA: deoxyribonucleic acid

The ANOVA test revealed a highly significant difference (p<0.001) between all studied groups regarding the mean values of the liver's tail length, tail DNA percentage, percentage of tailed nuclei, and percentage of untailed nuclei (Table 7).

By Least significance difference (LSD) test, comparing the quercetin-treated group to the negative control group revealed no discernible difference.

Comparing liver specimens from the TMX-treated and quercetin +TMX groups to those from the negative control group, however, revealed a very significant decrease in the percentage of untailed nuclei and a very significant increase (p<0.001) in the percentage of tailed nuclei, tail length, tail DNA percentage, and tail moment unit.

When compared to the TMX group, quercetin administration resulted in a very large rise in the percentage of untailed nuclei and a very substantial drop (p<0.001) in the percentage of tailed nuclei, tail length, tail DNA percentage, and tail moment unit.

Fluorescent photomicrographs, as seen in Figure (6), can be used to depict the comet assay results of liver tissues.

Table (7): Statistical comparison among different studied groups as regard mean values of Comet parameters in hepatic tissue for six weeks using ANOVA and LSD tests

Groups / Parameters	Negative control	Quercetin	Thiamethoxam	Quercetin+ Thiamethoxam	F	P-value
	Mean ± SD					
Tailed nuclei %	3.66±0.81	2.83±0.41	15.00 ±1.09 a,b	6.16 ±0.98 a,b,c	248.370	<0.001**
Untailed nuclei %	96.33±0.81	97.16± 0.41	85.00 ±1.09 a,b	93.83 ±0.98 a,b,c	248.370	<0.001**
Tail length	1.26±0.05	1.15±0.08	3.86 ±0.12 a,b	2.37 ±0.42 a,b,c	184.065	<0.001**
Tail DNA%	1.13±0.07	1.12±0.09	3.83 ±0.35 a,b	1.97 ±0.25 a,b,c	190.816	<0.001**
Tail moment	1.40±0.04	1.20±0.06	14.84 ±1.84 a,b	3.50 ±0.89 a,b,c	172.972	<0.001**

Number of rats in each group=6rats. All values are expressed as mean ± SD. (SD: standard deviation). **: very highly significant (P<0.001). DNA: deoxyribonucleic acid

a: significantly different as compared to control group ($p < 0.001$).

b: significantly different as compared to quercetin group ($p < 0.001$).

c: significantly different as compared to TMX treated group ($p < 0.001$)).

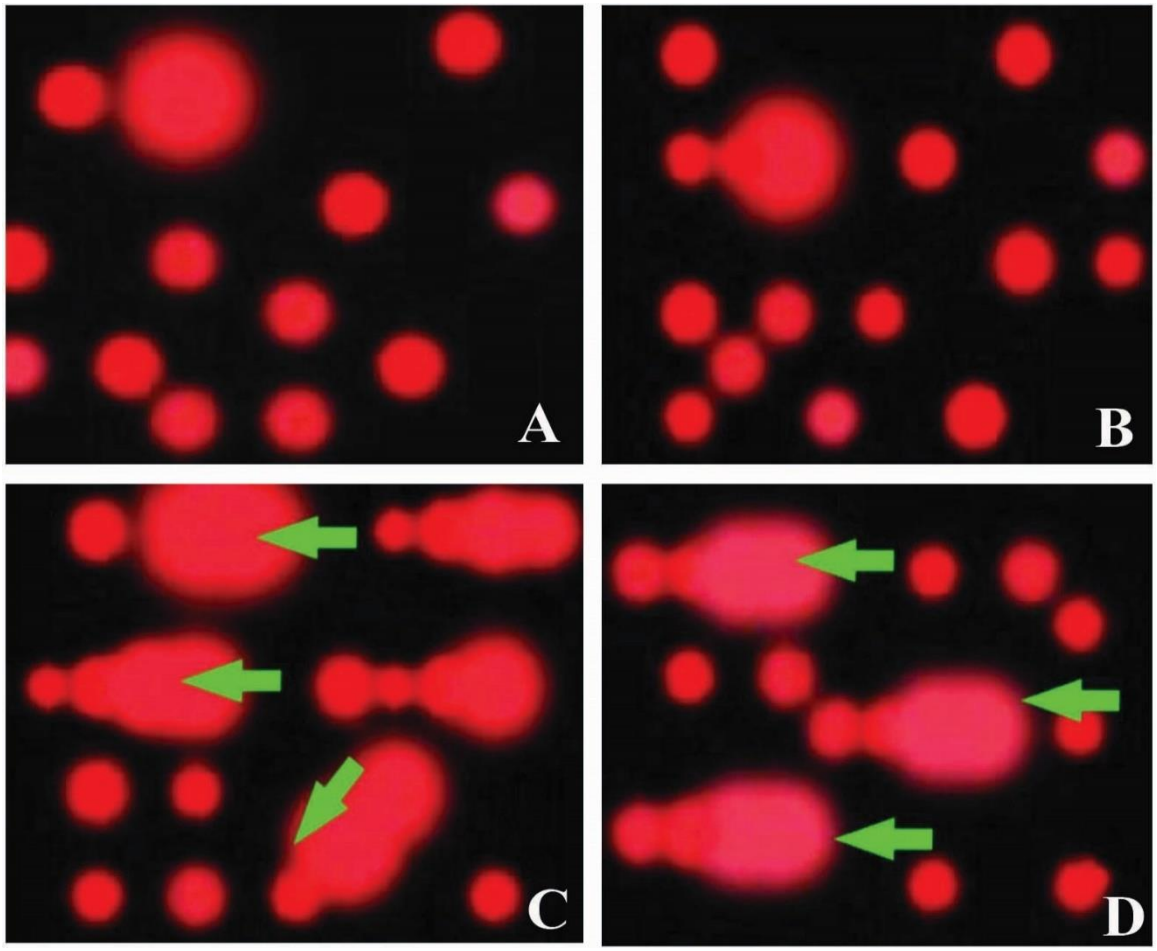

Figure (6): A photomicrograph by fluorescent microscope of liver cells nuclei of different studied groups. **(A&B)** represent normal and quercetin treated groups respectively showing showing DNA in most cells is tightly compressed and maintains the circular disposition of normal nucleus.(**C**) TMX-treated rats, the Comet has residual head and long dense tail pattern since most DNA migrated to tail (green arrow). **(D)** quercetin + TMX treated group, the comet has residual head but shorter and less dense tail pattern as compared to thiamethoxam treated group (green arrow).

Discussion:

The first example of a second-generation neonicotinoid with a distinctive structure and exceptional insecticidal activity is thiamethoxam, a new neonicotinoid insecticide (35).

Quercetin may help in prevention of a number of illnesses and has been used as a dietary supplement. Anticancer, antitumor, anti-ulcer, anti-allergy, antiviral, anti-inflammatory, anti-diabetic, gastroprotective, antihypertensive, immunomodulatory, and anti-infective are a few of the positive benefits (36).

The current study sought to identify the effects of TMX on the liver of adult male albino rats and determine the role of oxidative stress and apoptosis in the pathophysiology of TMX toxicity and to identify the protective role of quercetin against these toxic effects through biochemical, histopathological, and genotoxic studies.

Thiamethoxam caused hepatic toxicity in the current, as evidenced biochemically by a marked rise in the mean values of the liver enzymes GDH, AST and ALT in the serum coincided with the histopathological changes in hepatic architecture. The liver tissues displayed cytoplasmic density reduction, loss of the normal hepatic cord pattern, and lymphocyte infiltration in the portal region. Additionally, lymphocyte-induced sinusoidal constriction and congestion were induced. Most hepatocytes had little, dark, disintegrated cytoplasm. Higher numbers and hyperactivation of hepatic Kupffer cells were observed.

To the best of our knowledge, the impact of TMX on the GDH level was not discussed in any earlier research. However, Khalaf et al. (37) showed that 28 patients who arrived to Minia Poisoning Control Center with acute oral aluminum phosphide (AlP) poisoning between January 1st, 2015, and December 31st, 2015, had a marked rise in GDH levels as a result of AlP-induced hepatotoxicity.

Also, Savithri et al. (38) demonstrated a significant increase in GDH levels secondary to chlropyrifos-induced hepatotoxicity via oxidative stress. In the same way, TMX induced hepatotoxicity via oxidative stress so, elevated GDH levels may be found secondary to TMX toxicity.

The findings of the present study are similarly consistent with those of Elhamalawy et al. (2), Abouelghar et al. (39) Khaldoun-Oularbi et al. (16) and Hataba et al. (40) who found that administration of TMX in rats showed significant increases in ALT and AST along with disruption of normal hepatic architecture, as evidenced by clogged blood vessels, activated Kupffer cells, liver necrosis, lymphocytes infiltration sinusoid dilatation, bleeding, vacuolization, and necrosis.

Also, El-Sheikh et al. (41) reported dose-dependent increases in ALT and AST following oral treatment of TMX to adult male albino rats with focal necrosis and bleeding in hepatic tissues which are consistent with the findings of the current study.

Because TMX enhances the incidence of lipid peroxidation and modifies the antioxidant enzyme systems as reported by the current work, Abdel-Razik et al. (9) demonstrated that it can have a significant impact on the hepatic tissue. Triggering a variety of oxidative processes and lipid peroxidation, oxygen free radicals produced as a result of TMX.

In comparison to the negative control group, the current study's findings showed a significant positive caspase-3 immunoreactivity in the cytoplasm of the TMX-treated group's hepatocytes following oral gavage of TMX.

The results of present study are consistent with those of Nassar et al. (1), who found that TMX-treated rats' hepatocytes had positive caspase-3 immunoreactivity.

Increased oxidative stress that disrupted mitochondrial membrane permeability may have contributed to the strong positive reaction for Caspase-3 in this study. This resulted in the leakage of cytochrome-c and free radicals from the mitochondria to the cytosol, which binds to another protein called apoptotic protease activating factor 1 (Apaf-1) and promotes activation of the caspase cascade, ultimately resulting in cell death (42).

The histopathological findings are corroborated by morphometric research, which shows that, in comparison to the negative control and quercetin-treated groups, the TMX-treated hepatic tissues had a highly significant increase in Kupffer cells, Caspase-3-positive cells, and injury scores, as well as a significant decrease in the mean values of hepatocytes.

These findings are consistent with those of El Okle et al. (8), who documented similar alterations in liver sections of rabbits treated with TMX. Their computerized histo-morphometric analysis showed a significant decrease in the area of parenchymal elements and a significant increase in the area of non-parenchymal elements in comparison to the control group. Significant increases in intra-lobular necrosis and patchy infiltrates, as well as chronic inflammatory cell infiltration and fibrous tissue proliferation, may account for these findings in rabbits given TMX.

The TMX-treated group's liver specimens showed a very substantial drop in the percentage of untailed nuclei and a very significant rise ($p<0.001$) in the percentage of tailed nuclei, tail length, tail DNA percentage, and unit tail moment in comet assay.

According to Nassar et al. (1) and Elhamalawy et al. (2), TMX significantly increased the rates of DNA damage in hepatic cells as determined by the comet assay. These findings were consistent the findings of the present study.

Additionally, Also, Jameel et al. (43) reported that TMX interacts directly with DNA and greatly affects various biological and biochemical parameters of treated animals.

Lipid peroxy radicals, which harm cells by altering the fluidity and permeability of the cell membrane or by attacking the cellular DNA molecule, causing DNA strand breaks, oxidation of its bases, and other intracellular molecules like proteins, may be the cause of DNA damage with TMX. (1).

The administration of thiamethoxam resulted in a considerable increase in serum malondialdehyde (MDA) and a significant decrease in hepatic glutathione peroxidase and catalase levels in comparison to the controls ($P<0.001$) which could explain the hepatotoxicity and DNA damage reported by the current study.

The oxidative stress findings of the present study are consistent with those of Alsadee et al. (44), who reported that thiamethoxam administration reduced the levels of glutathione peroxidase and catalase enzymes and markedly increased the serum MDA levels in the albino rats' livers when compared to the control group.

Additionally, the current study's findings were consistent with those of Abdel-Razik et al. (9), El-Sheikh et al. (41), who showed that rats given TMX had significantly increase the levels of MDA and decreased the levels of glutathione peroxidase and catalase in their serum.

Numerous causes could be responsible for these oxidative stress marker values. First, by producing free radicals, TMX induce oxidative stress, which suggests a malfunctioning mitochondrial respiratory chain (45); second, they disturb antioxidant homeostasis, which results in antioxidant depletion; and third, they disturb redox processes, which changes the activities of antioxidant enzymes and raises lipid peroxidation (46).

According to this study, the antioxidant system is unable to handle the intake of free radicals brought on by TMX exposure, as evidenced by the reduction of antioxidant enzymes (catalase and glutathione peroxidase) and increase MDA which may be responsible for hepatotoxicity and DNA damage. Katić et al. (4) demonstated that H2O2 clumps together when enzymes involved in eliminating free radicals are suppressed, which encourages lipid peroxidation and DNA amendment.

When quercetin was administered with TMX, the mean values of serum GDH, AST and ALT decreased significantly ($P < 0.001$) with improvement of the hepatic architecture, as evidenced by normal central vein, low blood sinusoidal congestion, and low cellular infiltration. Few hepatocytes exhibited tiny, black nuclei, but the majority of liver nuclei were vesicular and brilliant with small amount of Kupffer cells.

Hassan et al. (12) demonstrated that pretreatment with quercetin with another neonicotinoid (imidacloprid) considerably reduced the elevated blood concentrations of liver enzymes, including AST and ALT, with noticeable improvement in hepatic changes caused by imidacloprid which is consistent with the findings of the present study.

The current study's findings also supported by those of Ghazanfari et al. (13) who found that pre-treatment of quercetin with another neonicotinoid (acetamiprid), reduced the elevated serum levels of liver enzymes (AST and ALT).

Furthermore, the findings of Gheshlaghi et al. (47) showed that multiple simultaneous treatment with quercetin intraperitoneally decreased the elevated ALT and AST and protected against pathological changes induced by another neonicotinoid (clothianidin) in the liver.

The findings of present study are in accordance with Nigam et al. (48) and Ghazanfari et al. (13) who found that quercetin reduced liver damage caused by neonicotinoids (TMX and acetamiprid respectively) in adult albino rats.

The current study's findings showed that quercetin with TMX significantly improved the hepatocytes' responses to Caspase-3.

These finding were coincided with Owumi et al. (49) who reported that co-treatment of quercetin with another insecticide (carbendazim) markedly suppressed the increase in caspase-3 activity, thus signifying the anti-apoptotic properties of quercetin.

Additionally, in adult male rats treated with another pesticide (cypermethrin), pretreatment with quercetin reduced the expression of Caspase 3 in the lung tissues, according to Ileriturk et al. (50).

This ameliorative effects of quercetin on biochemical, histopathologicl and immune-histochemical reactivity of caspase-3 can be explained with the quercetin's capacity to scavenge free radicals and provide an antioxidant barrier by chelating divalent cations and preventing ROS formation. helps prevention of apoptosis caused by lipid peroxidation by lowering hepatic lipid peroxides (51).

It is known that quercetin can be tethered to the polar head of phospholipids and easily disseminated in the lipid bilayer of the cell membrane, protecting it from oxidative damage at the cellular level in addition to distributing well in the aqueous phase to scavenge free radicals (36)

When compared to the TMX group, quercetin administration resulted in a very large rise in the percentage of untailed nuclei and a very significant decrease ($p<0.001$) in the percentage of tailed nuclei, tail length, tail DNA percentage, and unit tail moment in hepatic cells.

The results of the current study are coincided with those of Abdel moniem et al. (51) who showed that oral pretreatment with quercetin prior to another neonicotinoid (imidacloprid) treatment significantly decreased the mean of tail length, tail DNA%, and tail moment less than those of the imidacloprid-treated group which indicted that quercetin attenuated the imidacloprid -induced DNA damage in liver cells

The findings of current study are also in line with those of Refat et al. (52), who found that co-treating with quercetin and cadmium chloride in adult male albino rats reduced DNA damage of liver tissue in the comet assay.

According to experimental research, quercetin is a DNA-protective agent and is regarded as a significant antioxidant that scavenges free radicals because of its high hydroxyl group content and capacity to deactivate the metal iron, which is the cause of reactive oxygen species (53).

In comparison to the TMX-treated group, quercetin administration resulted in a very substantial drop ($p<0.001$) in the mean values of blood MDA and a very significant increase ($p<0.001$) in the mean values of hepatic glutathione peroxidase and catalase which could explain the ameliorative effects of quercetin on different parameters reported by the present study.

The results of current study are in agreement with Auwal et al. (11) who found that testicular glutathione peroxidase was considerably increased when quercetin and TMX were co-treated in adult male albino rats.

Additionally, the current study's findings were consistent with those of EL-Gendy et al. (54), Ghazanfar et al. (13) and Gooshki et al. (55), who found that administering quercetin along with another neonicotinoid (acetamiprid) significantly increased the mean values of glutathione peroxidase and catalase and significantly decreased the mean values of MDA.

Conclusion:

Male rats exposed to sub-chronic doses of thiamethoxam (TMX) demonstrated hepatotoxicity, as evidenced by a considerable rise in liver enzymes (GDH, AST and ALT) with severe histological alterations, increased caspase-3 immunoreactivity and substantial DNA damage in liver cells, as shown by the damaged nuclei in the comet assay which are indicative of TMX's apoptotic and genotoxic effects. Also TMX administration increase serum malondialdehyde as well as decrease in hepatic glutathione peroxidase and catalase levels which are indicators of oxidative stress.

By reversing the impact of thiamethoxam on these parameters, quercetin demonstrated an ameliorating effect in this investigation, as demonstrated in quercetin co-treated groups.

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