RESEARCH ARTICLE

# The Possible Hepatoprotective Effects of Combination of an Oral Krill Oil and Silymarin against Carbon Tetrachloride ($CCl_4$)-induced Liver Fibrosis/ Injury in White Albino Rats: Histopathological, and Biochemical Studies

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**ABSTRACT**

Liver fibrosis represents the final consequences of repeated occasions of hepatocellular necrosis and hepatic inflammation, regardless of the underlying causes of chronic hepatic disorders. It is manifested as a massive deposition of extracellular matrix (ECMs) within the damaged liver of different chronic hepatic disorders. Moreover, increase in the releases of radical moieties, e.g., superoxide, hydroxyl, and hydrogen peroxide radicals in living cells resulted in homeostatic deregulation and oxidative stress (OS) consequently damaging the biological molecules such as membrane lipids. Changing in cellular composition and level (amount) of antioxidant enzymes, e.g., superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) due to increased OS might result in chronic liver diseases. Hepatic fibrosis has been documented to be correlated with OS in numerous researches. In the current study 50 white albino rats were allocated as follows: Group I (Healthy Control): 10 rats received 1 mL/kg intraperitonealy (IP) olive oil (vehicle only group) twice a week for 6 weeks. Group II ($CCl_4$; Induction Group): 10 rats received 1 mL/kg of 50% $CCl_4$ solution in olive oil IP twice a week for 6 weeks. Group III (Silymarin Group): 10 rats received 1 mL/kg of 50% $CCl_4$ solution in olive oil IP twice a week + Silymarin (hepatoprotective agent) (100 mg/kg). Once daily orally for six weeks concurrently with $CCl_4$. Group IV (Krill Oil Group): 10 rats received 1 mL/kg of 50% $CCl_4$ solution in olive oil IP twice a week + krill oil (400 mg/kg) once daily orally for six weeks concurrently with $CCl_4$. Group V Combination of (Krill oil+ Silymarin Group): 10 rats received 1 mL/kg of 50% $CCl_4$ solution in olive oil IP twice a week + (krill oil (400 mg/kg) + Silymarin (100 mg/kg)) once daily orally for six weeks concurrently with $CCl_4$. This study reveals that orally administered krill oil in combination with oral Silymarin significantly ($p<0.001$) increase the hepatoprotective activity against $CCl_4$-induced hepatotoxicity in white albino rats through improvements in both liver tissue oxidative stress parameters, and histological features in both MT, and (H&E) stained sections.

**Keywords:** Carbon Tetrachloride, Hepatoprotection, Krill oil, Rats liver, Silymarin.

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## INTRODUCTION

Liver fibrosis represents the final consequences of repeated occasions of hepatocellular necrosis and hepatic inflammation, regardless of the underlying causes of the chronic hepatic disorder.[1] It is featured by excessive accumulation of extracellular matrix proteins (ECMs) in the injured liver of different forms of chronic hepatic diseases e.g., ethanol-induced hepatic disease (ALD), non-ethanol -induced fatty liver disease (NAFLD), and steatohepatitis (NASH), cirrhosis, and hepatocellular cancer (HCC).[2] Of the most crucial cells, hepatic stellate cells (HSCs) display a fundamental role in the initiation, progression, and regression in hepatic scaring via releasing fibrogenic factors that recruit portal fibrocytes, fibroblasts, and bone marrow-derived myofibroblasts to produce ECM and eventually propagate fibrosis.[3] The presence of HSC along with the space of disse allows for the direct contact of the HSC and other cells involving hepatocytes, endothelial cells, and Kupfer cells, therefore enhance the intercellular release of soluble mediators and cytokines. In addition to this proximity presence in the space of Disse,

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intercellular cross-talking of HSC and adjacent cell types have been shown to be encouraged due to the presence of dendritic cytoplasmic processes.[4]

Moreover, massive synthesis of e radical moieties, such as superoxide, hydroxyl, and hydrogen peroxide by the cells causes homeostatic deregulations in OS, causing damage to biological molecules, e.g., proteins, nucleic acids, and membrane lipids. Changes in cellular constituents and levels of antioxidant enzymes such as superoxide dismutase (SOD), CAT, and reduced GSH due to high oxidative stress might have resulted in chronic hepatic disorder.[5]Numerous Pathologies of hepatic disorders like chronic hepatitis, fibrosis have been shown to be correlated with OS in different researches.[6] Carbon tetrachloride ($CCl_4$) is an extensively researched hepatotoxin in studying the pathogenesis of hepatic damage in various animal models.[7] Metabolic activation of $CCl_4$ leads to the production of trichloromethyl and proxy chloromethyl radical moieties, resulting in an imbalance between reactive oxygen species (ROS) and antioxidants and initiating hepatic damage and necrosis. $CCl_4$ can also activate Kupffer cells by increasing $Ca^{2+}$ levels, initiating the intracellular production of harmful mediators that encourage the death of liver cells and the release of reactive oxygen species.[8]

However, to this date, there was no effective medicine in the clinic for the management of hepatic fibrosis. Accordingly, research on liver antifibrotic agents is a 'hot topic'. The main available treatment strategies for fibrosis focused on treatment of underlying diseases, suppressing the inflammation, targeting ECM synthesis and breakdown, attenuation of hepatic parenchyma cell damage, and apoptosis. Although there were no approved pharmacotherapies for liver fibrosis.[9] Some drugs and /or combination of two drugs are now in phase III clinical trials for attenuation of primary biliary cirrhosis such as Obetoticholic acid a farnesoid-X- receptor (FXR) agonist, Bezafibrate, an agonist of peroxisome proliferator-activated receptors (PPARs) in phase III clinical trials with ursodeoxycholic acid (UDCA) and other combination therapy.[10] The aim of this research is to investigate the possible potential hepatoprotective effects of krill oil alone and in combination with Silymarin in white albino rats by studying the liver tissues histopathological changes stained with masons trichrome (MT) and hematoxylin and eosin (H&E), and also assessing the oxidative stress markers in both serum and liver tissues samples.

## MATERIALS AND METHODS

### Chemicals

All chemicals used in the current study were of analytic grades. $CCl_4$ liquid was supplied by GCC ANALYST (UK), Silymarin by MADUAS® (Germany), and Krill Oil from Natrol® (USA). The kits for the estimation of serum total antioxidant capacity (TAC) microplate assay kit was purchased from (Cohesion Biosciences, UK), liver tissue homogenates kits: glutathione peroxidases (GPx) ELISA kit from (Elabscience, USA), malondialdehyde (MDA) microplate assay kit (Cohesion Biosciences, UK), myeloperoxidase (MPO) ELISA kit from (Abbexa, UK), superoxide dismutase (SOD) microplate assay kit from (Cohesion Biosciences, UK), and Masson's trichrome (MT) collagen staining kit from (COSM BIO CO, USA).

### Animals Handling and Experimental Design

Fifty healthy white albino rats of both genders (25 males and 25 females), weighing between (200–250 gm) were utilized in this study. An animal house supplied these animals at Biotechnology Center, Al Nahrain University, Baghdad, Iraq. These animals were placed as five animals in each separated cage. Animals were kept under standard conditions at a temperature between (23 ± 2) °C and 50–60% relative humidity, with 12/12 hours. Light-dark cycles were applied. Animals were subjected to acclimatization for approximately 2 weeks before starting the work. During the acclimatization period and during the whole experimental period, animals were fed a standard laboratory diet and free access to tap water *ad libitum*.[11,12] Such method was in accordance with approval committee of ethical of laboratory animals handing in the College of Medicine, Al-Nahrain University, Baghdad, Iraq. Animals were categorized for 5 groups (as ten animals per each group) as followed:

- *Group I (Healthy Control Group):* Ten rats received 1 mL/kg IP olive oil (vehicle only group) twice a week for 6 weeks. Group II ($CCl_4$; Induction Group): 10 rats received 1 mL/kg of 50% $CCl_4$ solution in olive oil IP twice a week for 6 weeks.[13]
- *Group III (Silymarin Group):* A total of 10 rats received 1 mL/kg of 50% $CCl_4$ solution in olive oil IP twice a week + Silymarin (hepatoprotective agent; standard treatment) (100 mg/kg)[14] once daily orally for six weeks concurrently with $CCl_4$.
- *Group IV (Krill Oil Group):* 10 rats received 1 mL/kg of 50% $CCl_4$ solution in olive oil IP twice a week + krill oil (400 mg/kg) once daily orally for six weeks concurrently with $CCl_4$.
- *Group V (Combination of (Krill oil+ Silymarin Group)):* 10 rats received 1 mL/kg of 50% $CCl_4$ solution in olive oil IP twice a week + (krill oil (400 mg/kg) + Silymarin (100 mg/kg)) once daily orally for six weeks concurrently with $CCl_4$.

Six weeks and 24 hours later, all animals were sacrificed under light anesthesia of Diethyl ether (ether). Liver weights were first documented separately, and tissue samples were collected for homogenization and measurement of proposed oxidative stress tissue parameters (glutathione peroxidase (GPx), malondialdehyde (MDA), myeloperoxidase (MPO), and superoxide dismutase (SOD)), and masson's trichrome (MT) collagen staining, in addition to hematoxylin & eosin staining.[15]

### Haematoxylin and Eosin (H&E), Masson Trichrome (MT) Staining, and Scoring of Fibrosis

Hepatic tissues were taken and fixed in 4% paraformaldehyde. Paraffin liver sections were dewaxed using xylene, dehydrated with ethanol, rinsed, and stained with hematoxylin for five minutes, washed quickly, and then differentiated by 1% acid alcohol (thirteen seconds) and washing for 10 minutes. Slides were stained with eosin solution for 2–3 minutes,

then washed and mounted. Photographs were acquired using a digital image-capture system.[16]

Regarding masson trichrome (MT) staining, the liver section was dewaxed, dehydrated, and rinsed. Firstly- Sections were first stained with Weigert's iron hematoxylin (5 minutes) and rinsed. Secondly- Sections were stained with Biebrich scarlet acid fuchsin solution (5 minutes) and rinsed. Thirdly- sections were differentiated in one percent phosphomolybdic-phosphotungstic acid solution (5 minutes), then transferred to aniline blue solution, and stained for 5 minutes. Fourth- Sections were differentiated in one percent acetic acid (AA) solution for one minute, rinsed, dehydrated, and mounted with an appropriate mounting medium. Images were captured by a digital camera connected to a light microscope. MT ( X400 magnification) was examined.[16] Scoring of fibrosis was done according to Ishak scoring system as below:

Grade 0: No fibrosis, Grade I: Fibrous expansion: was established in some of the portal areas, together with/or without short fibrous septa, Grade II: Fibrous expansion: of most portal areas, with or without short fibrous septa, Grade III: Fibrous expansion: was presumed in nearly most portal areas with an occasional portal to portal (P-P) bridging, Grade IV: Fibrous expansion: was seen in portal areas with marked bridging (P-P) as well as portal-central (P-C), Grade V: distinguished my marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis), Grade VI: Cirrhosis, probable /or definite.[17] The frozen tissues were weighed and immediately homogenized at an operating speed of 6000 rpm using homogenizer. For the determination of tissue parameters oxidative stress activity in the liver, the samples were homogenized in 0.1 M phosphate buffer, pH 7.4 for 1 minutes, according to the appropriate kit procedure. Homogenates were next used for biochemical assays.[18]

**Statistical Analysis**

Results from the current study were presented as means ± standard deviation (M ± SD) The difference among means had been analyzed by ANOVA using statistical package for social scientists (SPSS) version 20 where *p* values ≤ 0.05 were considered to be significant.

## RESULTS AND DISCUSSION

Results from the current study presented in Table 1 were revealed that addition of krill oil (Krill) to silymarin (Silym) oral combined therapy (Krill + Silym) as the hepatoprotective agent was produced the most significant result as represented in mean ± standard deviation (M ± SD) in Table 1 as compared to each of healthy, induction, Silym, and Krill groups. Regarding tissue homogenate level of glutathione peroxidase (GPx), a combination of (Krill+Silym) was produced most significant elevation in GPx level ($M \pm SD = 116.93 \pm 3.57$, $p < 0.001$) when compared to each of, induction, Silym, and Krill groups. Glutathione peroxidase (GPx) catalyzes the reduction of a variety of hydroperoxides (ROOH and $H_2O_2$) to water or the corresponding alcohols using GSH to protect mammalian cells against oxidative damage.[19] Therefore, replenishment of GPx level with oral (Krill+ Silym) administration contributed to significant hepatoprotection and /antioxidant ability of such combination.

Moreover, MDA level in rat liver tissue homogenate in (Krill + Silym) combination group was shown to be the most significant also as being presented with ($M \pm SD = 8.51 \pm 0.77$, $p < 0.001$) as compared to when compared to each of, induction, Silym, and Krill groups Table 1. MDA is a product of lipid peroxidation and the most representative indicator of oxidative stress (OS) in the body, reflecting OS after liver injury. MDA is one of the most commonly used representative indicators of OS injury. It is able to activate Kupffer cells to secrete numerous cytokines, activate hepatic stellate cells (HSC), and mediate HSC differentiation, proliferation, and collagen synthesis.[20] In in (Krill + Silym) combination group lowest level was achieved among all other treated groups suggesting excellent hepatoprotection and/ or antioxidant properties against $CCl_4$-induced liver injury.

Myeloperoxidase (MPO) on the other hand, is a basic metal-containing lysosomal enzyme and part of the organism's host-defense system. MPO uses hydrogen peroxide and halide ($Cl^-$, $Br^-$, $I^-$) or pseudohalide ($SCN^-$) ions to catalyze the production of hypochlorous (HOCl), hypobromous (HOBr), hypoiodous (HOI) or hypothiocyanous (HOSCN) acids. The enzyme is capable of catalyzing two types of redox reactions; specifically, the halogenation cycle and the peroxidase cycle.[21] Combination of (Krill + Silym) were shown to elicit the most significant dropping in liver tissues level of MPO ($2.03 \pm 0.15$, $p < 0.001$) when compared to each of, induction, Silym, and Krill groups Table 1. Furthermore, the combination of (Krill + Silym) was also shown to boost the superoxide dismutase (SOD) level and resulted in the most significant elevation in SOD level ($16.68 \pm 1.73$, $p < 0.001$) when compared to each of, induction, Silym, and Krill groups Table 1. Because SOD offers protection against the harmful effects of ROS,[22] therefore, a combination of (Krill + Silym) accordingly offered substantial hepatoprotection and/or antioxidant against $CCl_4$-induced hepatic injury/fibrosis. Due to its beneficial effects on hepatotoxicity, SOD has also been proposed as an antidote against carbon tetrachloride ($CCl_4$) intoxication. The $CCl_4$ metabolic process in the liver gives rise to two active microsomal radicals or peroxides ($CCl_3$ or $CCl_3OO$), via the cytochrome P450 pathway, thus causing lipid peroxidation and undermining the integrity of liver-cell membranes.[23]

Cheong *et al*., 2017 were revealed that dietary Krill oil administration was found to protect against oxidative damage, lipid peroxidation and neurodegenerative diseases by significantly improved OS biomarkers e.g., serum SOD and GPx levels of aging mice supplemented.[24] such findings support that obtained from the current study.

Krill oil from *Euphausia superba* rich in astaxanthin, a carotenoid antioxidant, belongs to the group of xanthophylls responsible for the color of some plants, animals, and microorganisms, and crustaceans.[25] Astaxanthin had been shown to be the most potent antioxidant against lipid peroxidation than D-tocopherols and for single- O2 neutralizing

than β-carotene.[26] Moreover, Krill oil contains high proportions of eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) engaged to phospholipids.[27]

Regarding the anti-inflammation actions of both EPA, and DHA, it was shown that they lowered serum levels of the acute phase mediators, C-reactive protein (CRP), and also decreased expression of pro-inflammatory mediators, tumor necrosis factor (TNF)-α and interleukin (IL)-6. Furthermore, both EPA and DHA also been documented to lower the blood levels of pro-inflammatory eicosanoids, e.g., thromboxane $B_2$, and leukotriene $B_4$.[27] The composition of phospholipid classes in krill oil extracted from Euphausia superba were separated by HPLC to yield standard mixtures, (phosphatidylethanolamine (PE) and phosphatidylcholine (PC).[28] Krill oil has taken an excellent interest as the PL-bound fatty acids are most highly absorbed and incorporated into cell membranes.[29] Therefore, ensuring excellent delivery and distribution to body tissue and maintaining membrane integrity.

*Note:* Score 0 corresponds to (1.9%), Score 1 corresponds to (3%), Score 2 corresponds to (3.6%), Score 3 corresponds to (6.5%), Score 4 corresponds to (13.7%), Score 5 corresponds to (24.3%), and Score 6 corresponds to (27.8%) according to (Pradhan S., 2013).

Regarding histopathological features in both MT and (H&E) stained rats liver tissue sections, following 6 weeks (twice weekly) of IP administration of 50% (V/V) $CCl_4$ in Olive oil, there were significant collagen deposition in $CCl_4$ treated (induction group) ($10.92 \pm 8.36$, $p \leq 0.001$) when compared to each of healthy ($M \pm SD, 3 \pm 0.00$), Silym ($M \pm SD, 4.52 \pm 1.82$), Krill ($M \pm SD, 3.24 \pm 0.33$), and combination of (Krill+Silym) ($M \pm SD, 3.82 \pm 1.52$) groups respectively as seen in Table 2. Furthermore, both Krill, and a combination of (Krill+Silym) groups were shown to exhibit the lowest

**Table 1:** Showed liver tissue homogenate levels of glutathione peroxidase (gpx), malondialdehyde (mda), myeloperoxidas (mpo), and superoxide dismutase (sod) among the studied groups

*p value (sig ≤ 0.05)*

| | | GPx (Pg/mL) | | | |
|---|---|---|---|---|---|
| **Group** | *M ± SD* | *Induction* | *Silym* | *Krill* | *(Krill+ Silym)* |
| **Healthy** | 119.72 ± 3.5 | <0.001 | <0.001 | <0.001 | 0.15 |
| **Induction** | 70.93 ± 4.68 | | <0.001 | <0.001 | <0.001 |
| **Silym** | 83.71 ± 4.98 | | | <0.001 | 0.001 |
| **Krill** | 95.64 ± 6 | | | | <0.001 |
| **(Krill+ Silym)** | 116.93 ± 3.57 | | | | |
| | | *MDA (ηmole/g)* | | | |
| **Group** | M ± SD | Induction | Silym | Krill | (Krill+ Silym) |
| **Healthy** | 6.58 ± 0.47 | <0.001 | <0.001 | <0.001 | 0.15 |
| **Induction** | 14.98 ± 1.18 | | <0.001 | <0.001 | <0.001 |
| **Silym** | 13.57 ± 0.61 | | | 0.059 | <0.001 |
| **Krill** | 12.93 ± 0.47 | | | | <0.001 |
| **Krill+ Silym))** | 8.51 ± 0.77 | | | | |
| | | *MPO (ηg/mL)* | | | |
| ***Group*** | *M ± SD* | *Induction* | *Silym* | *Krill* | *(Krill + Silym)* |
| **Healthy** | 1.16 ± 0.15 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Induction** | 4.66 ± 0.26 | | <0.001 | <0.001 | <0.001 |
| **Sylim** | 3.41 ± 0.19 | | | 0.62 | <0.001 |
| **Krill** | 3.38 ± 0.15 | | | | <0.001 |
| **(Krill+ Silym)** | 2.03 ± 0.15 | | | | |
| | | *SOD (U/g)* | | | |
| **Group** | M ± SD | Induction | Silym | Krill | Krill+ Silym |
| **Healthy** | 17.99 ± 1.88 | <0.001 | <0.001 | <0.001 | 0.15 |
| **Induction** | 5.18 ± 1.45 | | 0.002 | <0.001 | <0.001 |
| **Silym** | 8.05 ± 1.81 | | | 0.001 | <0.001 |
| **Krill** | 11.02 ± 2.93 | | | | <0.001 |
| **(Krill+ Silym)** | 16.68 ± 1.73 | | | | |

GPx: Glutathione peroxidase, MDA: Malondialdehyde, MPO: Myeloperoxidase, SOD: Superoxide dismutase, M: Mean, SD: Standard deviation,, Silym: Silymarin, Krill: Krill oil.

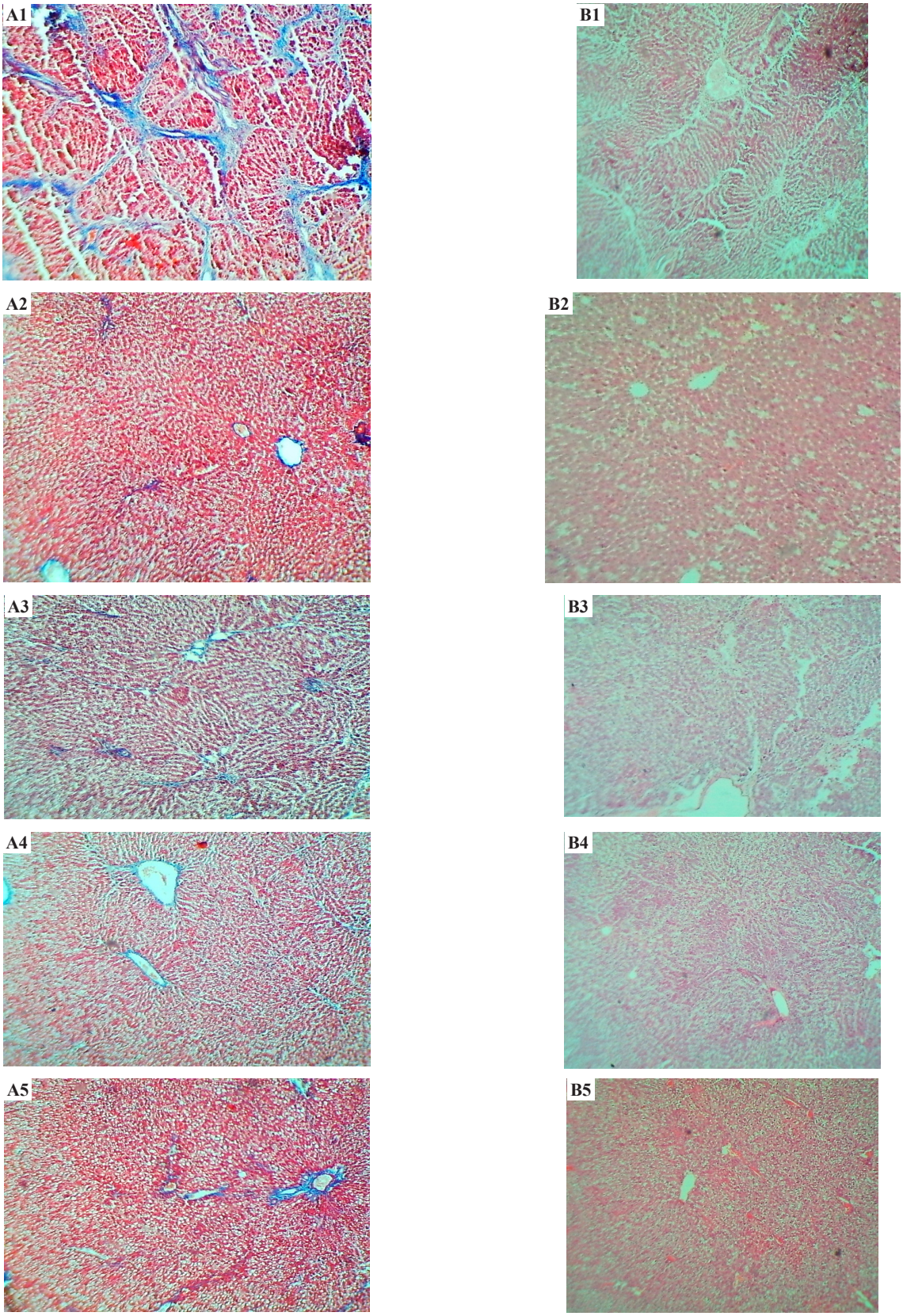


**Figure 1:** Representative photomicrographs showing in the column (A) Collagen deposition stained with Masson's trichrome stain (mt) within rats liver tissues, and (B) stained with hematoxylin & eosin (H&E); where [(A1,A2,A3,A4,&A5) corresponded to (Induction, Healthy, Silym, Krill, (Krill + Silym) groups stained with MT respectively], while, [(B1,B2,B3,B4,& B5) corresponded to (Induction, Healthy, Silym, Krill, & (Krill + Silym) groups stained with H&E, respectively].

**Table 2:** Showed liver tissue scores of collagen deposition (fibrosis scores) in masson's trichrome (MT) stained tissues among the studied groups

| *Fibrosis Scores* | | | | | |
|---|---|---|---|---|---|
| *Group* | *M ± SD* | *Induction* | *Silym* | *Krill* | *(Krill+Silym)* |
| Healthy | 3 ± 0.00 | ≤0.001 | 0.46 | 0.91 | 0.69 |
| Induction | 10.92±8.36 | | ≤0.001 | ≤0.001 | ≤0.001 |
| Silym | 4.5±1.82 | | | 0.53 | 0.73 |
| Krill | 3.24±0.33 | | | | 0.78 |
| (Krill+Silym) | 3.82±1.52 | | | | |

M: Mean, SD: Standard deviation, Silym: Silymarin,& Krill: Krill oil.

(M ± SD) regarding collagen deposition (fibrosis), suggesting the highest hepatoprotection activities as illustrated in Figure 1 in section A4, A5, B4, and B5.

## CONCLUSION

This study was revealed that orally administered krill oil in combination with oral Silymarin significantly increased the hepatoprotective activity against $CCl_4$-induced hepatotoxicity in white albino rats through improvements in both liver tissue oxidative stress parameters and histological features in both MT, and (H&E) stained sections.

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