'Silymarin' a potential treatment targeting hepatic fibrosis of schistosomiasis

Running title; Silymarin & schistosomiasis hepatic fibrosis

Abstract: Background: Despite all treatment and control efforts, schistosomiasis still thrives in humanity. It is endemic in 78 countries that are anchored by poverty and diseases. Until now, the broad-spectrum praziquantel (PZQ) drug is the only effective treatment of choice. However, reports documented some side effects for PZQ like haemorrhage in lung tissues, resistance, and inefficacy to treat fibrotic tissues. Therefore, alternative drugs that help in reducing the undesired effects of schistosomiasis are required. This study examined the efficacy of Silymarin in interfering with the fibrogenesis process using a mouse model. Silymarin is a herbal extract known to have flavonoids and polyphenols that help in reducing the inflammatory reaction, stimulating hepatocyte regeneration, and inhibiting the fibrogenesis process.

Methods: A total of thirty adult tBALB/c male mice were divided into negative, chronically infected control and treated groups. All were killed after 18 weeks from the initial infection. Different histopathological investigations and liver function tests were carried out to detect the difference between the groups.

Results: Administration of Silymarin exhibited a significant improvement in all associated histopathology with a considerable decline in the area percentage of collagen fibers. It restored the elevated level of serum ALT as well.

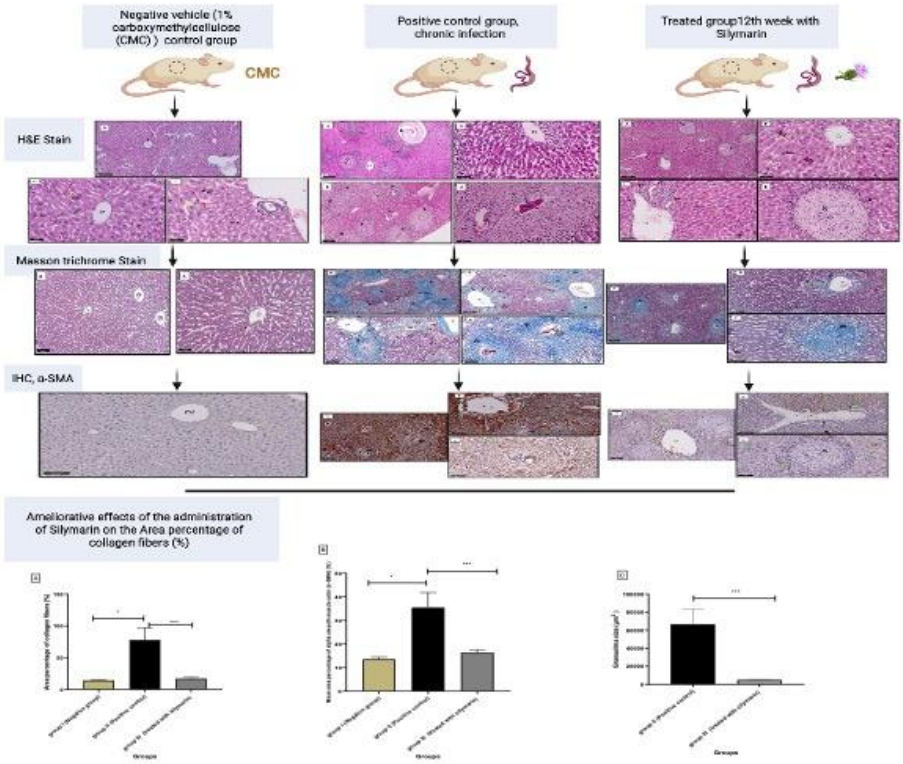
Conclusion: Silymarin as a treatment for chronic hepatopathies will only be successful if started during the acute phase of the disease.

Keywords: *Schistosoma mansoni*; Schistosomiasis; fibrosis; liver; Silymarin.

Graphical abstract

'Silymarin' a potential treatment targeting hepatic fibrosis of schistosomiasis

Maimonah Alghanmi and Aziza Alrafiah



Conclusion

Using a mouse model, the efficacy of Silymarin in inhibiting the fibrogenesis process was investigated. Silymarin treatment improved liver histology, particularly in terms of the decreased area % of collagen fibers. It is worth noting that Silymarin's capacity to inhibit fibrogenesis is limited to de novo fibrosis, not advanced fibrosis. As a result, using Silymarin as a treatment for chronic hepatopathies will only be successful if started during the acute phase of the disease. Silymarin has a considerable influence on parasite burden in Silymarin which worth a further investigation in future studies.

1. Introduction

Schistosomiasis is the second most predominant neglected tropical disease that till these days continues to thrive in humanity. It is estimated that the disease is endemic in 78 countries [1]. In 2018, at least 229 million individuals needed preventive treatment in 52 endemic countries [1]. In sub-Saharan Africa alone, the infection threat is over 193 million [2]. Besides, chronic schistosomiasis is accountable for losing 10.4 million disability-adjusted life years (DALYs) and cause over 280,000 deaths yearly [2].

The schistosomiasis infection is established when the infective stage (cercaria) penetrates the host skin in a poorly sanitized water surface contaminated by urine or faeces of infected individuals. The disease follows several pathological steps. It starts from the migrating schistosomula to the lodged eggs in various host tissues that ultimately cause several complications related to the disease. Of these, damage to vital organs such as urogenital, hepato-intestinal, pulmonary, or even to the central nervous system; neuro- schistosomiasis.

Despite these vast costs, schistosomiasis treatment depends on the broad-spectrum praziquantel drug (PZQ) [3]. The efficiency of PZQ reaches a 70-90% cure rate [4]. However, this magical treatment starts to raise scientists' concerns. The emerging resistance to treatment in some infected populations is not fully understood yet. It might be due to the low efficacy of PZQ on newly acquired immature parasite stages or the presence of genetic variations between parasite species [5]. Besides the reports of haemorrhage caused by PZQ in some patients' lungs, the drug only eliminates adult worms that dwell in the host's blood and not the histological damage caused by the trapped eggs [6].

Therefore, this study aimed to develop novel effective drugs to control schistosomiasis consequences deprived of side effects. We tested the ability of Silymarin to diminish liver fibrosis using a mouse model infected by *Schistosoma mansoni* cercariae.

Silymarin is an ancient herbal extract from *Silybum marianum* (milk thistle) known to treatliver diseases [7]. It consists of a combination of flavonoids and polyphenols, which act as membrane-stabilizing and an antioxidant. It helps in reducing the inflammatory reaction, stimulating hepatocyte regeneration, and inhibiting the fibrogenesis process. [7]. Previous studies showed the Silymarin's reduction ability of fibrosis deposition in the liver during acute schistosomiasis, which is linked to a reduction in the size of granulomas [8].
Accordingly, this study examined the efficacy of Silymarin in interfering with the fibrogenesis process using a mouse model.

2. Materials and Methods

2.1 Drug and kits:

Silymarin: (product number: S0292) Sigma-Aldrich, Chemical Co., St. Louis, MO, USA. High-pressure liquid chromatography (HPLC) was used to determine the constitution of Silymarin which is mainly composed of flavonolignans, including (22.6%) silicristin, (9.06%) silydianin, (21.3%) silybin A, (34.9%) silybin B, (8.26%) iso- silybin A, and (3.91%) isosilybin B. Carboxymethylcellulose (CMC): To prevent fast precipitation, silymarin extract was suspended in 1 percent carboxymethylcellulose (CMC) (Sigma-Aldrich, USA). It was given intraperitoneally (i.p.) every 48 hours until the study ended 18 weeks after the infection, at a dose of 10 mg kg-1 of body weight (P.I.).

Hydroxyproline Assay Kit (Colorimetric) (ab222941) was purchased from AbcamUSA.

Anti-mouse Alpha -smooth muscle actin (α-SMA) was purchased from LABVI-SION, USA (Catalog number: MS-185-R7).

Mouse Interleukin-13 (IL-13) (Catalog Number: RAB0257-1KT) enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich, USA).

Mouse Aspartate aminotransferase (AST) (Catalog Number: MAK055) and Alanine Aminotransferase (ALT) ELISA Kits (Catalog Number: MAK052) (Sigma-Aldrich, USA).

2.2 Animals:

Thirty adult t BALB/c male mice (7-8 weeks and 18- 25 gm).

2.3 Parasites:

S. mansoni cercariae (60 10/mouse) (obtained from infested *Biomphalaria Alexandrina* snails grown and stored at Theodor Bilharz Research Institute (TBRI), Giza, Egypt, injected subcutaneously) *S. mansoni* eggs in feces were discovered 42 days P.I., indicating an infestation. [9].

2.4 Experimental animals' groups (10 animals in each group):

Group I (negative vehicle control group) healthy liver group: Uninfected mice were given the vehicle (1% carboxymethylcellulose (CMC)) and tested alongside infected group II and infected treated group III before being killed after the trial (after 18 weeks P.I.).

Group II (positive control group) infected untreated group (chronic infection): killed after 18 weeks P.I. [9], [10].

Group III treated group12th week: The treatments were started from the 12th week to the 18th week PI. to allow the clinical manifestation of the disease and assess the efficacy of Silymarin in reducing histological alterations [10].

2.5 Histopathological studies:

For 48 hours, the liver was fixed in 10% buffered formalin. The samples were dehydrated for one hour in ascending alcohol grades (50, 70, 90, and 95 percent), followed by two changes of absolute alcohol (100 percent) for one hour each. After clearing the alcohol using xylene, 2 hours of embedding the samples in soft paraffin wax at 50 °C and another 2 hours of hard paraffin at 60 °C. Hematoxylin and eosin (H&E), Masson's trichrome stain, and immune-histochemical staining for alpha-smooth muscle actin (-SMA) were used to stain paraffin liver sections from each animal [11]. The immunoperoxidase technique of avidin-biotin was used as well. [12].

2.6 Parasitological parameters (Ova count):

Cheever methodology for hepatic tissue digestion was followed [13]. The egg count was calculated after 12-hour overnight incubation of liver tissue in 5% KOH at 37°C. Following that, samples were incubated for one hour at 37°C before counting in 50 l aliquots. The procedure was done three times, with the findings interpreted as ova/g tissue.

2.7 Liver homogenate (hydroxyproline content):

Phosphate buffer saline (PBS) PH=7.4 was used to clean liver tissues maintained at 80°C. The tissue was then chopped into small pieces and homogenized on ice with PBS (100 mg tissue/ ml). The suspension was treated to two freeze-thaw cycles to disrupt the tissues' cell membrane further. Then the suspension was centrifugated for 15 minutes at 1500×g), the supernatant was collected and stored at−80 °C till assayed. Hydroxyproline quantification was detected to evaluate the collagen volume from liver tissue as an index of liver fibrosis. The method of Woessner was used to estimate hydroxyproline in the liver [14].

2.8 Biochemical analysis:

Blood was collected for serum analysis. The colourimetric assay established serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) markers of hepato- cellular damage as per the Reitman and Frankel method [15]. IL-13 levels in serum were measured at 18 weeks Pi by an ELISA technique with capture and detection anti- bodies according to the manufacturer's instructions (Sigma, USA).

2.9 Morphometric analysis:

Each mouse in the subgroups (n=10) had ten specimens analyzed. Five different non-overlapping fields were photographed. Five distinct readings were counted from each taken photo, and the mean was determined. The measured parameters were as follows: (1) Masson trichrome dye was used to determine the mean area % of collagen fibers. (2) The percentage of (-SMA) positive brownish cells in the mean area. (3) Infected groups had their granuloma size (m2) quantified. The average width and length were used to calculate the mean size. Forty granulomas were evaluated and measured in each group in each animal using ImageJ software (National Institutes of Health, Bethesda, MD, USA), along with concomitant hepatic histological alterations. All the measurements were conducted at magnification fields of 40.

2.10 Statistical analysis:

Statistical Package for the Social Science software package (SPSS) version 26.0 (IBM Corp., Armonk / N.Y., USA) was used to conduct the statistical analysis. All data were analyzed using one-way analysis of variance (ANOVA) then by Tukey's post hoc test. Results presented as mean ± SEM Correlations between measured parameters were calculated using Pearson correlations. The significance was at ≤0.05. Graphs were drawn using GraphPad Prism software version 8 (2019), USA.

3. Results

3.1 Histopathological results:

3.1.1 H&E:

Analysis of H&E-stained liver sections of control mice revealed that the liver cells were radiated from the central vein in cords form toward the periphery of the lobules. They were separated from each other by irregular vascular spaces, hepatic sinusoids. Then, they were coated by thin flat endothelial cells and Von Kupffer cells. The hepatocytes appeared with acidophilic cytoplasm and a large central vesicular nucleus with a prominent nucleolus. Some hepatocytes contain two nuclei. At the border of the hepatic lobule, portal tracts were present; each tract contained branches of the portal vein, hepatic artery, and bile duct (Figure1).

In group II (positive control group), the liver revealed disturbed architecture and presence of cellular and fibrous granuloma with necrosis at the center, condensed fibrous connective tissue, and embedded ova in the center with infiltrated leukocytes surrounding the granuloma. Note the presence of adult *Schistosoma mansoni* inthe lumen of the dilated portal vein. Most of the hepatocytes had vacuolated cytoplasm. Many nuclei showed karyorrhexis, while other cells appeared with ill-defined nuclei: dilated central vein and portal vein with intense cellular infiltration around it.
Many hepatocytes appeared with dense nuclei and deeply acidophilic cytoplasm. The hypertrophied intra-sinusoidal (Von Kuepfer cells) in-between hepatocytes could be easily detected (Figure 2).

A noticeable enhancement was noticed in the Silymarin treated mice. The granuloma attenuation and the reduction in centrally localized ova with less condensed connective tissue were apparent. There was a decrease in the cellular infiltration around the central vein and portal area. The hepatocytes appeared nearly to the negative control group (Figure 3).

3.1.2 Masson trichrome stain:

Masson's trichrome was used to evaluate collagen deposition and liver fibrosis. Group I (negative control group) scanty fine collagen fibers were observed surrounding the central vein. No collagen fibers were observed between the hepatocytes (Figure 4). Moreover, the collagen fibres increased markedly in *S. mansoni*-infected liver (Positive control group II). There was a marked increase in the collagen fibers noticed around the central vein and between liver cells. The area of granulomas showed marked increased collagen fibers around the calcified egg (Figure 5). Interestingly, treatment with Silymarin caused a reduction in the granuloma area. Thin collagen fibers were noticed around the central vein and in between liver cells (Figure 6).

3.1.3 Immunohistochemistry Results

Immunostaining of α-SMA: In group I, no positive reaction was detected between the liver cells (Figure 7). There was a highly positive peri-sinusoidal reaction in group II and around the granuloma with an apparent increase of the granuloma size (Figure 8). There was an apparent decrease in the positive perisinusoidal reaction in group III and an apparent decrease in the granuloma size compared to group II (Figure 9).

3.2 Parasitological parameters (Ova count):

Hepatic tissues were digested, and there was a significant ($P < 0.001$) decrease in the number of the ova/gram liver tissue were observed in silymarin-treated mice group III as compared to the positive control group II (Figure10A, Table 1).

3.3 Hydroxyproline content:

The hydroxyproline levels in liver tissues were significantly reduced after treatment with Silymarin. This measurement indicates its ability to reverse hepatic fibrosis at the chronic stage (Fig. 10B). The reduced area percentage of collagen fibers in the liver tissue, mean area percentage of (α-SMA) positive brownish cells, and the granuloma size confirmed Silymarin's role in containing hepatic fibrosis.

that was observed in silymarin-treated mice and illustrated by the two images in Figs.11A, 11B & 11C & Table 1.

3.4 Morphometric results:

3.4.1 Collagen fibers mean area percentage using Masson trichrome stain.

Collagen area percentage highest peaks were measured in untreated mice (group II). Significant reduction in the area percentage in negative control animals, and group III Silymarin treated group) when compared to group II ($p \leq 0.001$ and $p \leq 0.001$), although a significant increase ($p < 0.001$) was recorded in subgroup B2 when compared to that in control mice (Fig 11A).

3.4.2 The mean area percentage of (α-SMA) positive brownish cells.

The highest peaks and significant increase in mean area percentage of α-SMA positive brownish cells was in infected untreated mice (group II) when compared to control negative group I & group III treated with Silymarin. On the other hand, a non-significant difference ($p = 0.107$) was measured in group III compared to that in group I(Fig. 11B).

3.4.3 Granuloma size (μm2)

The mean granuloma circumference hit its highest levels in group II. It showed asignificant increase ($p < 0.001$) compared to group III (Fig. 11C).

3.5 Biochemical results:

3.5.1 Markers of hepatocellular damage:

Mice infected with *S. mansoni* showed a marked increase in both serum AST ($P < 0.001$) and ALT ($P < 0.001$) levels when measured 18 weeks P.I. in comparison with those in the negative control group I. While those treated with Silymarin had a significant reduction in both serum AST ($P < 0.001$) and ALT ($P < 0.001$) levels (Figs. 12A, B & Table 1).

3.5.2 Serum IL-13 levels:

Serum levels of IL-13 showed a marked increase ($P < 0.001$) in the infected group compared to their corresponding negative control group I. The level of serum profibrogenic cytokine IL-13 decreased significantly ($P < 0.001$, $P < 0.001$) in silymarin-treated compared to negative control (group I) or non-treated mice group II (Fig12 C & Table1).

3.6 Figures and tables

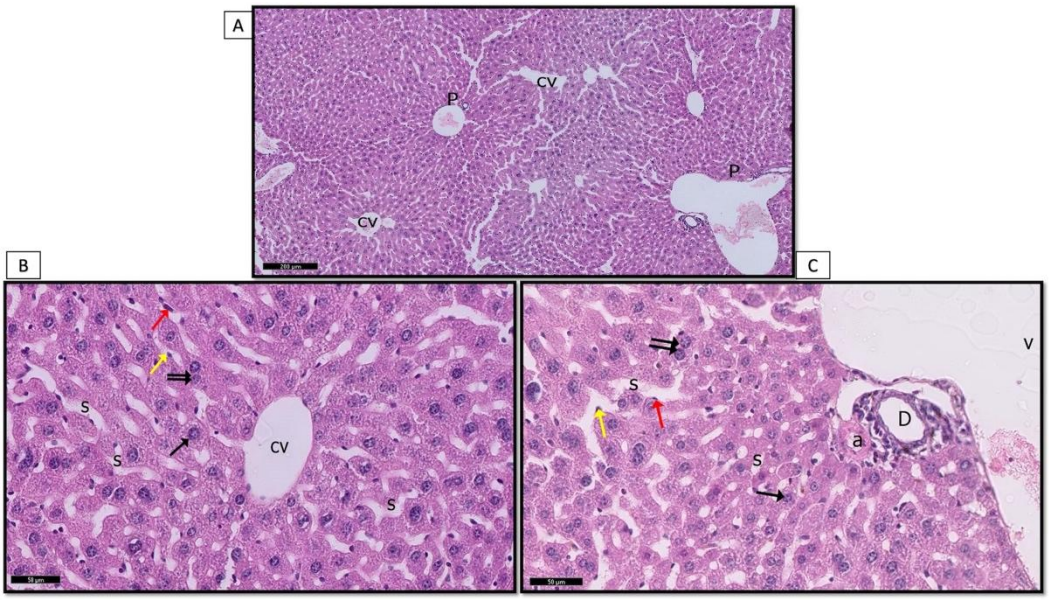


Figure 1. The polyhedral hepatocytes with normal vesicular basophilic nuclei (black ↑) distributed radially in cords around the central vein (cv). The blood sinusoids (red ↑) and Von Kupffer cells (yellow ↑) have a thin flat endothelial lining. The normal hepatocytes with their typical vesicular nuclei are shown in the portal area (P) around the portal vein (v), bile ducts (D), and hepatic artery (a). Hepatocytes appear to be binucleated in some cases (↑↑). **H&E Stain-Group I**

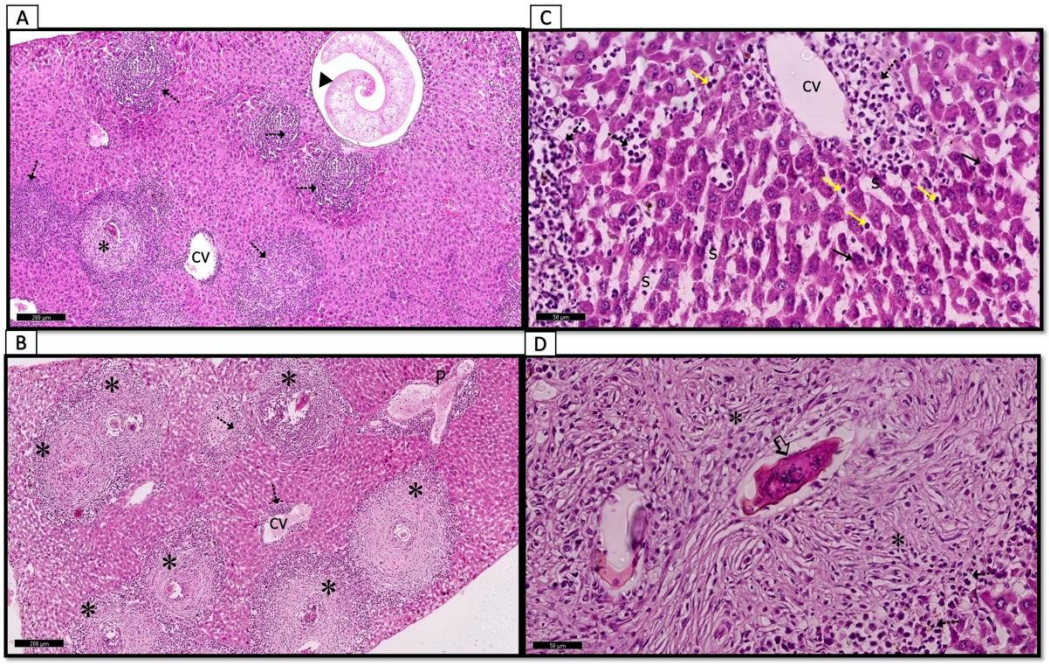


Figure 2. Infected positive control group demonstrating an apparent increase of number and size of fibrocellular granuloma (*) surrounding one intact egg (thick white arrow) and the peripheral zone of inflammatory cells (dot arrow) with only a few areas of hepatocytes. Adult *Schistosoma* (arrowhead) appear in the dilated portal vein. Notice few hepatocytes have deep acidophilic cytoplasm with loss of nuclei (↑) and dilated blood sinusoids (s) with hypertrophied Kupffer cells (yellow ↑). **H&E Stain-Group II**

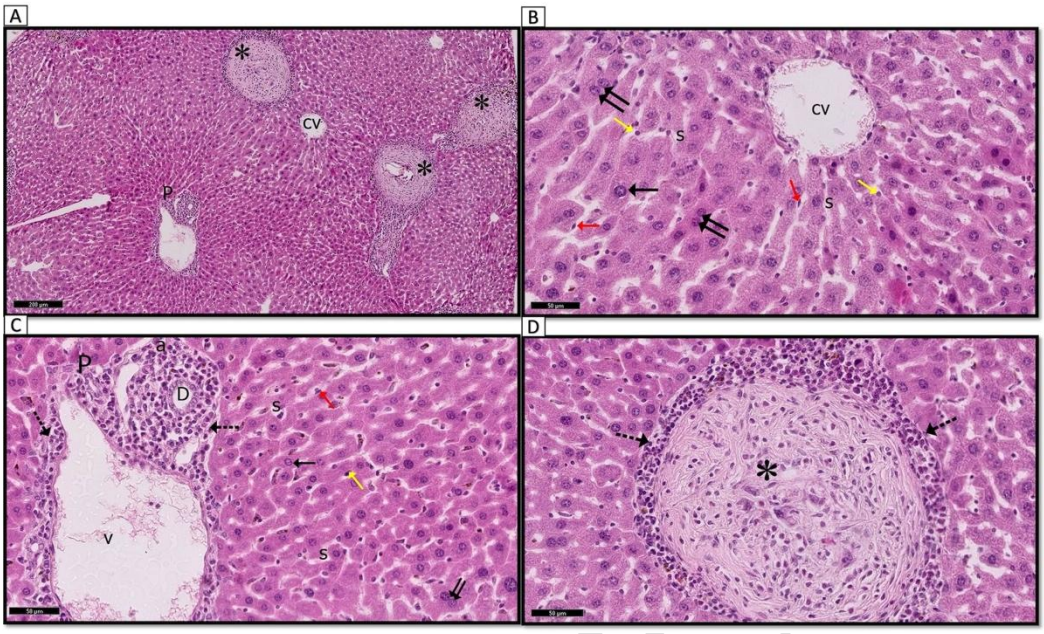


Figure 3. Infected mice treated with Silymarin (group III) demonstrated an apparent decrease of size and numbers of the granuloma with regularly arranged fibroblast (*) surrounding mild inflammatory cellular reaction (dot arrow). The polyhedral hepatocytes are arranged radially in cords around the central vein (CV) with normal vesicular basophilic nuclei and granular cytoplasm (↑). Notice Few hepatocytes appeared binucleated (↑↑), the thin endothelial lining of the blood sinusoids (red ↑) and Von Kupffer cells (yellow↑). Portal area (P) shows the normal hepatocytes with their normal vesicular nuclei around the portal vein (V), Bile ducts (D) and hepatic artery (a). **H&E Stain-Group II**

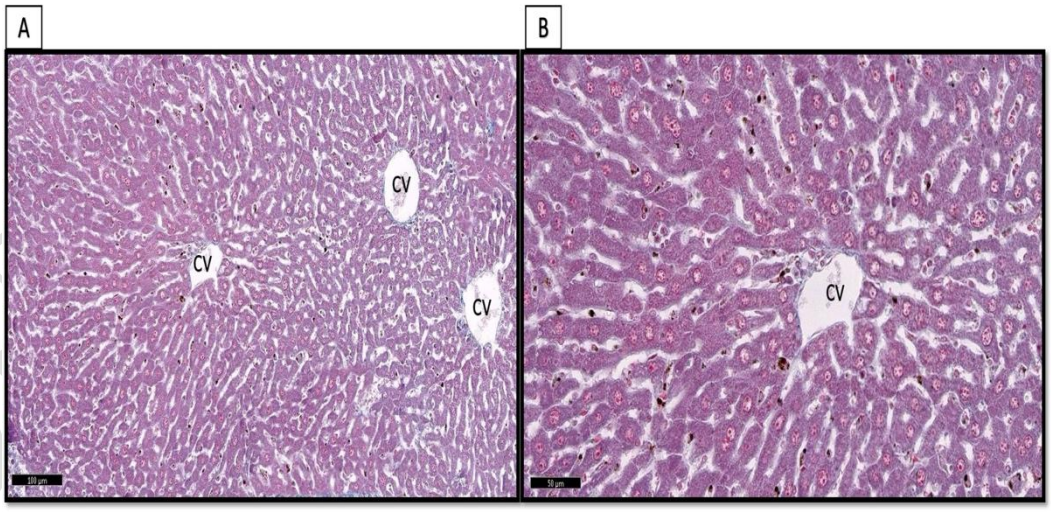


Figure 4. Around the central vein (cv) and the portal area, fine blue-stained fibers connect the radially organized hepatocytes (P). **Masson trichrome Stain-Group I**

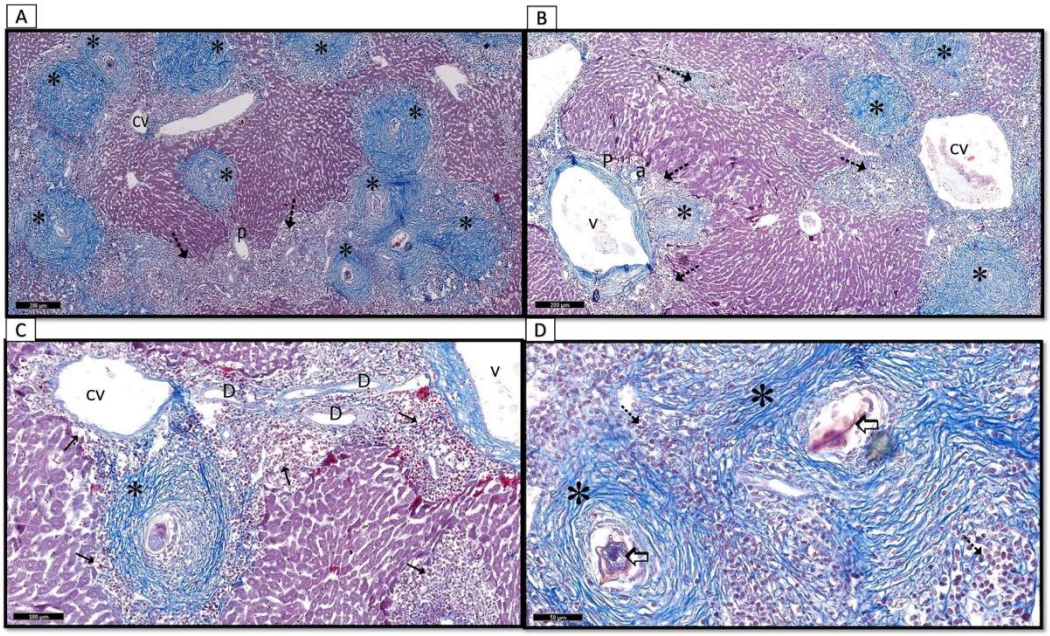


Figure 5. Infected positive control group demonstrating an apparent marked increase of the collagen deposition in the granuloma with marked compact fibrous reaction (*) intact egg (thick white arrow) and marked inflammatory reactions (dot arrow). Notice marked inflammatory reactions and collagen depositions around the central vein (cv) and portal area (P). **Masson trichrome Stain-Group II**

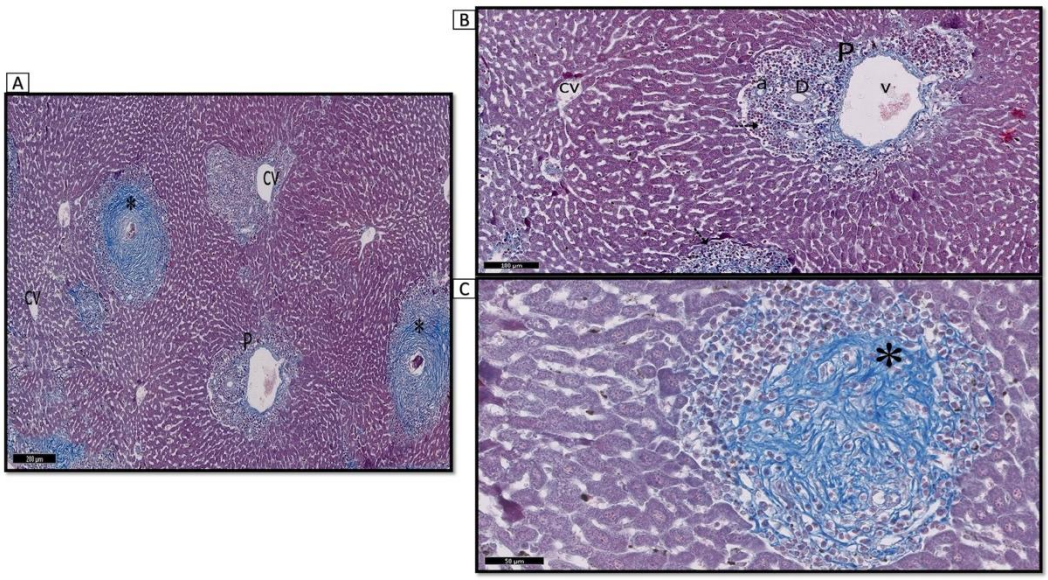


Figure 6. Infected mice treated with Silymarin (group III) demonstrated an apparent decrease of the granuloma (*) size and collagen deposition with well-organized fibrous tissue and sparing most of the hepatic tissue around the central vein (cv) and portal area (P). **Masson trichrome Stain-Group III**

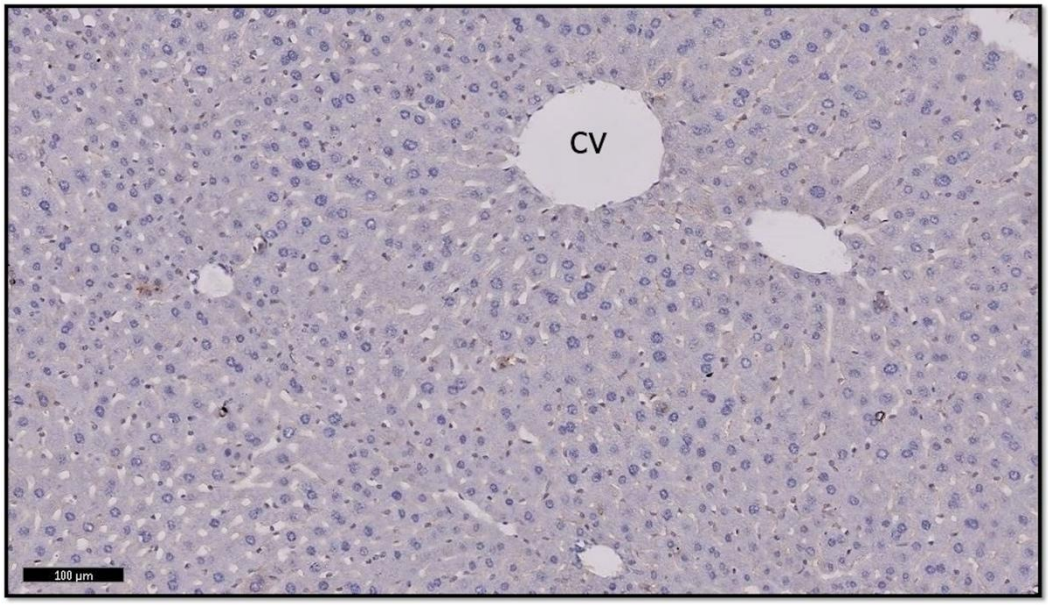


Figure 7. Immunostaining for alpha-smooth muscle actin (α-SMA) antibody in a liver section of group I (negative Control group) shows a negative expression of α-SMA. **IHC, α-SMA-Group I**

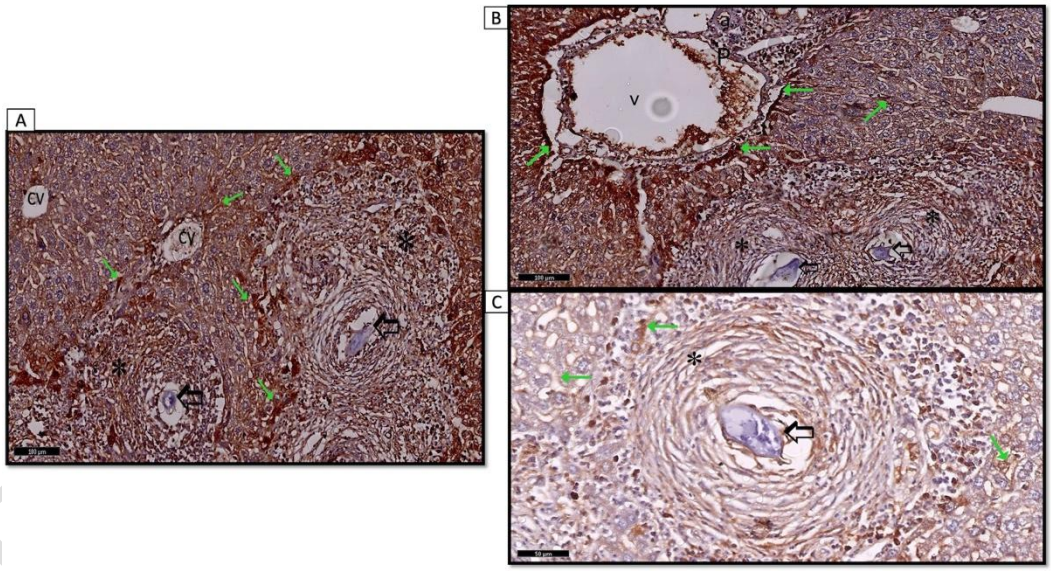


Figure 8. Infected positive control group demonstrating a marked increase expression of an α-SMA monoclonal antibody as cytoplasmic brownish color (green ↑) in-between hepatocytes around the central vein (cv), portal area (P), and granuloma cells (*) with dead calcified egg (thick white arrow). **IHC, α-SMA-Group II**

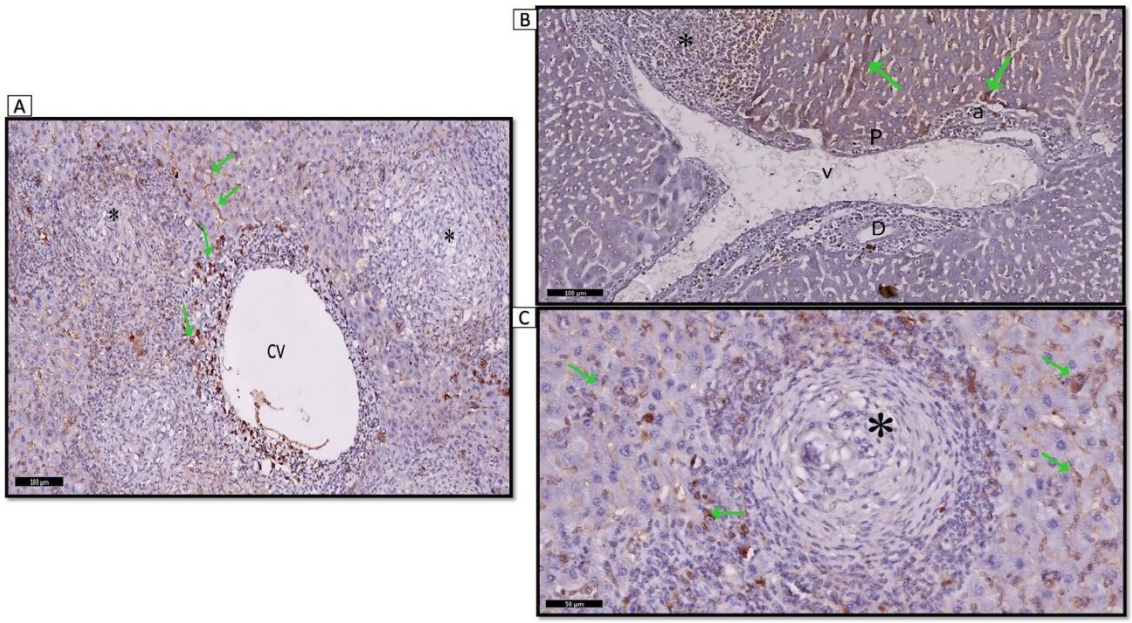


Figure 9. Infected mice treated with Silymarin (group III) demonstrated a marked decrease in expression of an α-SMA monoclonal antibody as cytoplasmic brownish color (green ↑) in-between hepatocytes around the central vein (cv), portal area (P), and granuloma cells (*). **IHC, α-SMA-Group III**

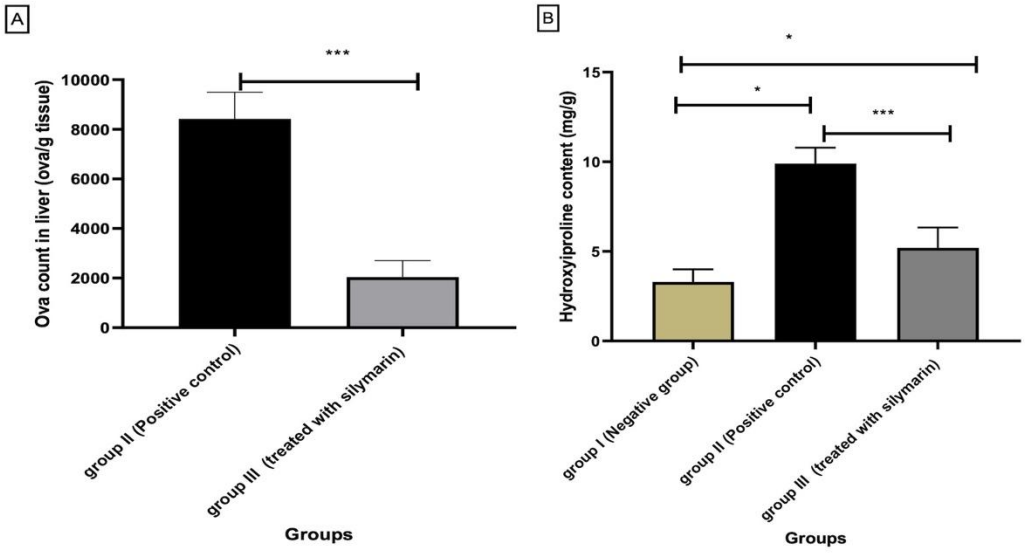


Figure 10. Ameliorative effects of the administration of Silymarin on the ova count in the liver **(A)** and hydroxyproline content on the liver tissue **(B)**

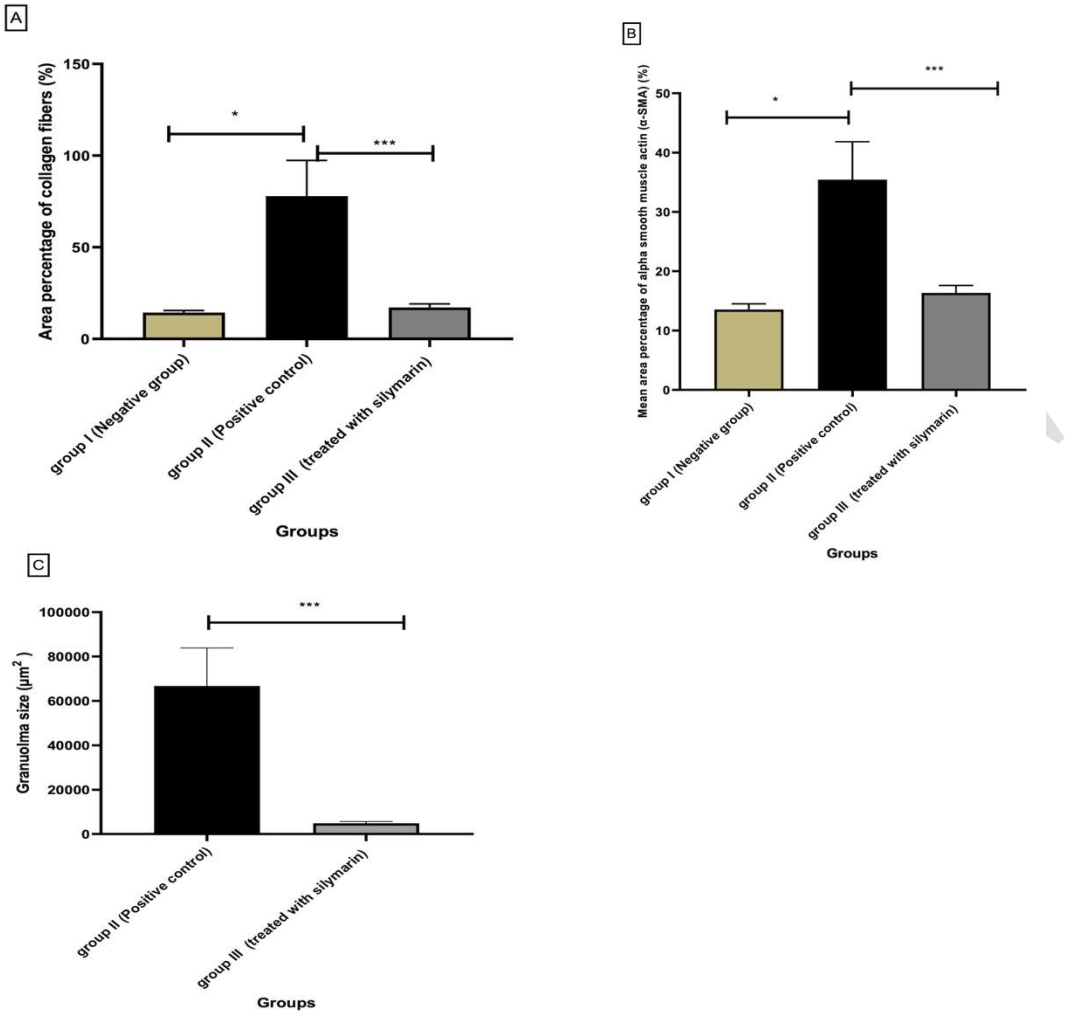


Figure 11. Ameliorative effects of the administration of Silymarin on the area percentage of collagen fibers (%) (A), the mean area percentage of alpha-smooth muscle actin (α-SMA) (%) (B), and granuloma size (μm2) (C)

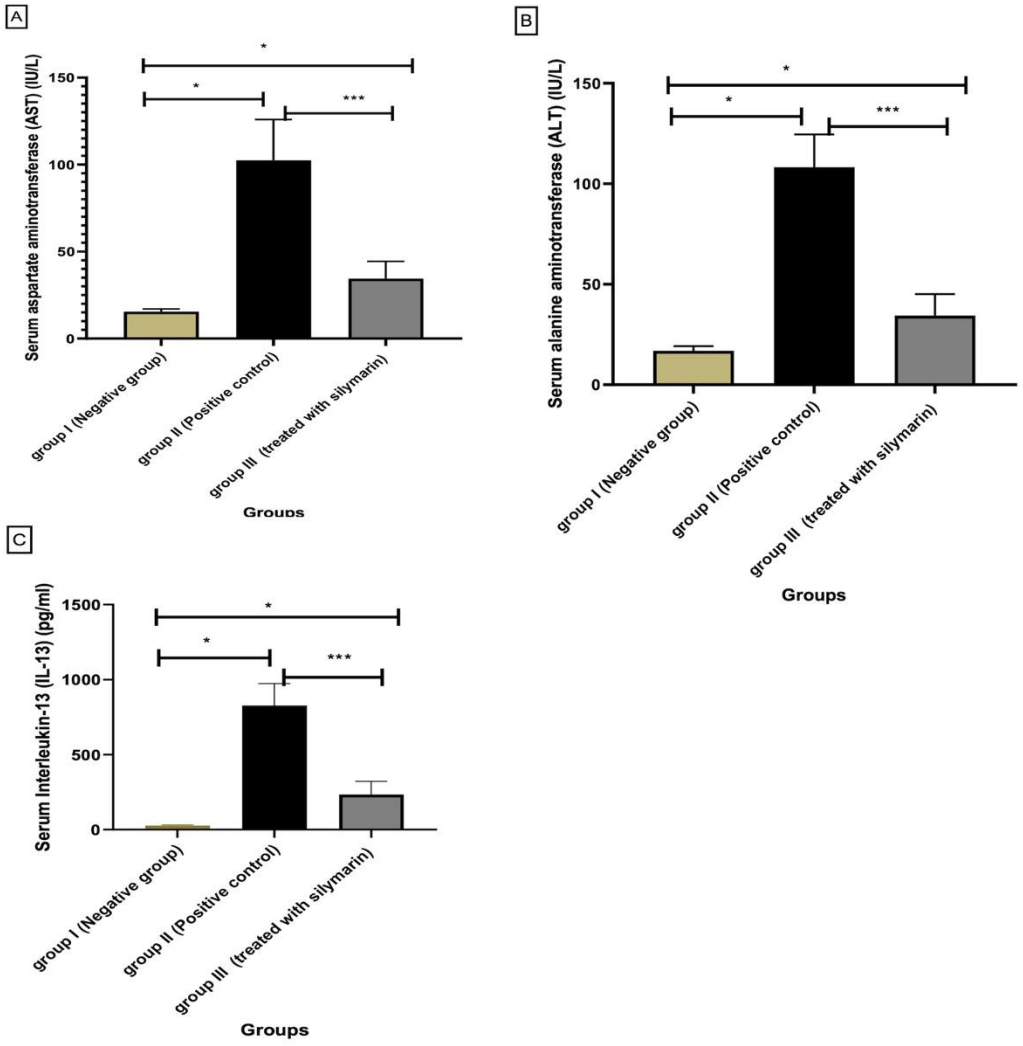


Figure 12. Ameliorative effects of the administration Silymarin on the serum aspartate aminotransferase (AST) (IU/L) (A), serum alanine aminotransferase (ALT) (IU/L) (B), and serum interleukin-13 (IL-13) (pg/ml) (C)

Table 1. Effect of the tested drug (Silymarin) on granuloma size (µm2), ova count in the liver (ova/g tissue), hydroxyproline content (mg/g), biochemical parameters, and morphometric result

Report									
Groups (N=10)		**Granuloma size (μm^2)**	**Ova count in the liver (ova/g tissue)**	**Hydroxyproline content (mg/g)**	**Serum alanine aminotransferase (ALT) (IU/L)**	**Serum aspartate aminotransferase (AST) (IU/L)**	**Serum Interleukin-13 (IL-13) (pg/ml)**	**Area percentage of collagen fibers (%)**	**Mean area percentage of alpha-smooth muscle actin (α-SMA) (%)**
Group I (negative control)	**Mean ±SEM**	-	-	3.30 ±0.22	16.97 ±0.70	15.47 ±0.50	27.0560 ±1.43	14.41 ±0.34	13.55 ±0.30
Group II (positive control)	**Mean ±SEM**	66737.78 ±54.60	8420.0 ±339.54	9.90 ±0.28 a	108.23 ±5.16 a	102.42 ±7.421 a	827.70 ±46.13 a	77.92 ±6.15 a	35.46 ±2.01 a
Group III Silymarin treated)	**Mean ±SEM**	4851.15 ±244.12 b	2036.0 ±211.36 b	5.20 ±0.35 a, b	34.40 ±3.38 a, b	34.50 ±3.13 a, b	234.20 ±28.07 a, b	17.12 ±0.618 a, b	16.36 ±0.39 a, b

[1] Data were expressed as mean ± SEM, N = number of rats. One-way ANOVA followed by Tukey multiple comparison tests. P <0.05 compared to negative control mice (a); P< 0.05 compared to positive control mice (b).

4. Discussion

Schistosomiasis comes second to malaria in its impact on humanity [1]. It causes an acute and chronic infection, resulting in granulomas formation around trapped eggs in the portal venules of the liver [1]. Trapped eggs release various antigens that lead to antigen-specific humoral and cell-mediated immune responses that cause fibrosis and portal hypertension [16], [17].

The drug that treating schistosomiasis relies on is praziquantel (PZQ). Although PZQ effectively eliminates adult worms and precludes eggs' accumulation, it is less effective in potentially reversing existing hepatic fibrosis [18]. Therefore, treating schistosomiasis hepatic fibrosis remains challenging. Moreover, some studies reported that PZQ induced hemorrhage in the host's lung tissue [9]. Several other studies documented that (PZQ)-resistant in some Schistosoma strains [9]. Therefore, it is vital to find effective alternative drugs to control the infection and prevent side effects. For centuries people used medicinal plants to maintain health and mitigate chronic diseases [19]. The present study established a mouse schistosomiasis model through *S. mansoni* cercariae infection and then treated them with Silymarin to diminish liver fibrosis. Previous studies demonstrated Silymarin's ability to reduce fibrotic deposition in the liver during acute schistosomiasis [8]. Accordingly, in this study, we tried to confirm these results further using different techniques for the different mice groups, starting from H&E stain, then using special stain (Masson's trichrome). Immunohistochemistry was used to give more specific results to localize the presence of the antigens in liver tissue sections.

Histopathological changes in liver tissues of sacrificed mice were detected by staining sections with haematoxylin and eosin and Masson's trichrome stains. Cure rate and healing degree in damaged tissues were assessed before and after treatment. The results demonstrated that tissues covered with granulomas were fused with eggs in the infected non-treated mice group. Also, deeply stained hepatocytes with dark pyknotic nuclei were diffusely seen. These results are coherent with previous studies as mentioned by [10], [17], [19], [21]– [23]. Treatment with Silymarin significantly improved liver morphology, especially on the decreased area percentage of collagen fibers. These findings agree with [10], [19], [24], [25]. Silymarin efficiently hinders the fibrogenesis process in hepatic tissue. Its administration at a dose rate equivalent to that used to treat hypertransaminasemia (50 mg/kg) for four weeks in experimental rats showed a reduction in hepatocyte damage [26]. In addition to reducing fibrosis score, markers of oxidative stress and tissue hyaluronic acid. It should be considered that Silymarin's ability to antagonize fibrogenesis is only to de novo fibrosis, not an advanced one. Consequently, administration of Silymarin as a treatment for chronic hepatopathies will be only effective if carried out from the early stages of the pathology [27], [28].

The excessive accumulation of several extracellular matrix (ECM) components is a significant observation in fibrosis. For example, in the fibrotic process, type III collagen deposition increases. Moreover, types I and II collagen, fibronectin, and proteoglycan accumulate during granuloma formation. The primary source of these ECM in liver fibrogenesis has been identified as the activated HSCs [29]–[31]. In the present study, Immunostaining of α-SMA revealed a highly positive peri-sinusoidal reaction and around the granuloma with an apparent increase of the granuloma size in infected untreated mice. Morphometric and statistical analyses were used to confirm the findings. Our results were consistent with previous investigations [32]– [34] have found a considerable rise in the area % of -SMA positive brownish cells. There was an apparent decrease in the positive reaction and granuloma size in group III treated mice compared to group II. These results are coherent with previous studies done by [32], [35], [36]. Silymarin inhibits the expression of various cell cycle targets, including p27, Akt, and sirtuin signaling, as shown by Ezhilarasan et al. on human HSCs [36]. According to convincing evidence, hepatic oval cells (HOC) are progenitors of the liver stem cell compartment [35]. After damage, oval cells with oval nuclei and basophilic and sparse cytoplasm form in the small portal zone. Alaca et al. concluded that Silymarin could treat the cholestatic liver injury in rats, decreased activated α-SMA positive brownish cells, and activation of hepatic stem cells (oval cells) [35].

Schistosomiasis is a relatively asymptomatic infection. Symptoms establish when the parasite starts oviposition 6 to 8 weeks after infection [37]. Some laid eggs are carried by mesenteric vessels and trapped in liver tissue. They cannot be exterminated, promoting a granulomatous reaction to isolate them from healthy hepatic parenchyma. Myofibroblasts deposit collagen around these eggs. The formed fibrosis, along with the resulting vascular damage, alters blood flow in the liver, causing portal hypertension [24], [25], [38]– [40].

Hydroxyproline level was measured in this study because it is a sensitive marker for liver fibrosis. This amino acid increase during the de novo synthesis of liver collagen [10], [41]. To assess the effects of Silymarin on hepatic fibrosis, we evaluated the reduction in granuloma area and fibrosis by measuring hydroxyproline levels. These changes were compared between infected silymarin-treated group III to non-treated positive control group II. Silymarin antioxidant, anti-inflammatory, anti-proliferative, and immunomodulatory effects enhanced liver health [42].

Silymarin benefits for liver health caught scientists' interest. This study verified that using Silymarin as treatment in experimental *S. mansoni* infection reduced granuloma area and hepatic fibrosis. Together with an observed reduction in the markers of hepatocellular damage and profibrogenic cytokines, these records reflect a reduction in morbidity rate in experimental schistosomiasis. El-Lakkany et al. (2012) reported that treating infected mice using Silymarin from 84 to 126 dpi caused a slight decline in parasite burden and fibrosis. However, the reduction was lower than that of praziquantel alone and possibly linked to the burden reduction [10]. Herein, we observed a significant decrease in the number of ova/gram liver tissue in silymarin-treated mice group III compared to positive control group II, suggesting a significant effect of Silymarin in parasite burden. As a result, silymarin may eliminate oxidative products and aids in the immune-mediated destruction of worms and eggs. Therefore, further investigating the effects of silymarin in future studies is noteworthy.

The observed elevation in ALT and AST reflects liver damage [43]. As observed by Naik and Panda (2007) [44] and Essam and Ashraf (2013) [45], our results showed a rise of these enzymes in serum. The hepatocytes damage causes this elevation due to the released parasite eggs proteases. Also, Naik et al. (2011) described that the hepatocyte membrane damage seems to be the leading cause for the upsurge liver function markers such as AST, ALT, and ALP after schistosome infection [46]. In addition, hypo-albuminemia may result from the malabsorption caused by intestinal mucosa damage. Mucosal damage could result from the extrusion of excessive numbers of eggs. Alternatively, diminished albumin synthesis following parasitic injury to hepatic cells [47]. Silymarin showed the ability to restore the elevation in serum ALT in CCl4 intoxicated rats [48] and diethylnitrosamine administered rats [49]. It reduced liver tissue damage and enzyme leakage by preventing liver damage and maintaining plasma membrane integrity. Silymarin's antioxidant action increased hepatic GSH concentration [50]. As a result, much lower intracellular ROS production in response to pro-oxidant stimuli [51]– [53]. Furthermore, Silymarin can protect liver cells directly by stabilizing membrane permeability by decreasing lipid peroxidation and limiting liver glutathione depletion, providing a combinatorial advantage of avoiding liver cell damage [54]– [56].

In mouse schistosomiasis, IL-13 insufficiency is linked to a reduction in hepatic fibrosis [57], and silybin has been shown to lower the amount of IL-13 [19], [58]. Furthermore, by blocking Kupffer cells, silybin inhibits the activation of stellate cells, which are responsible for collagen deposition [19]. In schistosomiasis, it was discovered that IL-13, not IL-1/IL-17, is responsible for hepatic fibrosis [59]. So far, there has not been any evidence of a link between oxidative stress and IL-13 production. As a result, we assume that Silymarin's previously documented ability to block IL-13 production and stellate cell activation is responsible for reducing fibrosis caused by therapy, a theory that is now being investigated in this study.

5. Conclusion:

Using a mouse model, we investigated the efficacy of Silymarin in inhibiting the fibrogenesis process. Silymarin treatment improved liver histology, particularly in the decreased area % of collagen fibers. It is worth noting that Silymarin's capacity to inhibit fibrogenesis is limited to de novo fibrosis, not advanced fibrosis. As a result, using Silymarin as a treatment for chronic hepatopathies will only be successful if started during the acute phase of the disease. Silymarin has a considerable influence on the parasite burden, worth further investigation in future studies.

Supplementary Materials: All figures and tables are included in the manuscript.

Institutional Review Board Statement: The study was designed with correspondence to the codes of the guidelines for Ethical Conduct in the Care and Use of Animals; experimental conduct and handling were authorized via the Animal Ethics division within the Ethics Committee of Biomedical Research-Faculty of medicine at King Abdul Aziz University, ethical approval number (438/290/610). The experiment was executed in consensus with the guidelines of dealing with experimental animals that are followed in KFMRC, KAU, Jeddah, Saudi Arabia, which are in accordance with the Canadian Council for animal safety and health care.

Informed Consent Statement: "Not applicable"

Data Availability Statement: Data is contained within the article

References

[1] https://www.who.int/news-room/fact-sheets/detail/schistosomiasis accessed 23/11/2020

[2] van der Werf, M. J., S. J. de Vlas, S. Brooker, C. W. N. Looman, N. J. D. Nagelkerke, J. D. F. Habbema, and D. Engels. (2003) Quantification of clinical morbidity associated with schistosome infection in sub-Saharan Africa. Acta Tropica 86: 125-139. doi:10.1016/S0001-706X(03)00029-9.

[3] Andrews P., H. Thomas, R. Pohlke, J. Seubert. (1983). Praziquantel. Med Res Rev 3(2):147–200. Epub 1983/04/01. https://doi.org/10.1002/med.2610030204 PMID: 6408323.

[4] Olliaro P.L., M.T. Vaillant, V.J. Belizario, N.J. Lwambo, M. Ouldabdallahi, O.S. Pieri. (2011) A multicentre randomized controlled trial of the efficacy and safety of single-dose praziquantel at 40 mg/kg vs 60 mg/kg for treating intestinal schistosomiasis in the Philippines, Mauritania, Tanzania and Brazil. PLoS Negl Trop Dis.; 5(6):e1165. Epub 2011/06/23. https://doi.org/10.1371/journal.pntd.0001165 PMID: 21695161; PubMed Central PMCID: PMC3114749.

[5] Gryseels B., A. Mbaye, S.J. de Vlas, F.F. Stelma, F. Guisse, L. van Lieshout. (2001) Are poor responses to praziquantel for the treatment of Schistosoma mansoni infections in Senegal due to resistance? An overview of the evidence. Trop Med Int Health. 6(11):864–73. Epub 2001/11/13. https://doi.org/10.1046/j.1365-3156.2001.00811.x PMID: 11703840.

[6] Aly H.F., M.M. Mantawy. (2013) Efficiency of ginger (Zingbar officinale) against Schistosoma mansoni infection during host-parasite association. Parasitology International 62: 380-389.

[7] Féher J. and G. Lengyel. (2012) Silymarin in the prevention and treatment of liver diseases and primary liver cancer. Curr Pharm Biotechnol. Jan;13(1):210-7. doi: 10.2174/138920112798868818. PMID: 21466434.

[8] Mata-Santos H. A., F. G. Lino, C. C. Rocha, C. N. Paiva, M. T. L. C. Branco, and A. dos Santos Pyrrho. 2010. Silymarin treatment reduces granuloma and hepatic fibrosis in experimental schistosomiasis. Parasitol. Res. vol. 107, no. 6, pp. 1429–1434.

[9] Raafat M. H., S. Abdel Gawad, and H. Fikry. (2017) Histological study on the possible therapeutic role of bone marrow-derived mesenchymal stem cells in a model of Schistosoma mansoni infestation of spleen of mice. Egypt. J. Histol. vol. 40, no. 3, pp. 388–404.

[10] El-Lakkany N. M., O. A. Hammam, W. H. El-Maadawy, A. A. Badawy, A. A. Ain-Shoka, and F. A. Ebeid. (2012) Anti-inflammatory/antifibrotic effects of the hepatoprotective silymarin and the schistosomicide praziquantel against Schistosoma mansoni-induced liver fibrosis. Parasit. Vectors. vol. 5, no. 1, p. 9.

[11] Bancroft J. D. and M. Gamble. (2008). Theory and practice of histological techniques. Elsevier health sciences.

[12] Ahmed S. K., S. A. Mohammed, G. Khalaf, and H. Fikry. (2014). Role of bone marrow mesenchymal stem cells in the treatment of CCL4 induced liver fibrosis in albino rats: A histological and immunohistochemical study. Int. J. Stem Cells. vol. 7, no. 2, pp. 87–97.

[13] Cheever A. W. (1968). Conditions affecting the accuracy of potassium hydroxide digestion techniques for counting Schistosoma mansoni eggs in tissues. Bull. World Health Organ. vol. 39, no. 2, p. 328.

[14] Woessner Jr J. F. (1961) The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch. Biochem. Biophys.vol. 93, no. 2, pp. 440–447.

[15] Reitman S. and S. Frankel. (1957) A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases," Am. J. Clin. Pathol. vol. 28, no. 1, pp. 56–63.

[16] Hegab M.H., S.H. Abd-Allah, M.S. Badawey, A.A. Saleh, A.S. Metwally, G.M. Fathy, S.M. Nada, S.A. Abdel-Rahman, A.A. Saleh, A., Fawzy, M.A. El-Magd. (2018) Therapeutic potential effect of bone marrow-derived mesenchymal stem cells on chronic liver disease in murine Schistosomiasis Mansoni. J. Parasit. Dis. vol. 42, no. 2, pp. 277–286.

[17] Fikry H., S. A. Gawad, and W. Baher. 2016. Therapeutic potential of bone marrow-derived mesenchymal stem cells on experimental liver injury induced by Schistosoma mansoni: A histological study. Int. J. stem cells. vol. 9, no. 1, p. 96.

[18] Andrade Z. A. 2008. Schistosomiasis and hepatic fibrosis regression. Acta Trop. vol. 108, no. 2–3, pp. 79–82.

[19] El-Hawary S. S., K. F. Taha, F. N. Kirillos, A. A. Dahab, A. A. El-Mahis, and S. H. El-Sayed. (2018) Complementary effect of Capparis spinosa L. and silymarin with/without praziquantel on mice experimentally infected with Schistosoma mansoni. Helminthologia. vol. 55, no. 1, pp. 21–32.

[20] Abdul-Ghani R. A. and A. A. Hassan. (2010) Murine schistosomiasis as a model for human schistosomiasis mansoni: similarities and discrepancies. Parasitol. Res. vol. 107, no. 1, pp. 1–8.

[21] Emam M. H., A. El-Rahman, I. S. Gamil, and M. A. Muselhy. (2009) Studies on the effect of antioxidant Selenium-ACE after treatment with Praziquantel and Mirazid in Schistosoma mansoni infected mice. Egypt. J. Hosp. Med. vol. 37, no. 1, pp. 709–725.

[22] Abdel-Rahman S. A. (2009) Monitoring Th1 and Th2 cytokine patterns after praziquantel and mirazid treatment in experimental mansoniasis. PUJ. vol. 6, no. 1, pp. 66–77.

[23] El-Sisi A., W. Awara, T. El-Masry, S. El-Kowrany, and R. El-Gharbawy. (2011) Effects and mechanism of action of immunomodulating agents against schistosomiasis-induced hepatic inflammation and fibrosis in mice. Res. Pharm. Biotechnol. vol. 3, no. 4, pp. 32–45.

[24] Mata-Santos H.A., F.G. Lino, C.C. Rocha, C.N. Paiva, M.T. Castelo Branco, A.S. Pyrrho. (2010) Silymarin treatment reduces granuloma and hepatic fibrosis in experimental schistosomiasis. Parasitol. Res. 107:1429–1434.http://dx.doi.org/10.1007/s00436-010-2014-8.

[25] Mata-Santos, H. A., F. F. Dutra, C. C. Rocha, F. G. Lino, Xavier, F. R., L. A. Chinalia, B. H. Hossy, M. T. Castelo-Branco, A. J. Teodoro, C. N. Paiva, and A. dos Santos Pyrrho. (2014) Silymarin reduces profibrogenic cytokines and reverses hepatic fibrosis in chronic murine schistosomiasis. Antimicrob. Agents Chemother. vol. 58, no. 4, pp. 2076–2083.

[26] Clichici S., D. Olteanu, A.L. Nagy, A. Oros, A. Filip, P.A. Mircea. (2015) Silymarin inhibits the progression of fibrosis in the early stages of liver injury in CCl4-treated rats. J. Med. Food. vol. 18, no. 3, pp. 290–298.

[27] Muriel P., M.G. Moreno, C. Hernández Mdel, E. Chávez, L.K. Alcantar. (2005) Resolution of liver fibrosis in chronic CCl4 administration in the rat after discontinuation of treatment: effect of silymarin, silibinin, colchicine and trimethylcolchicinic acid. Basic Clin. Pharmacol. Toxicol. vol. 96, no. 5, pp. 375–380.

[28] Federico A., M. Dallio, C. Loguercio .2017. Silymarin/silybin and chronic liver disease: a marriage of many years. Molecules. vol. 22, no. 2, p. 191.

[29] Carson J.P., G.A. Ramm, M.W. Robinson, D.P. McManus, G.N. Gobert. (2018). Schistosome-induced fibrotic disease: the role of hepatic stellate cells. Trends Parasitol. vol. 34, no. 6, pp. 524–540.

[30] Kamdem S.D., R. Moyou-Somo, F. Brombacher, J.K. Nono. (2018) Host regulators of liver fibrosis during human schistosomiasis. Front. Immunol.vol. 9, p. 2781.

[31] Almeer R.S., M.F. El-Khadragy, S. Abdelhabib, A.E. Abdel Moneim. (2018) Ziziphus spina-christi leaf extract ameliorates schistosomiasis liver granuloma, fibrosis, and oxidative stress through downregulation of fibrinogenic signaling in mice. PLoS One. vol. 13, no. 10, p. e0204923.

[32] Saber S., R. Goda, G.S. El-Tanbouly, D. Ezzat. (2018)

[33] Lisinopril inhibits nuclear transcription factor-kappa B and augments sensitivity to silymarin in experimental liver fibrosis. Int. Immunopharmacol. vol. 64, pp. 340–349.

[34] Sokar S.S., M.E. El-Sayad, M.E. Ghoneim, A.M. Shebl. (2017) Combination of Sitagliptin and Silymarin ameliorates liver fibrosis induced by carbon tetrachloride in rats. Biomed. Pharmacother.vol. 89, pp. 98–107.

[35] Sharaf El-Deen S.A., R.M. Brakat, A.S.E.D. Mohamed .(2017) Artichoke leaf extract protects liver of Schistosoma mansoni infected mice through modulation of hepatic stellate cells recruitment. Exp. Parasitol. vol. 178, pp. 51–59.

[36] Alaca N., D. Özbeyli, S. Uslu, H.H. Şahin, G. Yiğittürk, H. Kurtel, G. Öktem, B. Çağlayan Yeğen. (2017) Treatment with milk thistle extract (Silybum marianum), ursodeoxycholic acid, or their combination attenuates cholestatic liver injury in rats: Role of the hepatic stem cells. Turk J Gastroenterol. vol. 28, no. 6, pp. 476–484.

[37] Ezhilarasan D., J. Evraerts, B. Sid, P.B. Calderon, S. Karthikeyan, E. Sokal, M. Najimi. 2017. Silibinin induces hepatic stellate cell cycle arrest via enhancing p53/p27 and inhibiting Akt downstream signaling protein expression. Hepatobiliary Pancreat. Dis. Int.vol. 16, no. 1, pp. 80–87.

[38] Neva F. A. and H. W. Brown. (1994) Basic clinical parasitology. no. Ed. 6. Appleton & Lange.

[39] El Scheich T., L. Hofer, G. Kaatano, J. Foya, D. Odhiambo, J. Igogote, N. Lwambo, H. Ekamp, K. Karst, D. Häussinger, J. Richter. (2012) Hepatosplenic morbidity due to Schistosoma mansoni in schoolchildren on Ukerewe Island, Tanzania. Parasitol. Res. vol. 110, no. 6, pp. 2515–2520.

[40] Cheever A. W. and Z. A. Andrade. (1967). Pathological lesions associated with Schistosoma mansoni infection in man. Trans. R. Soc. Trop. Med. Hyg. vol. 61, no. 5, pp. 626–639.

[41] Tousson E., D. M. Beltagy, M. A. Gazia, and B. Al-Behbehani. (2013) Expressions of P53 and CD68 in mouse liver with Schistosoma mansoni infection and the protective role of silymarin. Toxicol. Ind. Health. vol. 29, no. 8, pp. 761–770.

[42] Ahmad S.B., M.U. Rehman, B. Fatima, B. Ahmad, I. Hussain, S.P. Ahmad, A. Farooq, S. Muzamil, R. Razzaq, S.M. Rashid, S. Ahmad Bhat, M.U.R. Mir. 2018. Antifibrotic effects of D-limonene (5 (1-methyl-4-[1-methylethenyl]) cyclohexane) in CCl4 induced liver toxicity in Wistar rats. Environ. Toxicol. vol. 33, no. 3, pp. 361–369.

[43] Polyak S. J., P. Ferenci, and J.-M. Pawlotsky.(2013) Hepatoprotective and antiviral functions of silymarin components in HCV infection. Hepatology. vol. 57, no. 3, p. 1262.

[44] Naik S. R. and V. S. Panda. (2007) Antioxidant and hepatoprotective effects of Ginkgo biloba phytosomes in carbon tetrachloride-induced liver injury in rodents. Liver Int. vol. 27, no. 3, pp. 393–399.

[45] Essam A. M. and A. E. Ashraf. 2013. Effect of curcumin on hematological, biochemical and antioxidants parameters in Schistosoma mansoni infected mice. International Journal of Sciences. Office ijSciences. vol. 2(03), pages 1-14, March.

[46] Kadry S. M., A. M. Mohamed, E. M. Farrag, and D. B. Fayed. (2013) Influence of some micronutrients and Citharexylum quadrangular extract against liver fibrosis in Schistosoma mansoni infected mice. African J. Pharm. Pharmacol. vol. 7, no. 38, pp. 2628–2638.

[47] Naik S. R., V. N. Thakare and S. R. Patil. (2011) Protective effect of curcumin on experimentally induced inflammation, hepatotoxicity and cardiotoxicity in rats: evidence of its antioxidant property. Exp. Toxicol. Pathol.vol. 63, no. 5, pp. 419–431.

[48] Oliveira S.A., B.S. Souza, C.A. Guimaraes-Ferreira, E.S. Barreto, S.C. Souza, L.A. Freitas, R. Ribeiro-Dos-Santos, M.B. Soares. (2008). Therapy with bone marrow cells reduces liver alterations in mice chronically infected by Schistosoma mansoni. World J. Gastroenterol. WJG, vol. 14, no. 38, p. 5842.

[49] Cho Y.K., J.W. Yun, J.H. Park, H.J. Kim, D.I. Park, C.I. Sohn, W.K. Jeon, B.I. Kim, W. Jin, Y.H. Kwon, M.K. Shin, T.M. Yoo, J.H. Kang, C.S. Park. (2009). Deleterious effects of silymarin on the

expression of genes controlling endothelial nitric oxide synthase activity in carbon tetrachloride-treated rat livers. Life Sci. vol. 85, no. 7–8, pp. 281–290.

[50] Pradeep K., C. V. R. Mohan, K. Gobianand, and S. Karthikeyan. (2007) Silymarin modulates the oxidant-antioxidant imbalance during diethylnitrosamine induced oxidative stress in rats. Eur. J. Pharmacol. vol. 560, no. 2–3, pp. 110–116.

[51] Gazak R., D. Walterova, and V. Kren. 2007. Silybin and silymarin-new and emerging applications in medicine," Curr. Med. Chem. vol. 14, no. 3, pp. 315–338.

[52] Loguercio C. and D. Festi. (2011) Silybin and the liver: from basic research to clinical practice. World J. Gastroenterol. WJG. vol. 17, no. 18, p. 2288.

[53] Surai P. F. (2015) Silymarin as a natural antioxidant: an overview of the current evidence and perspectives. Antioxidants. vol. 4, no. 1, pp. 204–247.

[54] Trappoliere M., A. Caligiuri, M. Schmid, C. Bertolani, P. Failli, F. Vizzutti, E. Novo, C. di Manzano, F. Marra, C. Loguercio, M. Pinzani. (2009). Silybin, a component of silymarin, exerts anti-inflammatory and anti-fibrogenic effects on human hepatic stellate cells. J. Hepatol. vol. 50, no. 6, pp. 1102–1111.

[55] Theodosiou E., K. Purchartová, H. Stamatis, F. Kolisis, and V. Křen. (2014) Bioavailability of silymarin flavonolignans: drug formulations and biotransformation. Phytochem. Rev. vol. 13, no. 1, pp. 1–18.

[56] Zhang W., R. Hong, and T. Tian. (2013) Silymarin's protective effects and possible mechanisms on alcoholic fatty liver for rats. Biomol. Ther. (Seoul). vol. 21, no. 4, p. 264.

[57] Kiruthiga P. V., R. B. Shafreen, S. K. Pandian, and K. P. Devi. (2007) Silymarin protection against major reactive oxygen species released by environmental toxins: exogenous H2O2 exposure in erythrocytes. Basic Clin. Pharmacol. Toxicol. vol. 100, no. 6, pp. 414–419.

[58] Fallon P. G., E. J. Richardson, G. J. McKenzie, and A. N. J. McKenzie. (2000) Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. J. Immunol. vol. 164, no. 5, pp. 2585–2591.

[59] Tyagi A., R.P. Singh, K. Ramasamy, K. Raina, E.F. Redente, L.D. Dwyer-Nield, R.A. Radcliffe, A.M. Malkinson, R. Agarwal. (2009). Growth inhibition and regression of lung tumors by silibinin: modulation of angiogenesis by macrophage-associated cytokines and nuclear factor-κB and signal transducers and activators of transcription 3. Cancer Prev. Res. vol. 2, no. 1, pp. 74–83.

[60] Wilson M.S., S.K. Madala, T.R. Ramalingam, B.R. Gochuico, I.O. Rosas, A.W. Cheever, T.A. Wynn. 2010. Bleomycin and IL-1β–mediated pulmonary fibrosis is IL-17A dependent. J. Exp. Med. vol. 207, no. 3, pp. 535–552.