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Evaluation of the possible ameliorative effect of spirulina on hepatotoxicity induced by methomyl in male albino rats.

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Abstract

Methomyl is a well-known insecticide that induces several side effects. Spirulina is a rich source of antioxidant and bioactive agents. The target of this experiment was to evaluate the possible ameliorative role of spirulina on some biochemical and histological alterations in rat livers caused by methomyl. Forty adult albino rats were split into 4 groups, 10 rats for each, as follows: control group (GI): rats of this group served as control rats, spirulina-treated group (GII): rats of this group received spirulina orally at a dose of 500 mg/kg b.wt daily for three weeks, methomyl-treated group (G III): rats of this group received methomyl orally at a dose level of 1/20 of LD_{50} daily for three weeks and methomyl and spirulina-treated group (GIV): rats of this group orally received both methomyl and spirulina at the same doses in GII and G III for three weeks. After 24 hours from the end of the treatments, animals of all groups were sacrificed. Biochemical analyses were performed, including serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and albumin (ALB), total protein (TP), cholesterol (CHOL) and triglycerides (TG) levels. Additionally, liver tissues were used for the determination of malondialdehyde (MDA), interleukin-6 (IL-6), glutathione (GSH) levels, and catalase (CAT) activity. Liver tissues were also used for histological and immunohistochemical examinations. From the obtained results in the current study, it is concluded that spirulina antioxidant contents reduced methomyl-induced hepatotoxicity through biochemical, histological, and immunohistochemical observations.

Keywords: Methomyl; Spirulina; Hepatotoxicity; Rats

Introduction

Methomyl is a carbamate insecticide usually used to treat farmland crops. It has a high contamination possibility in the environment because of its high water solubility **(1)**. It is classified as a restricted-use pesticide due to its low adsorption to soil and toxicity to non-target species; methomyl could flow into the groundwater and cause drinking water contamination (**2**).

Methomyl primarily works on the reversible suppression of acetylcholinesterase which breaks down acetylcholine. When acetylcholinesterase is suppressed, acetylcholine accumulates in the synaptic clefts, leading to continuous and

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uncontrolled nerve impulse transmission causing muscle paralysis, defect breathing problems, and even death **(3)**.

Methomyl is a moderate-to-highly toxic substance under oral or inhalation intake. Methomyl causes several human body side effects, so its usage has been limited by government agencies and manufacturers **(4)**.

Spirulina is a type of blue-green algae that is multicellular, filamentous, and prokaryotic **(5)** and it is also a cyanobacterium with the capacity to reproduce by using carbon dioxide that has been dissolved in saltwater as a source of nutrients **(6)**. Spirulina contains a high amount of proteins, carbohydrates, essential fatty acids, vitamins, minerals (calcium and potassium), carotenes, chlorophyll and phycocyanin **(7)**.

Spirulina has an elevated content of bioactive substances, such as phenols, phycocyanin pigment, and polysaccharides, which have antioxidant and anti-inflammatory effects **(5)**. Several studies documented the biological activities of spirulina such as antioxidant and immunomodulatory **(8)**, antitumorgenic **(9),** and neuroprotective effects **(10)**.

Material and Methods

Materials and chemicals:

Methomyl was purchased as Lannate 90% SP from USA and spirulina was obtained in the form of tablets from Puritans Pride, USA.

The present study assay kits and chemicals were of high purity and quality and purchased from Chemical Kits Companies in Egypt.

Animals:

Forty male albino rats (*Rattus norvegicus*) weighing about 150g were obtained from Animal House Colony of the National Research Centre, Dokki, Giza, Egypt. The rats were lived at 23 ± 2 °C in plastic cages and allowed free access to a standard diet and tap water. After two weeks of rat adaption, they randomly divided into four groups, 10 rats in each group, as follows:

Control group (GI): rats of this group orally received distilled water.

Spirulina-treated group (GII): rats of this group received spirulina orally at a dose level of 500 mg/kg b.wt daily for three weeks **(11)**.

Methomyl-treated group (GIII): rats of this group received methomyl orally at a dose of 1/20 of methomyl LD_{50} **(12)** daily for three weeks.

Methomyl and spirulina-treated group (GIV): rats of this group received both methomyl and spirulina at the same doses as the previous groups (GII & GIII) for three weeks.

After three weeks, the animals were fasted for 12 hours then they were anesthetized and dissected. Blood was withdrawn from the heart and centrifuged to separate clear sera. Sera were used for the estimation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and albumin (ALB), total protein (TP) cholesterol (CHOL) and triglycerides (TG) levels.

Liver tissues were collected from rats of all groups and divided into two parts. The first part was homogenated for the determination of malondialdehyde (MDA), glutathione (GSH), and interleukin-6 (IL-6) levels and catalase (CAT) activity. The second part was fixed in 10% neutral formalin for histological and immunohistochemical staining of tumor necrosis factor- α (TNF-α**)** studies.

Histological studies

The liver tissues were processed for histological technique and stained with hematoxylin and eosin (H&E) **(13)**. Finally, they were examined and photographed by a light microscope with a digital camera (Olympus, Tokyo, Japan).

Immunohistochemical studies:

TNF-α immunoreactivity

Immunohistochemical staining of TNF-α was carried out according to **Hsu *et al.* (14)**. Sections were washed in distilled water and counterstained with hematoxylin. The stained sections were examined and photographed by a light microscope with a digital camera (Olympus, Tokyo, Japan). The intensity of TNF-α expression was quantitatively evaluated by using Image J software **(15).**

Ethical approval

This study received ethical approval from the Faculty of Science, Damanhour University, Egypt (DMU-SCI-CSRe-24 02 07).

Statistical analysis:

The obtained data in the current study are expressed as mean ± SE and the data were analyzed using the one-way ANOVA test and Tukey's test (HSD) of SPSS software program version 20.0 **(16)**. The statistical significance levels between the experimental groups were $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$.

Results

Biochemical results

Serum transaminases (AST and ALT)

Data in Figure 1 represented the activities of both AST and ALT in the sera of rats in all studied groups. There was an insignificant decrease at p = 0.510 for AST and p = 0.972 for ALT activities between the control and spirulina-treated groups. When rats were treated with methomyl a significant increase at p = 0.002 and p = 0.000, respectively for AST and ALT activities was recorded compared with the control rats. On the other side, when rats were treated with both methomyl and spirulina, an insignificant increase at p = 0.066 and p = 0.735, respectively for AST and ALT activities compared with the control rats was recorded, however, they recorded an insignificant decrease (p = 0.294) in AST activity and a significant decrease (p = 0.001) in ALT activity comparing with methomyl-treated rats.

ALB and TP

Data in Figure 2 showed ALB and TP levels in the different studied groups. Spirulina-treated animals didn't record a significant difference (p = 1.000 & 0.981) in both ALB and TP levels, respectively compared with the control animals. In contrast, methomyl-treated rats recorded a significant decrease (p = 0.002 & 0.000) in both ALB and TP levels, respectively compared with the control rats. As a result of spirulina treatment with methomyl, the rats in GIV recorded an insignificant decrease (p = 0.249 & 0.532) in both ALB and TP levels compared with the control rats, while ALB level recorded an insignificant increase (p = 0.082) and TP level recorded a significant increase (p = 0.001) comparing with methomyl group.

CHOL and TG

The obtained results of CHOL and TG of the different studied groups are demonstrated in Figure 3. Compared with the control group, the spirulina-treated group recorded an insignificant difference (p = 0.633 & 0.878) in both CHOL and TG levels, while the methomyl-treated group recorded a significant increase (p = 0.000) in both CHOL and TG levels. Besides, rats treated with both methomyl and spirulina recorded an insignificant increase (p = 0.892) in CHOL level and a significant increase (p = 0.000) in TG level compared with the control group, while compared with methomyl-treated group, they recorded a significant decrease (p = 0.002) in CHOL level and an insignificant decrease (p = 0.129) in TG level.

Oxidative stress and inflammatory biomarkers in the liver tissues:

MDA and IL-6

Data of both MDA and IL-6 levels in the liver tissues of the different studied groups were represented in Figure 4 A&B. From the obtained data, spirulina-treated animals recorded an insignificant difference (p = 0.988 & 0.938) in both MDA and IL-6 levels, while methomyl-treated rats recorded a significant increase (p = 0.000) in both MDA and IL-6 levels comparing with the control group. When rats were treated with both methomyl and spirulina, they recorded an insignificant increase (p = 0.103) in MDA level and a significant increase (p = 0.006) in IL-6 level compared with the control group but compared with methomyl-treated rats a significant decrease (p = 0.007 & 0.004) was recorded in both MDA and Il-6 levels.

Antioxidant biomarkers (GSH and CAT) in the liver tissues

The obtained results of GSH level and CAT activity in the liver tissues of the different experimental groups were demonstrated in figure 4 C&D. Compared with the control rats, spirulina-treated rats recorded insignificant increases at p = 0.312 and p = 0.577, respectively of both GSH level and CAT activity, while methomyl-treated rats recorded a significant decrease at p = 0.004 in GSH level and p = 0.000 in CAT activity. But, rats treated with both methomyl and spirulina recorded an insignificant decrease (p = 0.125) in GSH level and a significant decrease (p = 0.002) in CAT activity as compared with the control group, while they recorded an insignificant increase (p = 0.109) in GSH level and a significant increase (p = 0.024) in CAT activity comparing with methomyl-treated rats.

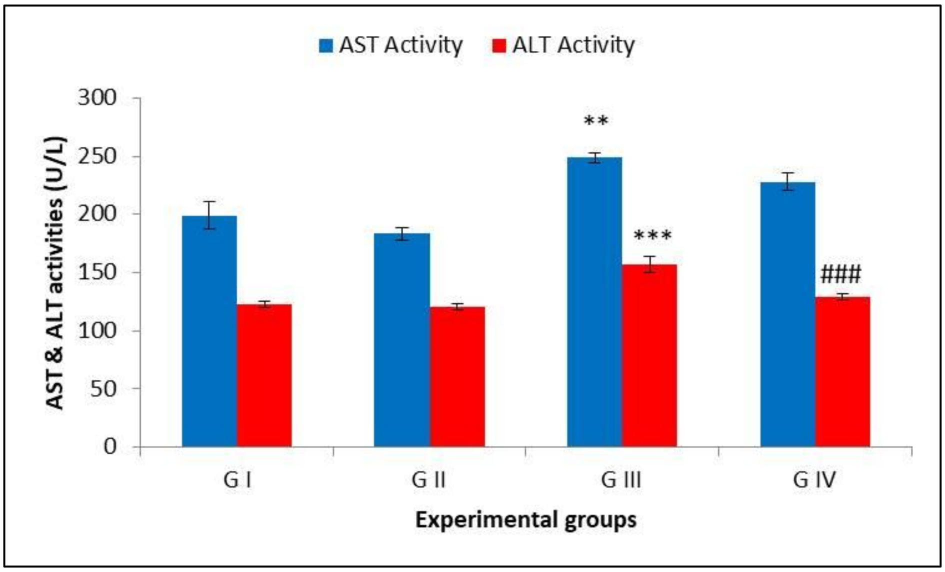

Figure 1: Column chart of AST and ALT activities in all studied groups.
***Significant at $p \leq 0.001$ compared with the control group.
**Significant at $p \leq 0.01$ compared with the control group.
###Significant at $p \leq 0.001$ comparing with methomyl group.
GI: Control group; GII: Spirulina group; GIII: Methomyl group; GIV: Methomyl and spirulina group.

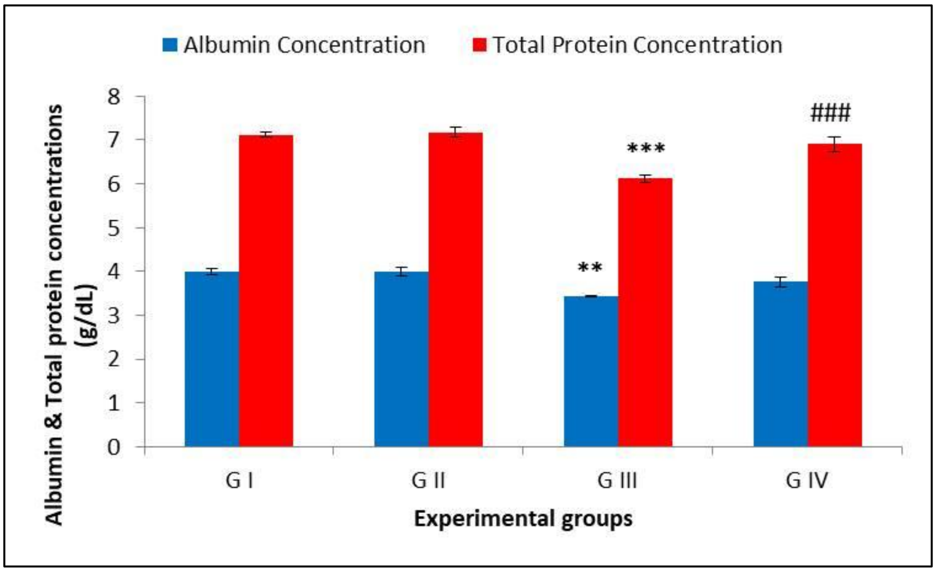

Figure 2: Column chart of albumin and total protein levels in all studied groups.
***Significant at $p \leq 0.001$ compared with the control group.
**Significant at $p \leq 0.01$ compared with the control group.
###Significant at $p \leq 0.001$ comparing with methomyl group.
GI: Control group; GII: Spirulina group; GIII: Methomyl group; GIV: Methomyl and spirulina group.

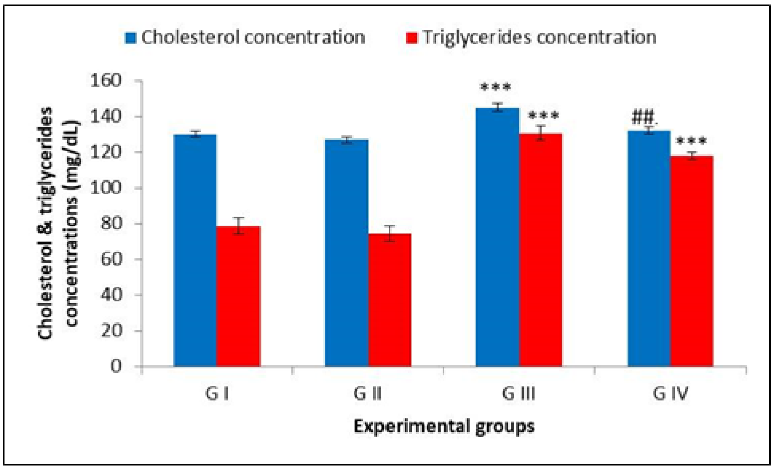

Figure 3. Column chart of cholesterol and triglyceride levels in all studied groups.

***Significant at $p \leq 0.001$ compared with the control group.

##Significant at $p \leq 0.01$ compared with methomyl group.

GI: Control group; GII: Spirulina group; GIII: Methomyl group; GIV: Methomyl and spirulina group.

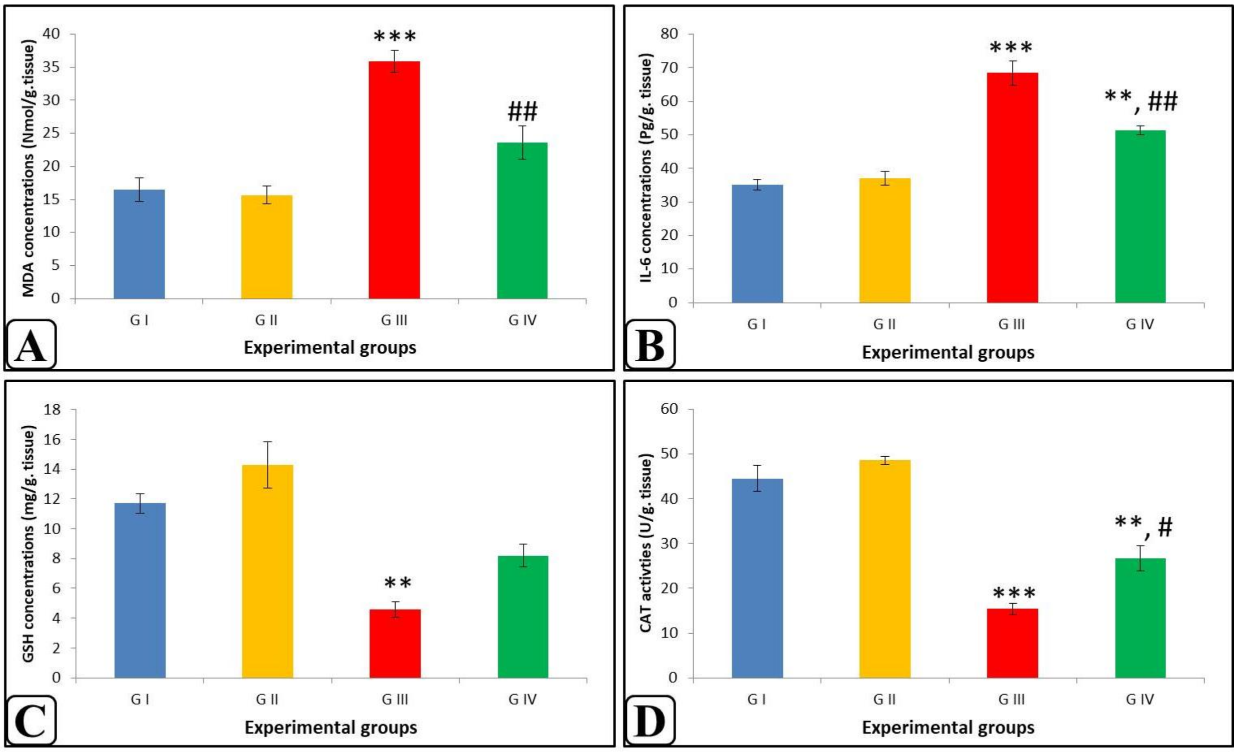

Figure 4: Column charts of (A) MDA, (B) IL-6, and (C) GSH levels and (D) CAT activity in the liver tissues of all studied groups.

***Significant at $p \leq 0.001$ compared with the control group.

**Significant at $p \leq 0.01$ compared with the control group.

##Significant at $p \leq 0.01$ compared with the methomyl group.

#Significant at $p \leq 0.05$ compared with the methomyl group.

GI: Control group; GII: Spirulina group; GIII: Methomyl group; GIV: Methomyl and spirulina group.

Histological observations:

When liver sections of the control rats were examined, normal histological architecture was noticed. The hepatocytes have normal cytoplasm and possess round nuclei (Fig. 5A). The portal area of connective tissue contains a branch of the bile ductule, a branch of the hepatic vein (Fig. 5B), and a branch of the hepatic artery. Spirulina-treated rats liver tissues examination revealed normal liver tissue structure, which was nearly similar to that of the control rats (Figs. 5C & D).

Methomyl-treated rat liver section examination showed several histological abnormalities. A marked dilation and congestion of some central veins, as well as dilation of some blood sinusoids were observed (Figs. 6A & B). Most of the hepatocytes displayed obvious degenerative features characterized by highly vacuolated cytoplasm and pyknotic nuclei, in addition to some hepatocytes being necrotic and swollen (Figs. 6A-D). Most of the portal areas also showed severely dilated, thickened, and congested portal veins and proliferated bile ductules (Fig. 6C). Moreover, numerous inflammatory leucocytic infiltrations were observed (Figs. 6C & D).

When methomyl and spirulina-treated rats' liver sections were examined, clear improvement features were observed. Liver tissues partially restored their normal architecture with nearly normal hepatocyte cytoplasm and nuclei (Fig. 6E). The portal areas contained improved branches of portal veins and bile ductules in addition to a few inflammatory leucocytic infiltrations (Fig. 6F).

Immunohistochemical observations:

Immunohistochemical expression of TNF-α has appeared as a brown color in the cytoplasm of the hepatocytes of the different experimental groups.

Liver sections of the control group examination showed a weak positive expression of TNF-α (Fig. 7A) and its intensity was quantitatively measured using NIH Image J software (Fig. 7E).

Spirulina-treated rats' liver tissues also showed a weak positive expression of TNF-α (Fig. 7B). Using NIH Image J software, TNF-α expression recorded an insignificant difference (p = 0.664) compared with the control group (Fig. 7E).

When methomyl-treated rats liver sections were examined, a strong positive expression as a dense brown color of TNFα was observed (Fig. 7C). This expression was confirmed by NIH Image J software, which revealed that TNF-α expression recorded a significant increase (p = 0.004) compared with the control group (Fig. 7E).

Examination of liver sections of rats treated with both methomyl and spirulina showed a mild positive expression of TNF-α (Fig. 7D) with an insignificant increase (p = 0.286) compared with the control group and a significant decrease (p = 0.05) compared with methomyl group (Fig. 7E).

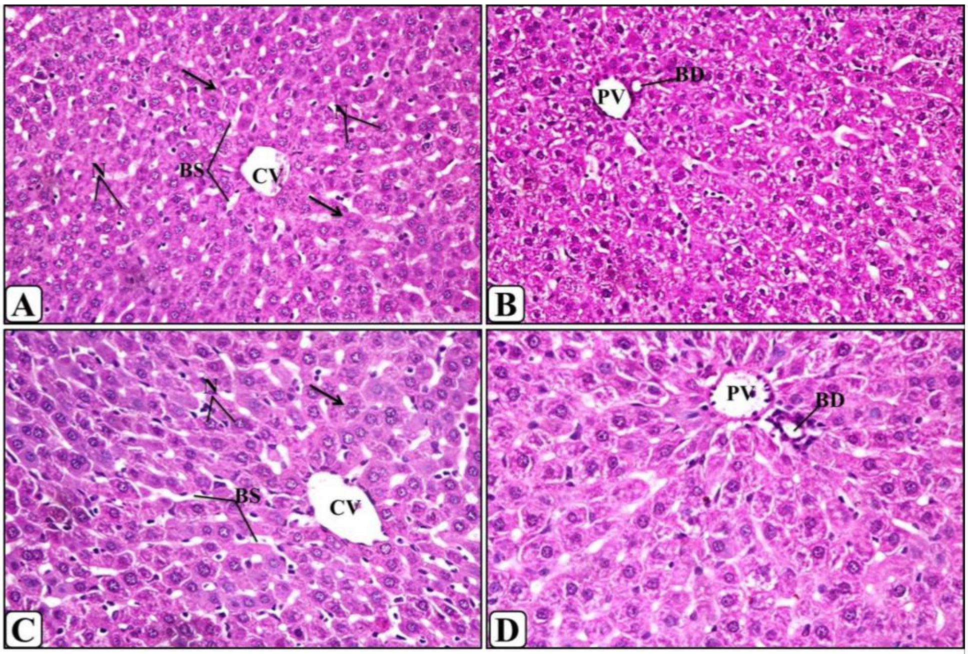

Figure 5: Photomicrographs of liver sections of both control **(A&B)** and spirulina-treated rats (**C&D)** showing normal histological structure, central veins (CV), hepatocytes with normal cytoplasm (arrows) and round nuclei (N), blood sinusoids (BS), portal areas with normal portal veins (PV) and bile ductules (BD), (H&E, X400).

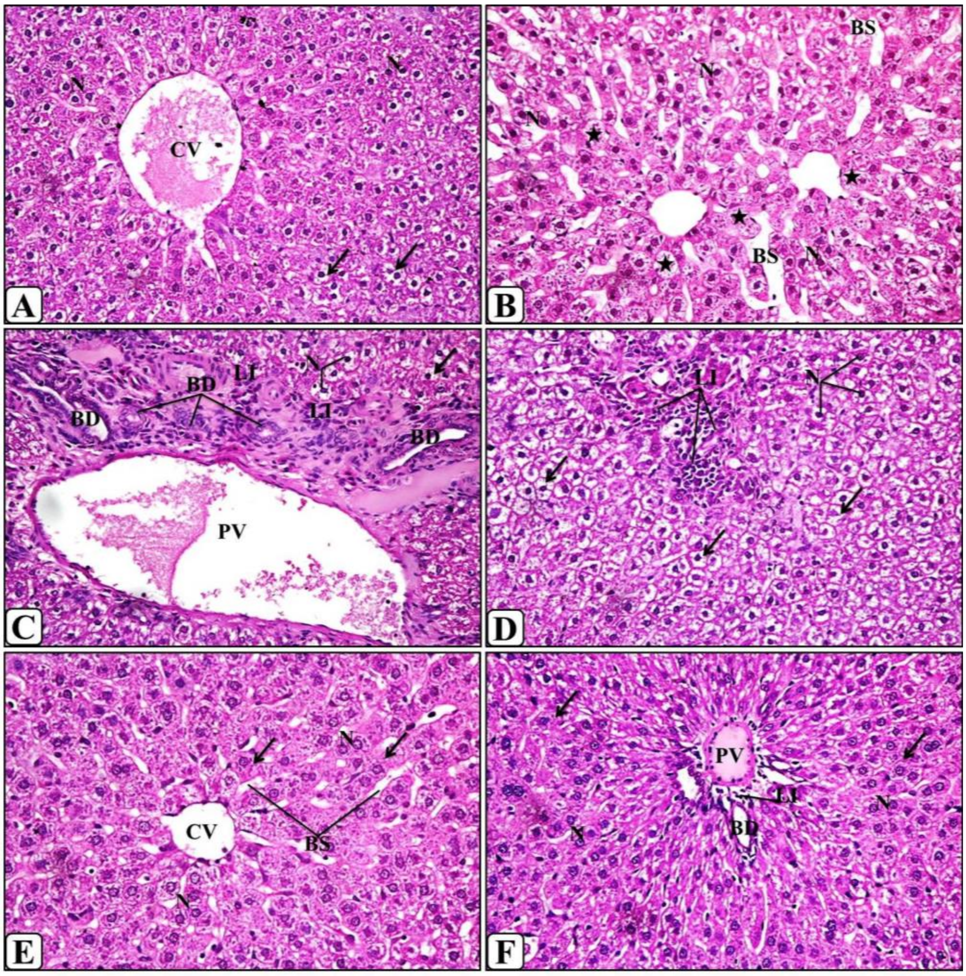

Figure 6: Photomicrographs of liver sections of methomyl-treated rats **(A-D)** showing dilated and congested central vein (CV), dilation of some blood sinusoids (BS), some degenerated hepatocytes with highly vacuolated cytoplasm (arrows) and pyknotic nuclei (N), another necrotic and swollen hepatocytes (stars), severe dilated, thickened and congested portal vein (PV), proliferated bile ductules (BD) and numerous inflammatory leucocytic infiltrations (LI) and methomyl and spirulina-treated rats **(E&F)** showing nearly normal histological architecture, normal central vein (CV), hepatocytes with normal cytoplasm (arrows) and round nuclei (N), blood sinusoids (BS), portal area with nearly normal portal vein (PV), bile ductule (BD) and few inflammatory leucocytic infiltrations (LI), (H&E, X400).

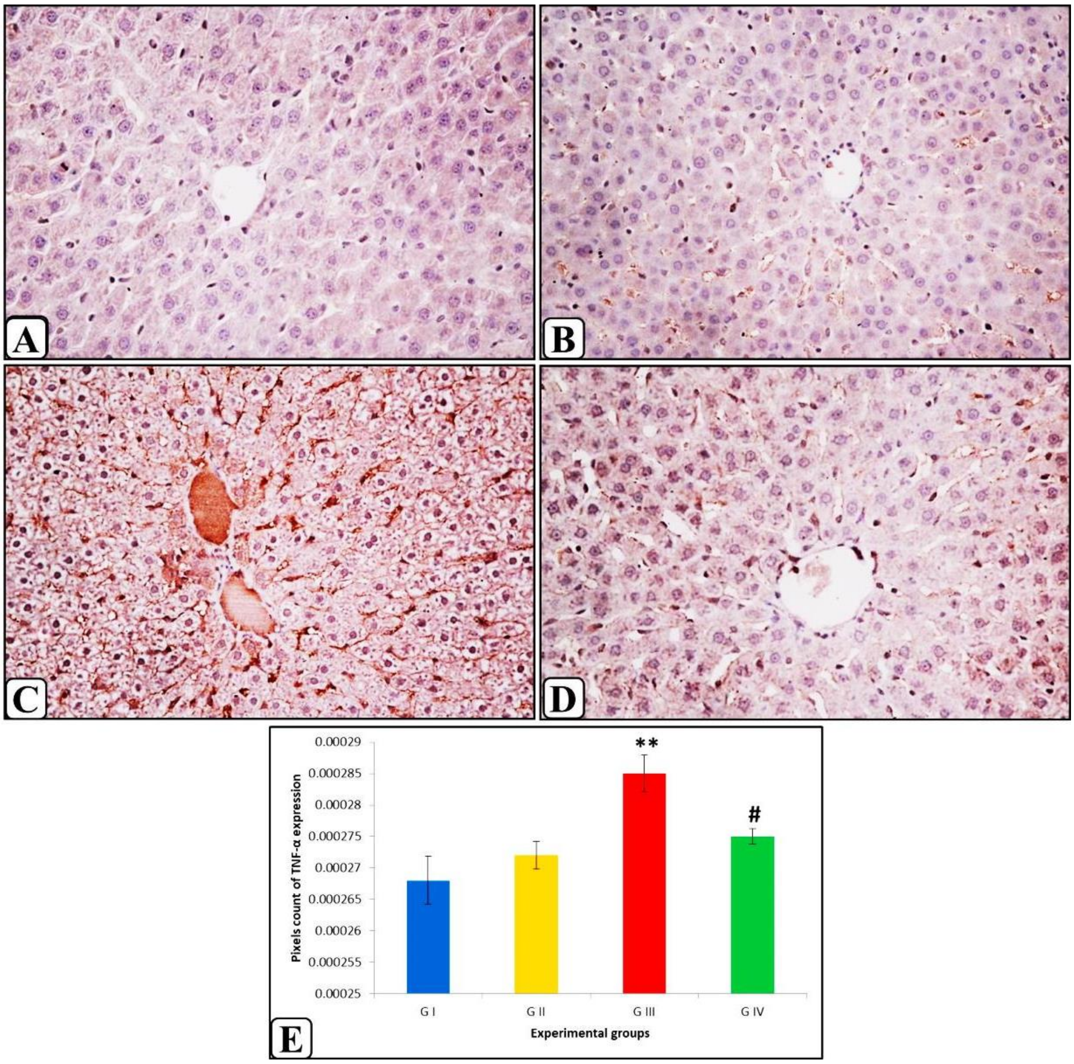

Figure 7: **(A-D)** Photomicrographs from liver sections showing cytoplasmic TNF-α expressions in the hepatocytes of all studied groups. **(A&B**) control and spirulina-treated rats, respectively showing week positive TNF-α expressions, (**C**) methomyl-treated rat showing a strong positive TNF-α expression, **(D**) methomyl and spirulina-treated rat showing a mild expression of TNF-α (TNF-α immunostain, counterstained with hematoxylin, X400) and **(E**) Histogram of TNF-α immunohistochemical expression in all studied groups.

**Significant at $p \leq 0.01$ compared with the control group.

[#]Significant at $p \leq 0.05$ compared with the methomyl group.

GI: Control group; GII: Spirulina group; GIII: Methomyl group; GIV: Methomyl and spirulina group.

Discussion

The current study evaluated the role of spirulina treatment on hepatotoxicity induced by methomyl administration.

It has been noted that methomyl induced oxidative stress in rats, which causes an excess of free radicals production and has negative effects on the kidney, liver, and brain **(17, 18)**.

Methomyl administration in the present study resulted in liver damage, as seen by the marked rise in AST and ALT activities, which may be due to methomyl-induced oxidative stress that harms liver tissue. **Rajesh and Latha (19)** reported that increased enzyme activity is a sign of cellular leakage and a breakdown in the functional integrity of the liver's cell membranes. Our findings were in agreement with **Singh *et al*. (20) and Shah *et al*.**

(21) regarding methomyl; they proposed that enzyme levels are sensitive markers of tissue injury and noted that the increase in serum AST and ALT was caused by liver cell necrosis and degeneration, which was accompanied by cytolysis and cell membrane damage.

In the current investigation, methomyl caused CHOL and TG levels elevation. According to **Karami-Mohajeri and Abdollahi (22),** carbamate insecticides typically cause an increase in CHOL and TG levels. Pesticides impact liver cell membrane permeability that cause elevation in serum CHOL and TG levels (23, 24). A potential membrane lipid peroxidation may be indicated by the observed elevation in CHOL and TG levels (25).

In the current study, methomyl-treated animals recorded a significant decrease in both ALB and TP levels, which may be related to liver injury induced by methomyl. The toxic consequences, which included the development of liver cell necrosis, were the cause of this drop in TP and ALB. The oxidative enzyme alterations were most likely a secondary cause of this depression, which may have resulted from changes in the intracellular processes governing protein synthesis. The impact of pesticides on the synthesis of proteins and nucleic acids was studied by **Shah *et al*. (21).**

Methomyl has been found to increase oxidative stress in rat tissues, which causes liver harm by free radicals (18, 26). The current study revealed that methomyl-treated rats exhibited a significant elevation in MDA level, indicating that methomyl stimulated the production of free radicals in the hepatic tissues. These findings concur with research showing that methomyl-induced erythrocyte reactive oxygen species (ROS) production (26).

In the current study, the methomyl-treated group recorded a significant increase in IL-6 levels. IL-6 plays a significant role in the liver in inducing the acute phase response and infection defense. Moreover, IL-6 is a potent hepatocyte mitogen and is vital in hepatocyte homeostasis. It is connected to both the liver's metabolic processes and liver regeneration. On the other hand, prolonged stimulation of the IL-6 signaling pathway harms the liver and may cause liver tumor growth (27, 28).

Decreased CAT activity and GSH levels that were observed in the current study were indicative of increased oxidative stress and cellular damage induced by methomyl. Consistent with this observation, several publications have noted both *in vitro* and *in vivo* observations of these effects (29, 30).

When rats were treated with both methomyl and spirulina, a decrease in serum ALT and AST activities and CHOL and TG levels in addition to an increase in ALB and TP levels were recorded. These findings support the findings of **Bashandy *et al*. (31)**, who revealed that spirulina has a potent antioxidant activity and activates an enzyme system that scavenges free radicals. The present data are consistent with the research conducted by **El-Desouki *et al*. (32),** which showed that spirulina can protect rats against liver damage.

Spirulina treatment with methomyl caused a significant decrease in MDA and IL-6 levels and an increase in GSH and CAT activities in the current experiment, which may be related to spirulina's antioxidant qualities. Accordingly, spirulina has been shown to have hepatoprotective capability (33, 34). Spirulina has been shown to decrease lipid peroxidation and boost antioxidant system activity (35). Spirulina's active ingredients have the potential to work in concert to provide a potent antioxidant impact (36). Spirulina's active components include phenolic compounds, phycocyanin, β-carotene, tocopherol, and selenium with anti-inflammatory and functional antioxidant properties (37, 38). According to **Ismail *et al*. (39)**, the antioxidant capacity of phycocyanin, a significant water-soluble antioxidant component of spirulina, is twenty times more potent than that of vitamin C.

In the current study, the observed histopathological alterations in methomyl-treated rat's liver tissues were obvious signs of inflammation and hepatotoxicity which may be resulted from methomyl-induced oxidative stress. **Djeffal *et al*. (40)** reported that antioxidant enzyme activity inhibition and lipid peroxidation level elevation probably cause the intracellular accumulation of ROS with subsequent development of tissue damage. Increased oxidative stress can induce cellular structure destruction **(41)**. Both inflammation and oxidative stress are interrelated since one could activate the other, causing a toxic feedback system **(41)**. Recently, **Groswald *et al*. (4)** documented that methomyl treatment led to liver injury and oxidative stress in mice. Our histopathological observations were in agreement with **Mansour *et al*. (42)** and **Aboushouk *et al*. (43)**, who noticed similar histopathological changes in the liver tissues of rats treated with methomyl.

On the other side, rats treated with both methomyl and spirulina showed an obvious degree of improvement in the liver tissue structure, which may be due to the antioxidant capacity of spirulina. In harmony with our results, **Mohamed *et al*. (44)** documented that spirulina with CCL_4 showed a partially normal appearance of liver tissues, increased liver organization, and reduced hepatocyte necrosis through its ability to decrease ROS and anti-inflammatory effects.

In the present study, methomyl-treated rats showed a strong positive immunohistochemical expression of TNF-α in liver tissues with a significant increase compared with the control rats. This observation may be attributed to methomyl-induced inflammatory effects. Increased TNF-α signaling activation is related to chronic inflammation and can cause pathological complications **(45,46)**.

On the other side, treatment of spirulina with methomyl resulted in a mild positive immunohistochemical expression of TNF-α in liver tissues with an insignificant increase compared with the control group and a significant decrease compared with methomyl group, which may be due to the anti-inflammatory effect of spirulina. **Abu-Taweel *et al*. (47)** reported that spirulina extract has strong anti-inflammatory and analgesic activities lowering TNF-α, IL-1β, IL-6, PGE2, and NO production and COX-2 and iNOS activities inhibition. Our immunohistochemical results of TNF-α expression in the liver tissues were in harmony with **Behairy *et al*. (11)**, who noticed that spirulina treatment led to a remarkable reduction in TNF-α expression with methotrexate in the liver tissue of rats.

Conclusion

Methomyl induces free radicals and changes the activity of antioxidant enzymes' defense mechanism. Spirulina contains essential and active components that can reduce induced oxidative stress and tissue damage. Based on the obtained results, spirulina has potential hepatoprotective properties.

CONFLICT OF INTEREST: No conflict of interest.

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