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Journal of Bioscience and Applied Research
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Modulating effect of milk thistle (*Silybum marianum*) oil on CD34 and vimentin expressions in fibrotic and cirrhotic liver tissues induced by CCl_4 in mice.

Running title: Protective effect of *Silybum marianum* oil on hepatic pathological changes induced by CCl_4 in mice.

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DOI: 10.21608/jbaar.2024.282043.1043

Abstract

Aim: evaluate the impact of milk thistle (Silybum marianum) on CD34 and vimentin expression in hepatic fibrosis and cirrhosis induced with CCl_4 in experimental mice. **Methods:** GI: normal group given no therapy; control group. GII: received a daily dose (1mL/kg/bw/d) of milk thistle oil M.T.O for 4 weeks; GIII&GIV: injected i.p. with (1:1 ratio) mixture of CCl4 and olive oil (1mL/kg/bw) twice weekly for 4 weeks to induce fibrosis, and for 6 weeks to induce cirrhosis. Gp5 and Gp6: fibrotic and cirrhotic groups administered M.T.O as in Gp2. **The results** showed that liver sections of Gp1 and Gp2 showed normal moderate to strong CD34 expression in the endothelial cells of the blood sinusoids and many hepatocytes. The liver tissues of Gp3 and Gp4 expressed decrement CD34 immunoreactivity in many hepatic lobules. The liver sections of Gp5 or Gp6 showed restoration of CD34 expression in most of the hepatic tissues. In Gp1 and Gp2, the vimentin was expressed as weak or moderate immunostaining in the endothelial cells and connective tissues (wall of the blood sinusoids, central portal veins, and portal tract stroma). The liver sections of Gp3 and Gp4 showed overexpression of vimentin immunoreactivity. The treatment with M.T.O in Gp5 and Gp6 showed improvement and recovery of vimentin expression in the hepatic lobules. **Conclusion:** M.T.O. treatment improved the hepatic injury induced in fibrotic or cirrhotic tissues by CCl_4 injection and could be recommended for patients with fibrotic and cirrhotic liver diseases.

Keywords: Liver, Fibrosis, Cirrhosis, Milk thistle (*Silybum marianum*), IHC, CD34, Vimentin.

Received: April 7, 2024. Accepted: May 12, 2024. Published: May 23, 2024

Introduction

Hepatic damage is associated with changes in its structure and function [1]. All chronic liver illnesses, including HBV, HCV, alcoholic liver disease, non-alcoholic steatohepatitis, as well as prolonged exposure to environmental pollutants like CCl_4 cause liver fibrosis, leading to deposition of scar tissue including excessive extracellular matrix protein (ECM). ECM is composed of three types of proteins, collagens, non-collagenous glycoproteins (fibronectin, laminin); and proteoglycans [2]. Activated hepatic stellate cells (HSCs), portal fibroblasts, and fibrogenic cytokines are produced in the injured liver [3]. HSCs are primarily responsible for hepatic fibrogenesis, and these cells are also crucial in the aetiology of liver fibrosis. Advanced liver fibrosis leads to cirrhosis, in which extensive fibrosis and nodular regeneration of the liver, and even liver failure occurs.

The principal collagen-producing cells in the injured liver are activated HSCs, myofibroblasts (MFs), and portal fibroblasts. These cells are stimulated by a variety of fibrogenic cytokines, such as TGF-1, angiotensin II, and leptin [3]. MFs are the source of the ECM creating the fibrous scar, are unique to the wounded liver and they express α-SMA and type I collagen. Therefore, a crucial process in liver cirrhosis is the activation and proliferation of hepatic MFs [4]. Scar tissue can prevent blood from passing through the liver. The liver may not function when more scar tissue accumulates, which frequently leads to cirrhosis, liver failure, and portal hypertension. Numerous liver injury processes that increase collagen deposition and change the extracellular matrix's composition result in cirrhosis, which in turn leads to a noticeable deformation of the hepatic vascular architecture [5].

Milk thistle (*Silybum marianum*) belongs to the family Asteraceae. A milky sap is released from this plant when the leaves and stems are broken. *S. marianum* is a native of southern Europe, southern Russia, Asia Minor, and northern Africa who became a citizen of North and South America. Several phytochemical components including silibinin (50–70%), silychristin (20%), silydianin (10%), and silymarin were identified in *S. marianum* [6].

Silymarin has anti-inflammatory, immunomodulating, antifibrotic, and antioxidant characteristics that lower the formation of free radicals and lipid peroxidation, liver-regenerating properties, and is also used to treat fatty liver disease. Silymarin raises glutathione levels and stimulates DNA polymerase and accelerates the synthesis of intact rRNA polymerase, which results in the formation of new hepatocytes [7]. Silibinin is found in fruit milk thistle is very effective at preserving hepatocytes from injury [8].

Embryonic fibroblasts, immature hematopoietic stem/progenitor cells, and regenerating or migrating capillary endothelial cells have CD34 expressed on their surfaces which is a sign of angiogenesis when sprouts are proliferating endothelial cells [9]. No or only weak CD34 immunostaining was visible in the liver sinusoids affected by various levels of chronic hepatitis [10]. Liver tissue with cirrhosis exhibited negative CD34 staining [11]. It has been reported that HCC tissues expressed α-SMA, Ang-1, and micro-vessel density (CD34) at significantly higher levels than tumour-adjacent tissues and healthy liver tissues [12].

All animal mesenchymal cells express the intermediate filaments protein known as vimentin. Vimentin is widely acknowledged to be the cytoskeletal element in charge of preserving cell shape, cell flexibility, cell integrity of the cytoplasm, as well as stabilizing cytoskeletal interactions. Vimentin is connected to the nucleus, endoplasmic reticulum, and mitochondria, maintaining, and anchoring their positions in the cytoplasmic matrix of the cells [13]. Since it was discovered, cells without vimentin are extremely delicate when disturbed with a micro-puncture. Additionally, during HSCs activation, vimentin

expression is highly elevated, and in advanced fibrosis **[14]**. The present investigation is designed to study the modulating effect of milk thistle oil on the expression of CD34 and vimentin by using the immunohistochemical (IHC) method in the fibrotic and cirrhotic liver tissues induced experimentally by CCl_4 in mice.

Materials and Methods

Chemicals

Carbon tetrachloride (CCl_4) and olive oil were obtained from Al-Gomhouria Company (Tanta, Egypt). Milk thistle oil (M.T.O) was purchased from a local market of agricultural seeds, and medicinal plants with affair degree of quality assurance (Alexandria, Egypt). Monoclonal antibodies against CD34 or vimentin were purchased from Biocare Polymer Detection Biosciences (San Diego, USA).

Animals

Sixty male albino mice weighing 25±3 g were obtained from Vacsera (Agouza, Giza, Egypt). For a one-week acclimation period, the animals were housed in plastic cages with stainless steel wire-bar covers (10 per cage), with wooden dust-free litter used as bedding. The conditions included the same temperature and natural dark-light cycle. The National Research Centre's Ethics Committee and the National Institutes of Health's Guide for Care and Use of Laboratory Animals were followed when performing animal procedures. The experimentation and animal care were performed and handled in compliance with the ethical guidelines approved by the animal care and use committee, Faculty of Science, Tanta University, Egypt (IACUC-SCI-TU-).

Induction of hepatotoxicity by CCl_4 and treatment

Hepatic injury was induced by intraperitoneal (i.p.) injection of CCl_4 (1mL/kg) twice a week for 4 weeks to induce fibrosis, and for 6 weeks to induce cirrhosis using a concentration of 50% (v/v) of CCl_4 in suspended in olive oil at a ratio of (1:1) [3; 15].

Experimental design

Sixty male albino mice were divided into 6 groups (n=10). Group (Gp1): normal group, Gp2: mice administered with a dose of M.T.O oil (1mL/kg) orally daily for four weeks. Gp3 and Gp4: fibrotic and cirrhotic groups injected with CCl_4 (1mL/kg) i.p. twice a week for four weeks to induce fibrosis and for six weeks to induce cirrhosis in liver tissues. Gp5 and Gp6: fibrotic and cirrhotic groups administered M.T.O as in Gp2. At the end of the experiment, mice from all groups were euthanized. The liver tissue specimens were taken and cut into small pieces and processed for immunohistochemical (IHC) investigations [16].

Immunohistochemical studies and image analysis

The immunohistochemical technique was used to detect the expression of CD34 and vimentin in liver tissues [16]. Monoclonal antibodies against CD34 or vimentin were used [11]. Semi-quantitative software was used to assess digital photographs (Fiji-Image J software, a Java-based application for analysing images). The brown color of CD34 and vimentin-positive cells was immunohistochemically expressed in fibrotic and cirrhotic liver sections. The percentage-colored stained area (area fraction) per field area was determined by measuring five randomly photographed high-power fields (X400 magnifications) [17].

Results

Treatment with M.T.O increased CD34 expression in fibrotic and cirrhotic liver tissues

In the current study, the expression of CD34 was expressed in normal moderate to strong immuno-positive reaction to the endothelial cells of the blood sinusoids of the liver tissue and many hepatocytes of normal control sections (Gp1) (Figure 1). Similar results were observed in the liver sections of mice that were treated with M.T.O only (Gp2) (Figures 2a and b). However, the

intensity of the immunoreactivity to CD34 immunoreactivity was almost diminished in CCl_4-induced fibrotic or cirrhotic liver groups (Gp3 and Gp4). The reduction of the CD34 immunostain was expressed in endothelial cells of the blood sinusoids and many hepatocytes. Many hepatic lobules were expressed with no CD34 immunostain (Figures 3 and 4). Whereas sections of the liver of fibrotic or cirrhotic mice groups that were administered with M.T.O (Gp5 and Gp6) showed strong CD34 immunostain in sinusoidal epithelia of the hepatic sections and the appearance of numerous intense oval cells in the fibrotic liver (Figures 5 and 6) more than in cirrhotic one (Figure 7).

Image analysis of liver-positive cells by CD34 immunostain

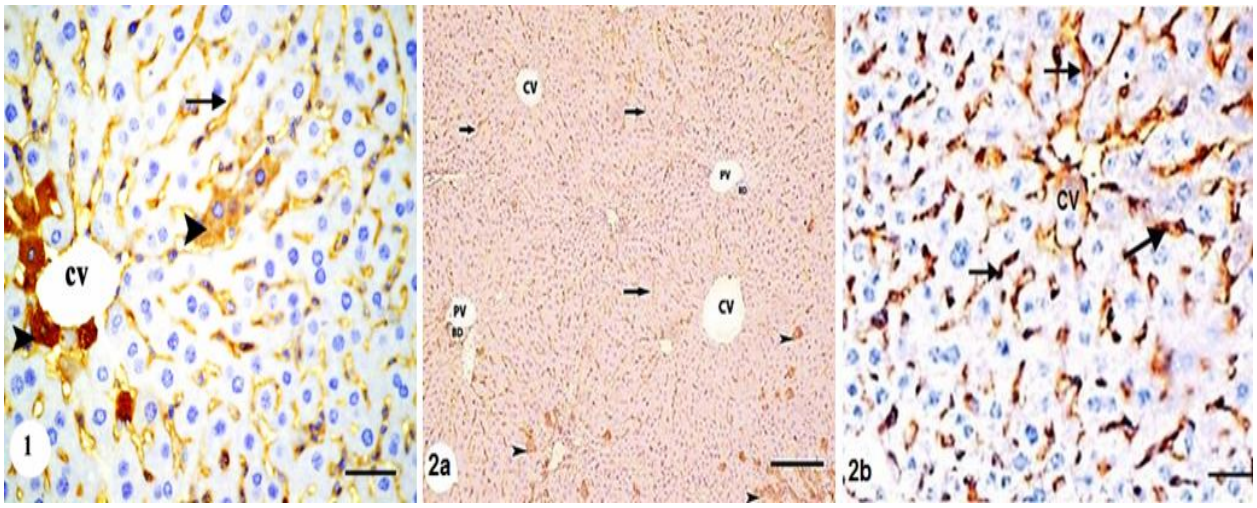
The CCl4-induced fibrotic or cirrhotic groups (Gp3 and Gp4) showed a significant decrease in positive CD34 cells comparable to the normal mice group (Gp1). Fibrotic and cirrhotic mice groups administered orally with M.T.O (Gp5 and Gp6) showed a significant increase in positive CD34 cells, in comparison to the groups of fibrotic and cirrhotic mice (Figure 8).

Treatment with M.T.O decreased vimentin expression in fibrotic and cirrhotic liver tissues

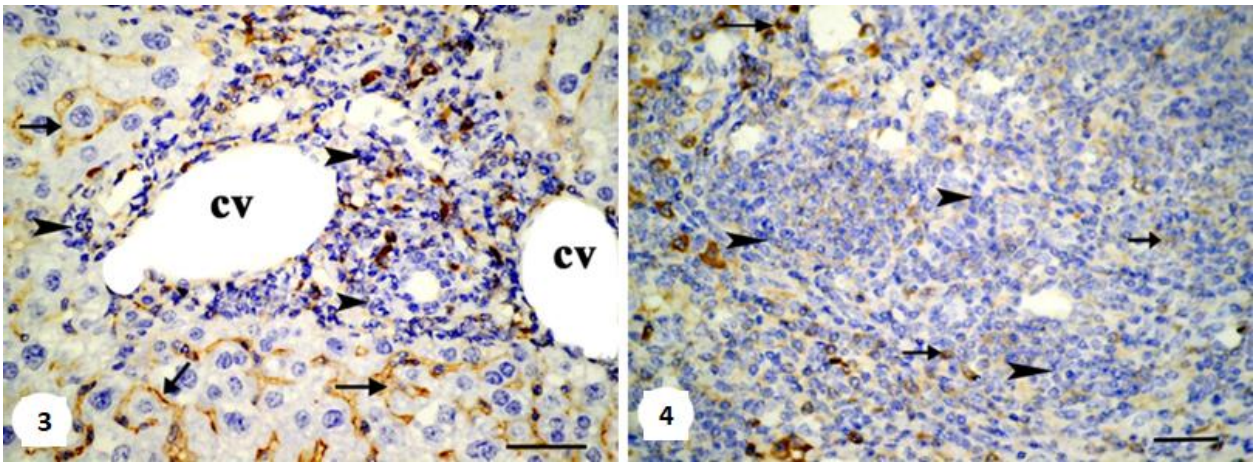
The liver sections of either the normal control group (Gp1) or mice treated with M.T.O (Gp2) expressed normal weak to moderate immunoreactivity to vimentin in the endothelial cells and the connective tissue periphery of the wall of the blood sinusoid, central and portal veins as well as in the cells of portal tract stroma (Figures 9 and 10). Fibrotic or cirrhotic liver sections of mice injected with CCl_4 for either 4 or 6 weeks (Gp3 and Gp4), respectively revealed overexpression and increased vimentin immunoreaction in the endothelia of blood sinusoids and the connective tissue periphery to the wall of the endothelia. Many hepatic lobules of cirrhotic liver expressed no or weak vimentin immunoreaction in the endothelia of blood sinusoids and the fibroblasts of connective tissues adjacent to the walls of sinusoids (Figures 11-13). The administration of M.T.O for four weeks to either fibrotic or cirrhotic mice groups (Gp5 and Gp6) expressed the improvement and decline of most vimentin protein filaments in hepatic lobules. Weak vimentin immunostain is expressed in the fibrotic-treated group, and the liver demonstrated approximately like a normal image. While the cirrhotic treated group expressed moderate to increased vimentin immunostain in endothelium and connective tissue peri-blood sinusoids walls (Figures 14 and 15).

Image analysis of liver-positive cells by vimentin immunostain

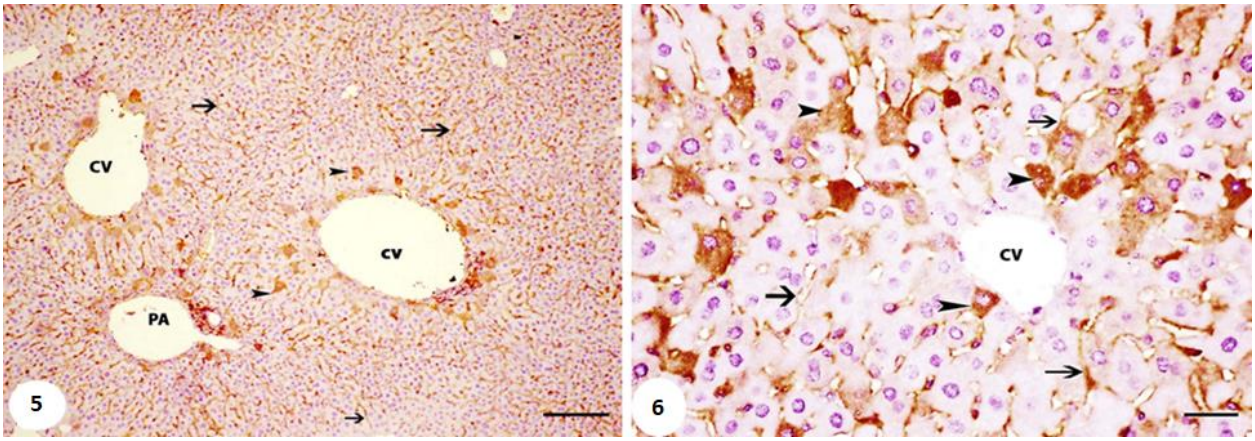
The CCl4-induced fibrotic and cirrhotic groups (Gp3 and Gp4) recorded a highly significant increase in areas of positive cells expressed vimentin compared to the normal mice group (Gp1). Fibrotic and cirrhotic mice groups administered orally with M.T.O (Gp5 and Gp6) expressed a significant decrease in areas of positive cells compared to the groups of fibrotic and cirrhotic mice (Figure 16).



Figures (1 and 2): (1) Liver section of a mouse of control group (Gp1) expressing normal moderate to strong immuno-positive reaction to CD34 in endothelial cells of the blood sinusoid (arrows) as well as many hepatocytes (arrowheads). CD34 immunostain, Bar =12.5 µm. **(2a and b):** Liver sections of the control mice group treated with M.T.O for 4 weeks (Gp2) expressing intense CD34 immunoreaction in endothelial cells of the blood sinusoids (arrows) and many hepatocytes (arrowheads) with the maintenance of normal architecture of liver hepatocytes, central vein (CV), portal vein (PV) and bile duct (BD). CD34 immunostain, Bar =25.0 & 12.5µm, respectively.



Figures (3 and 4): Liver section of fibrotic and cirrhotic mice injected with CCl_4 for four and six weeks, respectively; **(3):** A fibrotic liver section expressing a decrease of CD34 immunoreaction in endothelial cells of the blood sinusoids (arrows) and no CD34 immunostain in many lobules of the hepatic tissue (arrowheads). **(4):** A cirrhotic liver section showing a sharp decrease (arrows) to no CD34 expression in most lobules of the hepatic cirrhosis (arrowheads). CD34 immunostain, Bar =12.5 µm.



Figures (5 and 6): Liver sections of fibrosis tissues of mice administered with M.T.O for four weeks expressing approximately recovery normal moderate to strong CD34 immunoreactivity in endothelial cells of the blood sinusoids (arrows) and abundant appearance new oval cells (arrowheads) seeing intense to CD34 immunoreaction peripherally to the central vein (CV). PV is a portal vein.CD34 immunostain, Bar =25.0 & 12.5 µm, respectively.

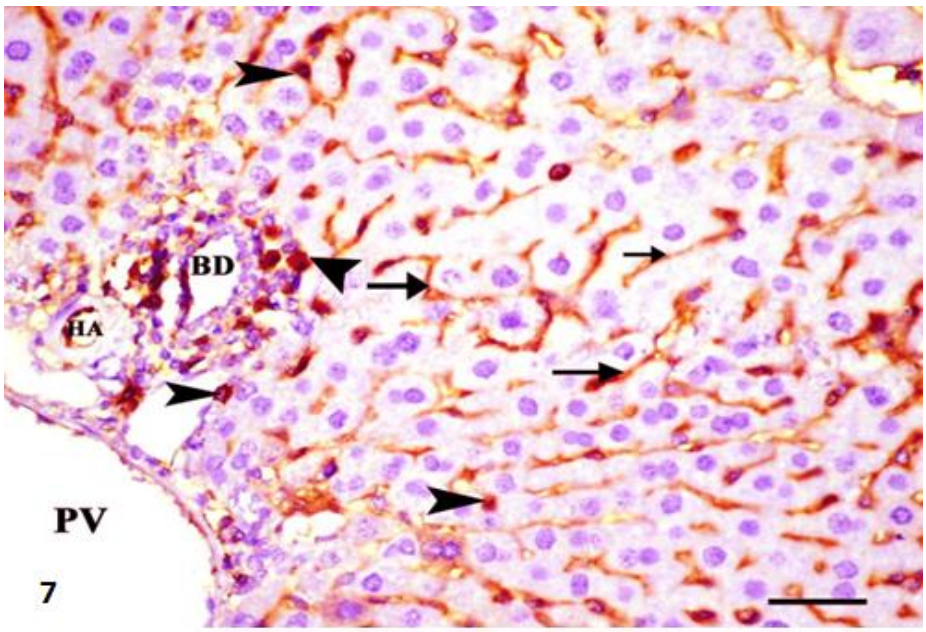


Figure (7): Section of the cirrhotic liver of a mouse given M.T.O at a dose of 1mL/kg/bw for 4 weeks showing the recovery of a moderate to strong immunostain to CD34 in the endothelia of the blood sinusoids (arrows**),** and appearance of a few new dense oval cells in the area of the portal vein (PV). HA is a hepatic arteriole, and BD is a bile duct**.** CD34 immunostain, Bar =12.5 µm.

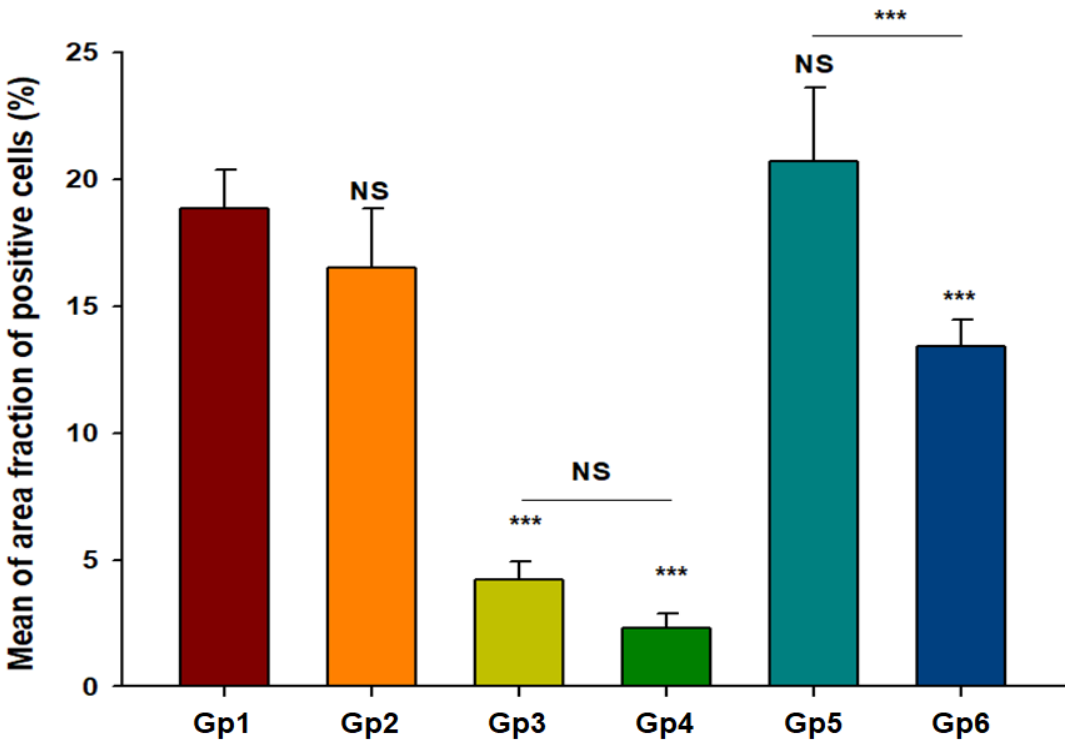
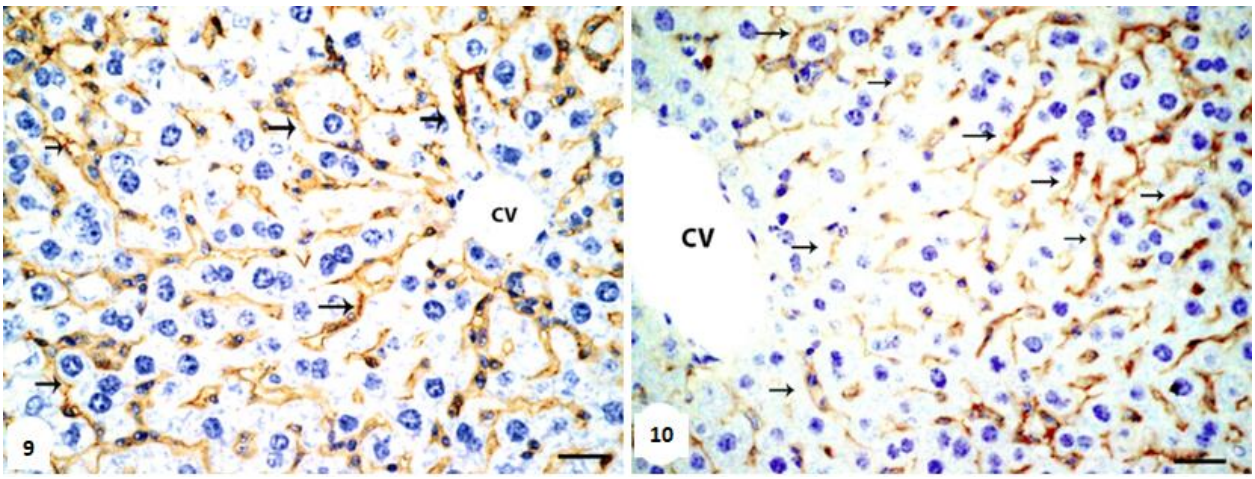
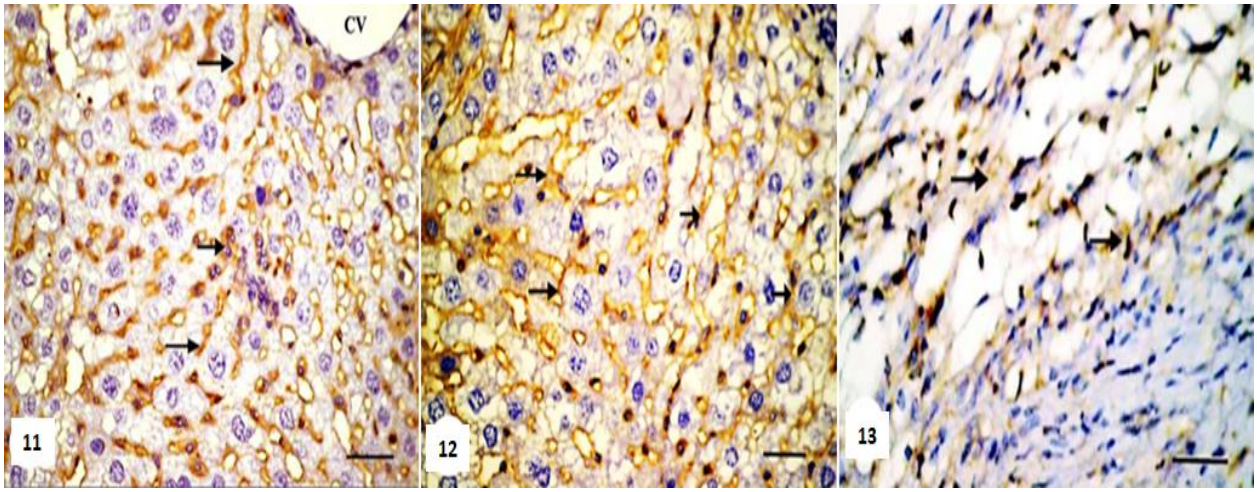


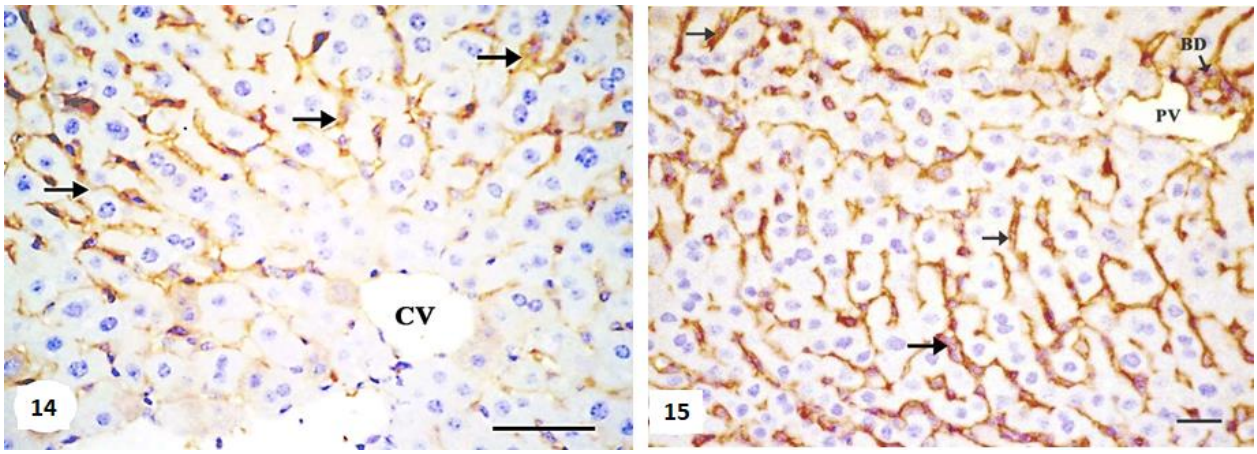
Figure (8): The mean of area fraction of positive cells (%) to CD34 of all groups. 5 replicas for each group, data were expressed as mean ±SE. Statistical significance was set at $p \leq 0.05$.



Figures (9 and 10): Liver sections showed control normal mice **(9)** or treated with M.T.O for four weeks **(10)** Liver sections showed normal weak to moderate vimentin immunoreaction in the endothelial cells and the connective tissue periphery to the wall of sinusoids (arrows). Vimentin immunostain, Bar =12.5 µm.



Figures (11-13): (11): Fibrotic liver section of a mouse injected with CCl_4 for four weeks revealing overexpression and intense vimentin in the endothelia of blood sinusoids and the connective tissue adjacent to the walls of the endothelia (arrows). Vimentin immunostain, Bar =12.5 µm. **(12):** Cirrhotic liver section of mice injected with CCl_4 for six weeks revealing overexpression and intense vimentin in the endothelia of blood sinusoids and the connective tissue adjacent to the walls of sinusoids (arrows) in many hepatic lobules; **(13):** while other lobules of cirrhotic liver section expressing no or weak vimentin immunoreaction. Vimentin immunostain, Bar =12.5 µm.



Figures (14 and 15): Liver Sections of fibrosis and cirrhosis of mice administered M.T.O expressing decrement of most hepatic vimentin density and seeing like normal image in the fibrotic treated group **(14)** more than in cirrhotic treated mice group **(15)**. Weak vimentin immunostain (arrows) is expressed in the fibrotic treatment group while vimentin in cirrhotic treatment is shown moderate to increase immunostain in endothelia and connective tissue peri-blood sinusoids walls (arrows). CV is a central vein, PV is a portal vein and BD is a bile duct. Vimentin immunostain, Bar =12.5 µm.

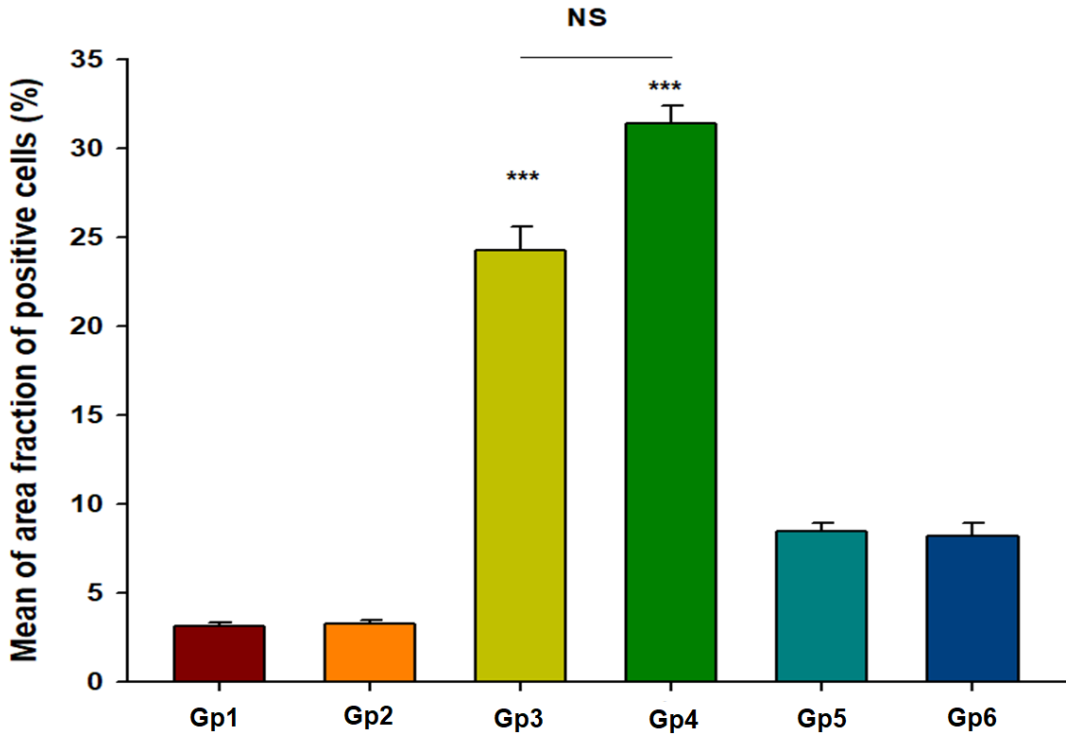


Figure (16): The mean of area fraction of positive cells (%) to vimentin of all groups. 5 replicas for each group, data were expressed as mean ±SE. Statistical significance was set at $p \leq 0.05$.

Discussion

In the present study, CD34 immunostain is expressed as a normal moderate to strong immuno-positive reaction to the endothelial cells of the blood sinusoids and many hepatocytes of the liver of the normal group. Similar results were observed in the control group that was treated with M.T.O for four weeks. The intensity of the immunoreactivity to CD34 was diminished in CCl_4-induced fibrotic or cirrhotic liver groups. The reduction of the CD34 immunostain was expressed in endothelial cells of the blood sinusoids and in many hepatocytes. Many hepatic lobules expressed with no CD34 immunostain. Whereas the livers of fibrotic or cirrhotic mice groups that were treated with M.T.O expressed a recovery of strong CD34 immunostain in most sinusoidal epithelia of the hepatic tissues and the appearance of numerous intense oval cells in the fibrotic liver more than in cirrhotic one.

Similar results were reported by [18] in the control and cirrhotic liver of mice. Administration of cirrhotic liver mice with green tea or moringa or a mixture of both improved the hepatic tissues and expressed strong CD34 immunostain in endothelia of blood sinusoids, and the appearance of many intense oval cells in the hepatic tissues [19]. Gligorijević et al., illustrated the link between sinusoidal capillarization or neo-vascularization, and the dedifferentiation of the liver tissue during cirrhosis was considered as being helped by CD34 immunostain [20].

CD34 immunostain was almost diminished in CCl_4-induced fibrotic or cirrhotic liver groups in the current study. The reduction of CD34 immunostain was also expressed in endothelial cells of the blood sinusoids and in many hepatocytes. Many hepatic

lobules expressed with no CD34 immunostain. It has been reported that HCC displayed significant positive for CD34 immunostaining in the endothelial cells [21; 22]. Researchers have shown that CD34 is expressed in chronic hepatitis at the level of progenitor oval cells [23]. Pusztaszeri et al**.,** reported that CD34 was expressed in the periportal area in normal human liver, but pathological conditions can change their phenotype and express this marker [24]. CD34 was positive in the blood sinusoids and restricted to the periportal area of the liver. [25] elucidated that immobilized-stressed rats for 5 and 30 days manifested an obvious decrement of CD34 expression in hepatocytes surrounding the central veins; these alterations were time-dependent.

Fibrotic or cirrhotic mice groups that were treated with M.T.O expressed strong CD34 immunostain in sinusoidal epithelia of the hepatic sections and the appearance of numerous intense oval cells in the fibrotic liver more than in cirrhotic one. The stimulatory effect of silymarin on the regeneration of liver tissue through boosting protein synthesis and DNA synthesis was linked to the increased expression of CD34-positive cells in rats after silymarin administration [26]. Almundarij et al., also came to similar conclusions that the combination of silymarin and bone marrow-derived mesenchymal stem cells can control the hepatotoxicity brought on by CCl4 in rats [27]. Silibinin, which is isolated from milk thistle, has chemo-preventive effects. It showed anti-angiogenic effects in human HCC cell lines by down-regulated CD34 [28]

Vimentin is an intermediate filament, found in the cytoplasm of mesenchymal cells [29] and one of the important cytoskeletal proteins for cell morphology, motility, and growth. However, later during hepatic fibrogenesis, the cellular responses are coordinated by vimentin-mediated cytoskeletal signaling transduction. Vimentin expressed in the liver sections of either normal or control mice as a normal weak to moderate immunoreactivity in the endothelial cells and the connective tissue periphery to the wall of the blood sinusoids, central and portal veins as well as in the cells of the portal tract. Where the fibrotic or cirrhotic liver of mice induced by CCl_4, revealed overexpression and increased vimentin immunoreaction *in situ***.** The protein expression of vimentin significantly enhanced many hepatic lobules compared with the control group. However, many hepatic lobules of cirrhotic liver expressed weak or no vimentin immunoreaction. The treatment with M.T.O to either the fibrotic or cirrhotic group expressed an improvement decline in vimentin protein filaments in most hepatic lobules, and the decrement in the fibrotic mice group was more than in the cirrhotic one.

The formation of liver fibrosis depends on HSCs. Migration and proliferation, which are considered the key events involved in hepatic fibrogenesis, are manifestations of HSC activation [30]. In the fibrotic liver of mice, the expression of vimentin was shown to be greatly enhanced**.** Increased vimentin expression has also been observed in a variety of tumor cells and tissues. Silibinin has a great *in vivo* capacity to block epithelial-to-mesenchymal transition (EMT). Since has been widely reported to inhibit mesenchymal markers like vimentin while boosting epithelial features as a means of combating EMT [31]. Moreover, the antiproliferative and apoptotic properties of silymarin resulted in a decrease in vimentin matrix metalloproteinase and muscle actin when used to treat various forms of cancer [32]. Newly, the transient receptor potential vanilloid 3 (TRPV3) significantly inhibited the of HSCs proliferation [33]

Conclusion

The treatment with M.T.O in mice with liver fibrosis or cirrhosis could play a role in the hepatic progenitor cells (CD34) differentiating into vimentin hepatocytes. Thus, M.T.O is recommended for patients with liver diseases.

Statements and Declarations

SOURCE OF FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

ETHICAL APPROVAL

The experimentation and animal care were performed and handled in compliance with the ethical guidelines approved by the animal care and use committee, Faculty of Science, Tanta University, Egypt (IACUC-SCI-TU-).

AUTHOR CONTRIBUTION

The authors confirm that all persons designated as authors qualify for authorship and have verified the article for plagiarism. If plagiarism is detected, all authors will be held equally responsible and will bear the resulting sanctions imposed by the journal thereafter. **S.O.** conceived and designed the study, and conducted research, **B.A**. provided research materials and collected and organized data, **M.B.** designed the study, analyzed and interpreted data, and wrote the initial and final drafts of the article, **R.H.** provided logistic support. **N.I.** conceived and designed the study, critically reviewed and approved the final draft, and are responsible for the content and similarity index of the manuscript. and accountable for all aspects of the work.

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