

Evaluation of *Plantago ovata* ethanolic extract on histopathological, immunohistochemical and biochemical indexes in the liver of BDL-induced cholestatic rats

Maedeh Rafiee

Islamic Azad University

Pejman Mortazavi (✉ Sp.mortazavi@srbiau.ac.ir)

Islamic Azad University

Ahmad Asghari

Islamic Azad University

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Abstract

Background and Purpose

Cholestasis is defined as inhibition of bile flow due to defective secretion of hepatocytes or obstruction of the bile flow pathway and accumulation of bile in liver cells and bile ducts and functional damage of the hepato-biliary system as decreased bile flow in canaliculi. This study investigated the effect of plant extract *Plantago ovata* on cholestasis-induced liver damage in rats of bile duct obstruction model.

Methods

In this study, extraction of *Plantago ovata* was performed. 48 male Wistar rats were divided into 8 groups (health control, BDL, treated with doses of 100, 200, and 400 mg/kg, BDL treated with doses of 100, 200, and 400 mg/kg) with 45 days treatment period (Once a day). After anesthesia and isolation of serum, biochemical ALT, ALP, AST, Total bilirubin, Total protein, and Albumin parameters were measured. Also, a histopathological and immunohistochemical study was performed by antibody. Data analysis was performed using the One Way ANOVA and Kruskal-Wallis test and the Prism statistical program ($P < 0.05$).

Results

The results showed that there was a significant increase in serum levels of ALT, ALP, AST, total bilirubin, and α -SMA expression in BDL groups, but the treatment of BDL groups with *Plantago ovata* could significantly reduce these factors in a dependent manner. Improve the dose. *Plantago ovata* also has a protective effect on the liver and the stability of liver cell membranes

Conclusion

Plantago ovata, in terms of histopathology, immunohistochemistry, and biochemistry, has a protective effect against liver damage caused by BDL and by regulating liver parameters can be useful in the treatment of cholestasis

1. Introduction

Cholestasis is defined as inhibition of bile flow due to defective secretion of hepatocytes or obstruction of the bile flow pathway and accumulation of bile in liver cells and bile ducts and functional damage of the hepato-biliary system as decreased bile flow in canaliculi. In time, this disease can cause liver fibrosis, liver cirrhosis, and eventually death (1).

Cholestasis of intrahepatic origin is caused by abnormal formation and secretion of bile in hepatocytes and has various causes; These include the use of certain medications (antibiotics such as rifampin,

glibenclamide, and cyclosporine), sex hormones (intrahepatic cholestasis in pregnancy), and the release of proinflammatory cytokines by hepatic Kupffer cells (infection, alcohol, medication, or Viral hepatitis), systemic disorders such as sarcoidosis and amyloidosis, autoimmune, metabolic and genetic diseases (mutations in carrier protein genes and cholestasis syndrome). In this type of cholestasis, the liver does not enlarge and the bile ducts inside the liver do not dilate (2, 3). While extrahepatic cholestasis occurs following bile duct obstruction in the bile ducts, bile duct stenosis in chronic pancreatitis, pancreatic and bile duct tumors, and gallbladder can cause jaundice, chronic inflammation, stress Oxidative, fibrosis, and cirrhosis of the liver (4).

In vivo models of liver fibrosis include chemical-based, diet-based, genetically modified, infectious, and surgical models (5). Bile duct ligation (BDL) is a surgical method used to induce liver fibrosis. BDL leads to an acute obstructive jaundice that progresses to cirrhosis with portal fibrosis (5). BDL in mice induces an increase in expression of pro-inflammatory cytokines such as TNF- α and IL-6 as well as in pro-fibrotic proteins such as collagen- α 1, MMP-2, and TIMP-1.60 Although K pffer cells are activated in livers of mice undergoing BDL,2 depletion of K pffer cells in these mice appears to increase the development of liver fibrosis, highlighting the role of K pffer cells and the synthesis of IL-6 in protection against this pathology (5). Obstruction of the bile duct by BDL increases bile pressure, mild inflammation, and secretion of cytokines by bile epithelial cells, thereby producing cholestasis. This leads to proliferation of bile epithelial cells, increased expression of fibrogenic markers including TIMP-1, α -SMA, collagen 1 and TGF- β 1, and accumulation of B cells and T cells in the portal area, causing ROS and liver damage (5).

Nowadays, using natural compounds and paying special attention to their healing properties, various studies and reports are available (6–9). *Plantago ovata* (2n = 8) belongs to the family Plantaginaceae, a large genus of plants and their sub-branches that are mostly distributed in temperate regions and a few in the tropics. This plant is composed of about 250 species and two important species of this genus *Plantago ovata* forsk and *Plantago psyllium*. Linn in Iran is called Asfarzeh (10). The shell of the economic part is that they are separated by a physical process. This shell contains colloidal mucilage (30%), which mainly consists of xylose, arabinose, galacturonic acid along with rhamnose and galactose, etc. The seeds also contain some oils and small amounts of acupuncture glycosides and tannins (11). *Plantago ovata* is traditionally used to treat constipation, diarrhea, hemorrhoids, irritable bowel syndrome, weight loss, obesity, high cholesterol, BDL, and diabetes, protecting the lining of the gastrointestinal tract (12).

The most important function of the liver is to detoxify various metabolites, synthesize proteins and produce the enzymes needed to digest food. Besides, it regulates red blood cells, metabolism, synthesis, and storage of glucose. Alanine transaminase (ALT) and aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), serum bilirubin, prothrombin time (PT), and international normalized ratio (INR) and Albumin are important indicators to be analyzed to diagnose liver damage (13, 14). On the other hand, in BDL, stellar cells gradually undergo a phenotypic change that results in the ability to proliferate and divide, losing large amounts of their fat and retinoid reserves and turning into myofibroblasts, which are high in α -SMA. They express. This process is called

transdifferentiation and is considered a key event in the pathobiology of fibrosis (13). Therefore, according to the contents of this article, we investigate the effect of plant extract *Plantago ovata* on cholestasis-induced liver damage in rats of bile duct obstruction model.

2. Materials And Method

2.1. Chemicals

Chloroform from Merck (Germany) and immunohistochemistry kit ABCAM, (USA) were purchased. The Pars Azmoun Company (Iran) was used to study changes in liver enzymes.

2.2. Extraction of the *Plantago ovata*

Three kg of *Plantago ovata* was purchased from perfumeri in Tehran, Iran. The plant experiments were performed in accordance with relevant guidelines and regulations and was sent to the herbarium laboratory of Razi Laboratory Complex of Islamic Azad University, Science and Research Branch to approval (Plant verification code: 12-67453). To prepare the ethanolic extract, *Plantago ovata* seeds were ground, resolved in 80% ethanol and then mixed for 72 hours on the basic shaker. Then, it was passed through a filter paper and placed in a rotary machine for 1.5 hours. Finally, the liquid was dried for 24 hours at 50 ° C and the resulting powder was mixed in a certain amount of distilled water as a solvent. This extract was used to treat animals (15).

2.3. Experimental Animals and Ethical aspects

In this study, 48 male Wistar rats (weighing 220–250 g) were purchased from the animal breeding center of Baqiyatallah Hospital in Tehran, Iran and transferred to the Veterinary Hospital. Animals care, and the experimental steps were performed in full accordance with the criteria of care and approved by institutional animals Medical Sciences Ethics Committee of Science and Research University, Tehran, Iran (Ethical code: IR.IAU.SRB.REC.1398.043). All methods were carried out in accordance with relevant guidelines and regulations and also all methods are reported in accordance with ARRIVE guidelines.. The number of rats was minimized as far as possible. Wistar rats were housed separately in cages and had free access to water and food. They were also kept under light / dark cycles (12.12 hours), constant temperature ($22 \pm 2^{\circ}\text{C}$), and humidity (55%). After 7 days of acclimatization, the Wistar rats were randomly divided into eight groups ($n = 6$).

2.4. Study design

The rats were randomly divided into eight groups of six animals each as follows:

Group 1: healthy control group without BDL surgery and treatment

Group (2–4): three healthy experimental groups plus *Plantago ovata* groups: rats underwent laparotomy without BDL, treated with *Plantago ovata* at dose levels of 100, 200 and 400 mg/kg b.w., respectively

Group (5) The BDL group: rats with BDL and treated with distilled water

Group (6–8) The BDL plus *Plantago ovata* groups: rats with BDL and treated with *Plantago ovata* at dose levels of 100, 200 and 400 mg/kg b.w., respectively via intragastric gavage for 45 consecutive days.

The *plantago ovata* doses were selected based on previous studies (16).

2.5. Bile Duct Ligation

BDL surgery was performed according to the standard protocols as follows (17):

I) Deep anesthesia was performed by intraperitoneal administration of intraperitoneal (IP) ketamine (90 mg/kg body weight) and xylazine (10 mg/kg body weight), then placed on the dorsal surface in the dissection tray. After shaving the hair in the abdomen, the skin of the abdomen was disinfected with 70% alcohol. II) After disinfection, a 3 cm longitudinal incision was made in the abdominal area using a scalpel blade 11 in the midline of the abdomen, and the skin and muscles of the abdominal wall were opened in two stages. III) The liver was then tilted slightly upwards, and after identifying the common bile duct, a pair of pliers was placed under it. The common bile duct was sutured and blocked by 4 – 0 silk sutures in two places (after the end of the intrahepatic bile duct and before the pancreatic duct) and then the space between the two sites was cut. IV) 3 µl of 10% normal saline solution was poured into the abdominal cavity, the muscle was closed with 4–4 absorbable vicryl sutures and the skin was closed with 3 – 0 non-absorbable nylon sutures. V) Oxytetracycline spray was used to prevent suturing of rats. After completion, the surgical site was disinfected with betadine. 2–3 days after BDL, the animals' urine and ears turned yellow, indicating that the surgery was successful. All animals survived the experiment.

2.6. Rats Treatment

Three days after BDL, the urine and ears color of the animals turned to yellow, which was a sign that the surgery was successful. From a day after the surgery, the treatment was started via intragastric gavage for 45 consecutive days (once per day). At the end of the treatment period and after 12 hours of fasting, the animals were euthanized with chloroform based in ethical principles. Blood samples were taken directly from their hearts for serum analysis. Also, part of the liver tissue was fixed in 10% formalin buffer solution for histopathology and immunohistochemical studies (18).

2.7. Serum analysis

At the end of 45 days from the time of the experiment, the animals fasted for 12 hours. They were then killed by inhaling chloroform-impregnated cotton. Immediately, the animals' chests were incised and, after the heart appeared, blood was drawn directly from inside the heart using a 5 ml syringe. After a 30-minute pause (for clotting), the blood samples were placed in a centrifuge at 2000 rpm for 5 minutes and stored at -20°C. The separated serum was used for estimation of some biochemical parameters like Serum ALT, ALP, AST, Total bilirubin, Total protein, and Albumin using relevant dedicated kit from Pars Azmoun Company (Iran).

2.8. Histological examination

At the end of the 45-day treatment period, the liver of mice in each group was isolated. The liver was dissected and fixed in 10% neutral buffered formalin solution for 24 h. The fixed tissues were processed routinely and were then embedded in paraffin, sectioned to 5 mm thicknesses, for pathological analysis, paraffin sections were stained with hematoxylin and eosin and examined by conventional light microscopy. Pathology scoring method include the Necrosis: [Lack of change: 0, Central (focal) necrosis in less than 25% of tissue: 1, Central necrosis in 25–50% of tissue: 2, Extensive but still focal and central necrosis: 3, Global necrosis: 4]. Inflammation: [Lack of inflammation: 0, Focal inflammation in less than 25% of tissues: 1, Focal inflammation in 25–50% of tissues: 2, Extensive but still focal inflammation: 3, Global inflammation: 4]. Fibrosis: [Lack of fibrosis: 0, Expansion of fibrosis portal: 1, Septum formation: 2, Prominent bridge fibrosis: 3, Cirrhosis: 4]. Ductal hyperplasia: [Lack of hyperplasia: 0, Hyperplasia in less than 25% of each liver lobule: 1, Hyperplasia in 25–50% of each liver lobe: 2, Extensive but still focal and central hyperplasia: 3, Total hyperplasia: 4].

2.9. Immunohistochemistry (IHC)

Immunohistochemistry is a method to show the presence and location of proteins in tissue sections and makes it possible to observe processes in the context of intact tissue. The immunohistochemical study was performed by obtaining 3 mm thick sections from paraffin-embedded tissue blocks and placing them on a silanized glass slide. The sections were deparaffinized, then washed in PBS and submitted to antigen retrieval. After treatment with normal serum, the sections were incubated with the anti α -SMA antibodies in a moist chamber at room temperature. Peroxidase activity was visualized by immersing the tissue sections in diaminobenzidine, which resulted in a brown reaction product. Finally, the sections were counterstained with Harris hematoxylin and coverslipped. Sections of human breast carcinoma were used as positive controls for the anti- α -SMA antibody, and sections of hepatic tissue without primary antibody were used for negative control. Immunohistochemical analysis was performed with an Olympus light microscope. Tissue sections were examined under light microscopy (100X magnification) to identify areas containing the largest number of immunoreactive cells, and the selected microscopic field were then analyzed at 400X magnification. The following aspects were considered in evaluating the α -SMA immunostaining: Presence (+) or absence (-) of immunostaining, type of immunopositive cells and their tissue distribution (focal or diffuse). Expression of α -SMA in hepatic tissues was evaluated on the base of the percentage of positive cells and classified as follows:

Zero-negative, α -SMA expression in less than 3% of cells

Grade 1, low, α -SMA expression in 3–33% of cells

Grade 2, moderate, α -SMA expression in 34–66% of cells

Grade 3, severe, α -SMA expression in more than 66% of cells

2.10. Statistical Analysis

All data reported as mean $\pm$ SD and analyzed using statistical variances (ANOVA) followed by post- Tukey post hoc test and graphs were plotted using Kruskal Wallis. P-value less than 0.05 was considered as a significant difference.

3. Results

3.1. Estimation of enzyme activity

In the study of AST, ALT and ALP indices, it was observed that the BDL group had a statistically significant increase of $P < 0.001$ compared to the control group. On the other hand, in both serum indices, BDL groups treated with *Plantago ovata* 100, 200, and 400 mg/kg compared to the BDL group showed a statistically significant decrease in the secretion of $P < 0.001$, which was the largest decrease in the dose of 400 mg/kg. Healthy and treated groups with doses of 100, 200 and 400 mg/kg *Plantago ovata* compared to BDL group also reported a statistically significant decrease of $P < 0.001$ (Fig. 1a, b and f). In the study of Total bilirubin index, the BDL group compared to the control group showed a statistically significant increase $P < 0.001$. A statistically significant decrease of $P < 0.001$ secretions of this index were observed only in the BDL group treated with a dose of 400 mg/kg of *Plantago ovata*. Healthy and treated groups with doses of 100, 200, and 400 mg/kg of *Plantago ovata* also showed a statistically significant decrease of $P < 0.001$ compared to BDL group (Fig. 1c). Total protein and Albumin indices did not show statistically significant changes in comparison with the studied groups (Fig. 1d and e).

3.2. Histopathology (Masson's Trichrome staining)

According to the results reported in (Table 1), after histopathological examination of liver tissues, lesions observed in the cholestasis group included hepatocyte necrosis and infiltration of inflammatory cells such as lymphocytes, bile duct hyperplasia, the formation of false bile ducts, and connective tissue infiltration. Necrotic and degenerated hepatocytes and the formation of defective liver lobules varied in degree and severity.

Table 1

Ranking of histopathological indicators; to convert qualitative data to quantitative data and use in Kruskal-Wallis test of statistical program.

Indicator		Point	Control	BDL	BDL 100	BDL 200	BDL 400	100	200	400
Necrosis	No necrosis	0	0	2	0	0	0	2	2	1
	Local damage less than 50%	1								
	Localized between 50 - 25%	2								
	Extensive but local	3								
	Extensive necrosis	4								
Infiltration of inflammatory cells	No inflammation	0	0	3	0	0	0	2	1	1
	Focal point less than 25%	1								
	Focal point between 50 - 25%	2								
	Extensive but local	3								
	Extensive inflammation	4								
Connective tissue	No connective tissue	0	0	3	0	0	0	2	2	2
	Increase collagen without walls	1								
	Incomplete wall	2								
	Full and thin wall	3								
	Full but thick wall	4								

Indicator		Point	Control	BDL	BDL 100	BDL 200	BDL 400	100	200	400
Bile duct hyperplasia	Lack of hyperplasia	0	0	3	0	0	0	3	1	1
	Less than 25% of each lobule	1								
	25–50% per lobule	2								
	Extensive but local	3								
	Wide	4								
Total			0	11	0	0	0	9	6	5

The results of Kruskal-Wallis test for histopathological indices reported that there were significant differences in different groups according to the significant values of histopathological indices. In addition to statistical analysis, the score obtained in each parameter also showed a significant difference in liver slides of different groups (Table 2).

Table 2
Kruskal-Wallis test results for histopathological indices

	Test statistics Chi-square	Degrees of freedom	Significant amount
Inflammation	33	7	0
Bile duct hyperplasia	33	7	0
Necrosis	33	7	0
Fibrosis	33	7	0

According to the results obtained from the study of liver tissue pathology of the studied groups, no significant tissue changes were observed in the livers of control healthy rats. The liver of this group of rats was healthy and did not have any special lesions. The entire structure of the liver was clearly identifiable. Hepatocytes, central arteries and even liver lobules can be detected (Fig. 2a). *Plantago ovata* treatment at doses of 100 mg/kg (Fig. 2b), 200 mg/kg (Fig. 2c), and 400 mg/kg (Fig. 2d) body weight did not cause any significant changes in the liver of animals compared to the healthy group. In this group, no necrotic changes were seen along with germinal nodules or fibrous bands that extend around the liver parenchyma, and the histopathological appearance of tissue samples due to the treatment of these doses was quite similar to the healthy control group. As seen in the (Fig. 2e), in the BDL group with *Plantago ovata* treatment at dose of 100 mg/kg severe histological changes were observed in the liver of cholestatic rats. Major lesions include bridge fibrosis, which can be detected by the deposition of collagen

(connective tissue) as thin walls. Bile duct necrosis and hyperplasia and infiltration of mononuclear inflammatory cells (lymphocytes) were also observed. These changes were seen in almost all lobules, but were more widespread in the central lobular regions. In BDL rats treated with *Plantago ovata* 200 mg/kg (Fig. 2f) and 400 mg/kg (Fig. 2g) body weight; respectively mild bile duct hyperplasia and infiltration of mononuclear inflammatory cells and mild bile duct hyperplasia and poor connective tissue infiltration seen as incomplete walls are seen.

Immunohistochemical staining

The α -SMA is normally found in myocytes and muscle cells around arteries. In cholestasis groups, in addition to these parts, it is seen in myofibroblasts in fibrous areas. The positive criterion is the browning of the cell nucleus.

According to the α -SAM expression statistical analysis results from immunohistochemical staining, α -SAM expression was evaluated in different areas of the hepatic tissues. (Table 3) shows the changes in the α -SAM index in different groups.

Table 3
The ranking of immunohistochemical indices α -SAM in different groups tested is as follows

Name	Control	BDL	BDL 100	BDL 200	BDL 400	100	200	400
α -SAM	0	3	3	2	2	0	0	0

In control healthy group α -SMA expression is positive only around arteries and sinusoidal spaces (Fig. 3a). Expression in the BDL group, in addition to around blood vessels and sinusoidal spaces (which is also positive in natural tissue), was positive in 90% of fibrous areas and around the false bile ducts (Fig. 3b). *Plantago ovata* treatment at doses of 100 mg/kg; (Fig. 3c), 200 mg/kg (Fig. 3d) and 400 mg/kg (Fig. 3e) α -SMA expression was positive only around arteries and sinusoidal spaces. α -SAM expression in BDL group with *Plantago ovata* treatment at dose of 100 mg/kg (Fig. 3f), 200 mg/kg (Fig. 3g) and 400 mg/kg (Fig. 3h) body weight, in addition to the areas around blood vessels and sinusoidal spaces that are also positive in normal tissue, in areas Fibrosis in and around the false bile ducts was also positive. However, the amount of α -SMA expression was positive in 80% of tissues in the group treated with *Plantago ovata* 100, positive in 70% of tissues in the group treated with *Plantago ovata* 200, and in 50% of tissues in the group treated with *Plantago ovata* 400.

Discussion

Among liver diseases, liver fibrosis is the most common disease. In response to the damaging agent, the accumulation of phenotypically activated fibroblasts causes excessive production of extracellular matrix (ECM) components, which can disrupt the structure and function of the organ (21). In this study, cholestasis and subsequent liver fibrosis were induced in rats by BDL surgery to evaluate the effect of *Plantago ovata* extract on the expression of α -SMA and AST, ALT, ALP, Albumin, Total protein, and Total bilirubin biochemical parameters changes in the liver.

Cholestasis refers to the inhibition or decrease in bile secretion and flow. Extrahepatic cholestasis or obstructive cholestasis means obstruction outside the liver, which is clinically associated with the bile ducts. Cholestasis leads to the retention of bile compounds in the blood. The two main constituents of bile are bilirubin and bile acids. Therefore, biochemically, cholestasis is characterized by an increase in ALP, and also an increase in ALT and AST enzymes is evident. Histologically, bilirubin retention in hepatocytes, bile ducts, or causes bilirubinostasis, which is clinically manifested as jaundice. Histologically, intrahepatic cholestasis generally indicates the presence of bile within hepatocytes and bile ducts with a pattern of diffuse cholestatic damage. In obstructive cholestatic patterns, histopathology shows bile duct obstruction between lobules, portal dilatation, and bile duct hyperplasia, which is mainly a central lobular pattern of cholestatic damage (3).

Since various studies aimed at investigating the effect of various compounds including Spring bark extract (22), Moringa Oliveira seed extract (6), Vitex negundo extract (23) was performed on BDL mice and the results showed that not only natural compounds can significantly reduce the serum level of AST, ALP, ALT, Total bilirubin but also by increasing the stability of cell membrane they prevent liver damage and failure. In this study, we also used the *Plantago ovata* extract and in line with the results of these researchers, we were able to provide successful reports on the beneficial effects of this plant on controlling liver damage in BDL mice.

Plantago ovata plant is rich in bioactive compounds and different primary and secondary metabolites such as polyphenols, fatty acids, amino acids, flavonoids (polyphenols and flavonoids possess a prominent antioxidant activity), alkaloids, terpenoids, and vitamin C. These compounds have good antioxidant and anti-inflammatory effects. In addition to these effects, *Plantago ovata* is a good source of carbohydrates and nutrients. This plant has also been approved as a laxative, anti-cholesterol, hypoglycemic, anti-inflammatory, anti-viral, analgesic, antioxidant, anti-cancer, immune modulator, and anti-hypertensive drug. It has traditionally been used to treat many ailments including colds, hepatitis, skin diseases, infectious diseases, gastrointestinal, respiratory, reproductive, gastric ulcer treatment, and circulatory problems (12, 24–26).

There have been specialized studies on the effects of *Plantago ovata* on liver disease, some of them are mentioned. One valuable study of *Plantago ovata* was the dramatic reduction in plasma fats by altering the metabolism of hepatic liver and bile acids (27). The rise of a wide range of nonalcoholic fatty liver (NAFLD) diseases, ranging from simple liver inflammation to steatosis, has become a serious concern today, eventually leading to fibrosis and cirrhosis. Therefore, studies have been able to report the successful effects of *Plantago ovata* through the regulation of liver enzymes in the treatment of this disease (28). Due to its antioxidant properties, the *Plantago ovata* extract may have a hepatoprotective effect and improve liver enzymes AST, ALP, ALT, Albumin as well as lipid profile in patients with non-alcoholic fatty liver (29). *Plantago ovata* acted as a safe agent for the treatment of ketoprofen-induced hepatotoxicity in rats and was recognized as a therapeutic insight into the carbohydrates and oils of *Plantago ovata* seeds. The antitoxic effects of this plant are related to the specific settings of liver enzymes to control toxicity (24). This plant was also able to prove its hepatoprotective effects against

liver damage caused by toxicity of carbon tetrachloride in rats. Treatment of mice with *Plantago ovata* significantly reduced the toxic effects of carbon tetrachloride. The presented data show the potential hepatic protective effect of this extract compared to the standard. This effect can be attributed to the antioxidant and anti-inflammatory effects of *Plantago ovata* extract (30). In agreement with the results of this study, a previous study on the therapeutic effect of *Plantago ovata* seeds against ketoprofen-induced hepatotoxicity in rats showed that the use of this plant with high concentrations of antioxidant compounds, improved indicators of liver damage, including the penetration of inflammatory cells, fibrosis, necrosis and also regenerated liver cells and stabilized their membranes (24). Also in another study on diabetic rats, it was shown that *Plantago ovata* has an antioxidant effect due to the phenolic compounds of the plant, which led to a reduction in oxidative stress (31). In other research about the anti-ulcer and hepatoprotective effects of aqueous extract of *Plantago ovata* on indomethacin-induced rats, which resulted in a reduction of microscopic and macroscopic wounds in treated animals (32). In this study, serum level was assessed in the different groups for AST, ALP, ALT, Albumin, Total protein, and Total bilirubin, which are widely used biomarkers to assess liver functions.

Bile duct obstruction (BDL) surgery to induce fibrosis in mice, changes stimulate morphological and time-dependent structure in the liver, including changes in liver cell damage and elevated serum enzyme levels during the first few days of surgery, to the severe inflammatory response in the liver after 5–7 days, and advanced hepatic fibrosis. It will be common three to four weeks after surgery and bile duct closure (33). It has been suggested that the surgical method be a model that is closer to the human condition and causes minimal cholestasis by producing minimal tissue damage and does not continue to chronic cholestasis (34). Therefore, in our study we used this method to induce cholestasis, and following the successful results of previous researchers, we were able to cause this disease in animals with minimal damage.

As we know, ALT, ALP and AST markers are naturally located in the cytoplasm, but in liver damage, following the accumulation of bile salts, oxidative stress, and damage to liver cells, they leave the pores of hepatocytes and enter the bloodstream, and its serum level increases, followed by a total increase in Total bilirubin (18). In several studies designed to induce inflammatory liver damage and fibrosis by obstructive cholestasis and measurement of levels ALT, ALP and AST, which are good serum markers of liver damage, their results showed that during 7 and 20 days after BDL increased significant increases occur in these markers, and consequently, the serum concentration of Total bilirubin increases steadily. Serum AST and ALT levels in the first week after BDL surgery can also be increased 5 or 10 times above normal levels (33, 34). In our study, in line with the results of previous researchers, a significant increase in the level of liver indices AST, ALP, ALT and Total bilirubin were observed in the serum of BDL groups.

The *Plantago ovata* effect on the improvement of non-alcoholic fatty liver in male rats showed that the aqueous extract of it due to its strong antioxidant properties can increase the serum concentration and profile of liver enzymes. Improve lipids in male rats and it has also been shown that a dose of 400 mg/kg has the greatest effect and the most effective dose is recommended for treatment. In other words, this aqueous extract with a concentration of 400 mg/kg has an effective role in reducing the serum levels of

ALT, AST, and ALP compared to other doses. *Plantago ovata* reduces the rate of liver damage and bile duct damage by preventing the direct discharge of bile into the lymph and blood and reduces the serum bilirubin level in BDL rats (29). Serum Total bilirubin levels in BDL rats increased significantly compared to the control group. As a result of obstruction of the bile ducts inside and outside the liver, conjugated bilirubin returns to the bloodstream and mainly plasma conjugated Bilirubin increases. BDL significantly increases Total bilirubin levels (34). According to previous researchers, our results showed an increase in ALT, ALP, AST, and Total bilirubin indices in BDL groups compared to the control group and on the other hand showed a significant decrease in the secretion of these indices in treatment groups compared to the BDL group, especially at a dose of 400 mg/kg. We also showed that increasing the therapeutic dose reduces liver tissue damage in cholestatic mice and also improves serum enzyme levels in liver enzymes. Other indicators studied in this study were Albumin and Total protein. Albumin is the most abundant protein in plasma that is synthesized by the liver and with a half-life of 20 days, is the best indicator of liver function and its serum level at any time indicates the rate of its synthesis, breakdown, and distribution. Serum Albumin levels are normal in acute liver disease but also in liver diseases such as cirrhosis and injury following reduced hepatic synthesis capacity. Decreased Albumin concentrations may be due to changes in protein synthesis or metabolism, and decreased protein levels in BDL may be due to decreased hepatocyte counts. Decreased Albumin levels usually follow the severity of the disease and are a reliable indicator of the prognosis of the disease. Significant improvement in the level of biochemical parameters indicates the beneficial effects of the selected plant extract (23). While in the present study we did not observe significant changes in Albumin and Total protein secretion in the *Plantago ovata* treated groups. This result was since the treatment period of BDL animals with *Plantago ovata* was 45 days and cholestasis damage in the treated animals did not reach cirrhosis, so we did not see any changes in these two indicators.

The results of a comparison of two types of cholestasis (BDL-induced cholestasis and Ethinyl estradiol-induced cholestasis) for canaliculus enzymatic changes showed that changes in canaliculus membrane proteins may play a role in the pathogenesis of cholestasis. This study was performed on three groups of male Sprague-Dawley rats.. In both species, cholestasis was induced in rats after 4–5 days. The abnormal structure and histochemical appearance of bile duct membranes suggest that changes in their composition, enzyme content, and protein turnover may be involved in cholestasis. Morphological features of cholestasis and bile duct hyperplasia were associated. Liver slices were stained with hematoxylin and eosin. Microscopic observation of rats receiving Ethinyl estradiol appeared normal and was similar to sections of mice treated with propylene glycol. However, liver sections obtained from mice undergoing BDL surgery showed the infiltration of inflammatory cells into the portal triad, bile duct hyperplasia, and parenchymal cell necrosis, which is consistent with the results of the present study in the untreated BDL group (35).

A group of researchers conducted a study to induce inflammatory liver damage and fibrosis by obstructive cholestasis. BDL surgery was performed on 40 male mice and the results were reported as follows. Persinusoidal fibrosis developed on day 10 postoperatively. The progression of liver fibrosis was assessed using a scoring system, and in animals that had been BDL, the fibrosis score steadily increased.

In all studied animals, perinosomoid fibrosis was visible after two weeks. Liver of animals undergoing sham surgery showed no signs of fibrosis, but the liver of animals undergoing BDL surgery had an irregular surface structure with the formation of edema and fibrotic nodules on the surface of the liver (33). In the present study, we also observed complete fibrosis after 45 days of completion of the experiment in the BDL group without treatment, which is consistent with the reports provided

Myofibroblasts appear to be the main cells in the process of fibrogenesis in all chronic liver diseases that produce new proteins, such as α -SMA, when activated in liver damage (36). Stellate cells (HSC) play a major role in liver fibrosis, which changes phenotype during liver injury and becomes myofibroblastic cells, which are activated during this deformation and express α -SMA (5). The time-dependent increase in intrahepatic collagen expression and its deposition is progressing as a result of fibrogenesis. Similarly, in fibrogenesis, high expression α -SMA, which represents fibroblasts (active hepatic stellate cells) and portal myofibroblasts, and hepatic hydroxyproline is significant (34). Studies have shown that a dose-dependent oral treatment protects the liver of adult rats against cholestasis by increasing the antioxidant capacity of the liver, inhibiting HSC activation, and inhibiting fibrotic signals. The dose-dependent effect of this plant was confirmed due to its antioxidant and anti-inflammatory properties in improving inflammation, bile duct hyperplasia, necrosis, and fibrosis. Also, this plant, due to its phenolic compounds and its antioxidant effect, has a protective effect on the liver and maintains the stability of hepatocyte membranes, and subsequently improves the increased serum indices during BDL. Among the doses used for asparagus, dose 100 mg/kg had no effect, and doses 200 and 400 mg/kg significantly reduced α -SMA expression in BDL rats and prevented liver fibrosis. Decreased expression of α -SMA in spheres-treated BDL groups indicates a decrease in oxidative stress in liver tissue, a decrease in stellar cell stimulation, and thus a reduction in the differentiation of these cells into myofibroblasts (37).

In the present study, the expression of fibrogenic factor α -SMA (alpha-smooth muscle actin) was positive in the control group and control groups treated with different doses of *Plantago ovata*, only around blood vessels and sinusoidal spaces. While α -SMA expression in the BDL group, in addition to these areas, was also positive in 90% of fibrous areas and around false bile ducts. In this section, due to the lack of expression of fibrosis markers by *Plantago ovata*, we evaluate the effect of other plant or chemical extracts on the expression of this marker. The most important cells involved in fibrosis are myofibroblasts, which are phenotypically modified fibroblasts that express the expression of contractile proteins such as α -SMA and can produce large amounts of matrix protein when activated. The α -SMA is a fibrogenic factor that accumulates in the extracellular matrix, especially in the portal area, and is responsible for the distribution of collagen in these areas. It is also a marker of myofibroblasts that increase its expression in tissue, indicating an increase in the differentiation of HSC cells into myofibroblasts. In normal liver tissue α -SMA is expressed in myocytes, muscle cells, around blood vessels, and in sinusoidal spaces. In the cholestatic liver α -SMA is also found in myofibroblasts and fibrous areas. Treatment of rats with asparagus improved histopathological indices compared to the BDL group but in the case of fibrosis, different doses had the same effect. *Plantago ovata* reduces oxidative stress and improves liver damage due to its antioxidant properties, it may be possible to prove a dose-dependent effect by increasing the

duration of treatment or increasing the dose. *Plantago ovata* could likely be used as an antifibrotic supplement for patients with cholestatic liver disease in the future.

Conclusion

According to the findings of this study, it can be concluded that *Plantago ovata*, in terms of histopathology, immunohistochemistry, and biochemistry, has a protective effect against liver damage caused by BDL and by regulating liver parameters can be useful in the treatment of cholestasis. Also, due to its phenolic compounds, this plant maintained the stability of hepatocyte membranes and subsequently improved serum indices increased during BDL. Among the doses used for *Plantago ovata*, dose 100 had no effect, and doses 200 and 400 significantly reduced a-SMA expression in BDL rats and prevented liver fibrosis.

Declarations

Ethics approval and consent to participate

There are no “human subjects” in this study

Consent for publication

Not applicable

Availability of data and materials

All data were analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

M.R., and P.M. developed the idea and designed the experiments. M.R., P.M., and A.A. conducted the experiments. A.A., and P.M. analyzed the data. M.R. wrote the manuscript. All authors confirmed the final manuscript before the submission.

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Figures

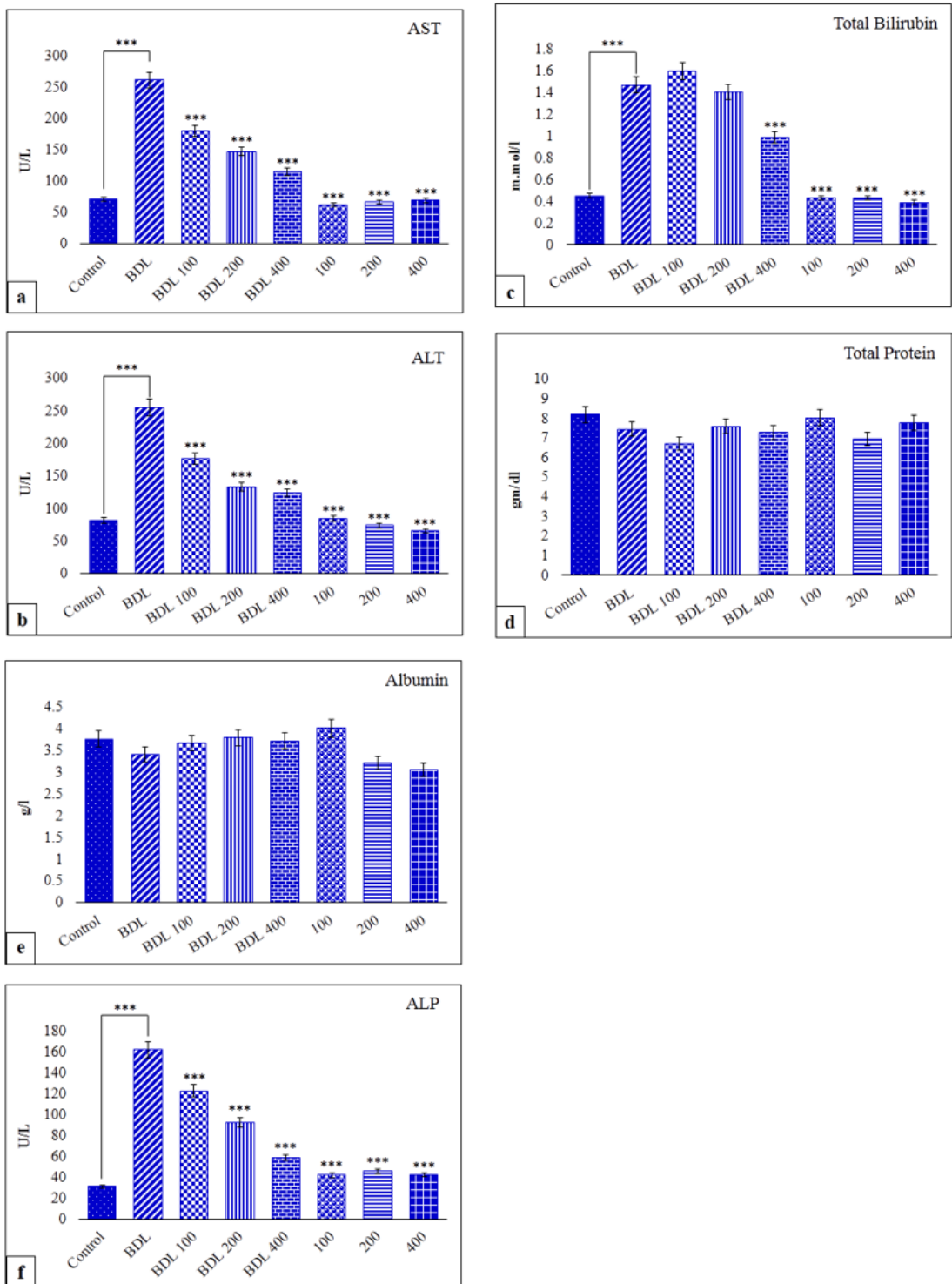


Figure 1

Comparison of liver indices in control group and *Plantago ovata* treated in Wistar rats. Mean and standard deviation (mean $\pm$ SD) of AST (a), ALT (b), Total bilirubin (c), Total protein (d), Albumin (e) and ALP (f) in serum of different enzymes after 45 days. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ show significant differences.

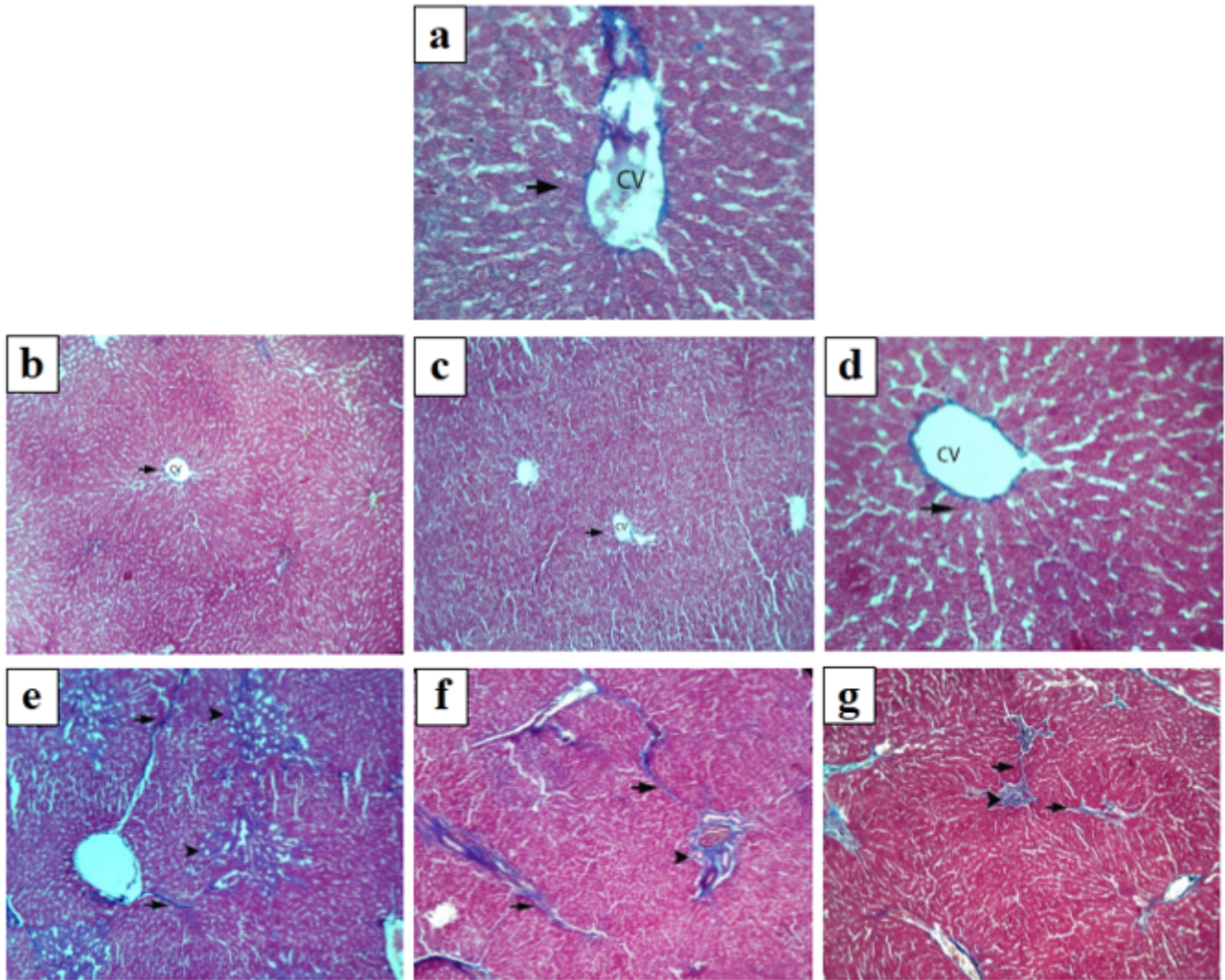


Figure 2

Representative H&E liver histology pictures of rats following respective treatments. Control healthy group; Central vein (CV) and healthy hepatocytes (arrowhead) (a), *Plantago ovata* treatment at doses of 100 mg/kg; Central vein (CV) and healthy hepatocytes (b), 200 mg/kg; Central vein (CV) and surrounding healthy hepatocytes and portal space (arrow) (c), and 400 mg/kg; Central vein (CV) and surrounding healthy hepatocytes and portal space (arrow) (d). BDL group with *Plantago ovata* treatment at dose of 100 mg/kg; Bile duct hyperplasia (arrowhead) and penetration of connective tissue into thin walls (arrow) (e), 200 mg/kg; Mild bile duct hyperplasia (arrowhead) and infiltration of mononuclear inflammatory cells (arrow) (f), and 400 mg/kg; Mild bile duct hyperplasia (arrow tip) and slight penetration of connective tissue as incomplete walls (arrow) (g). (Magnification X400).

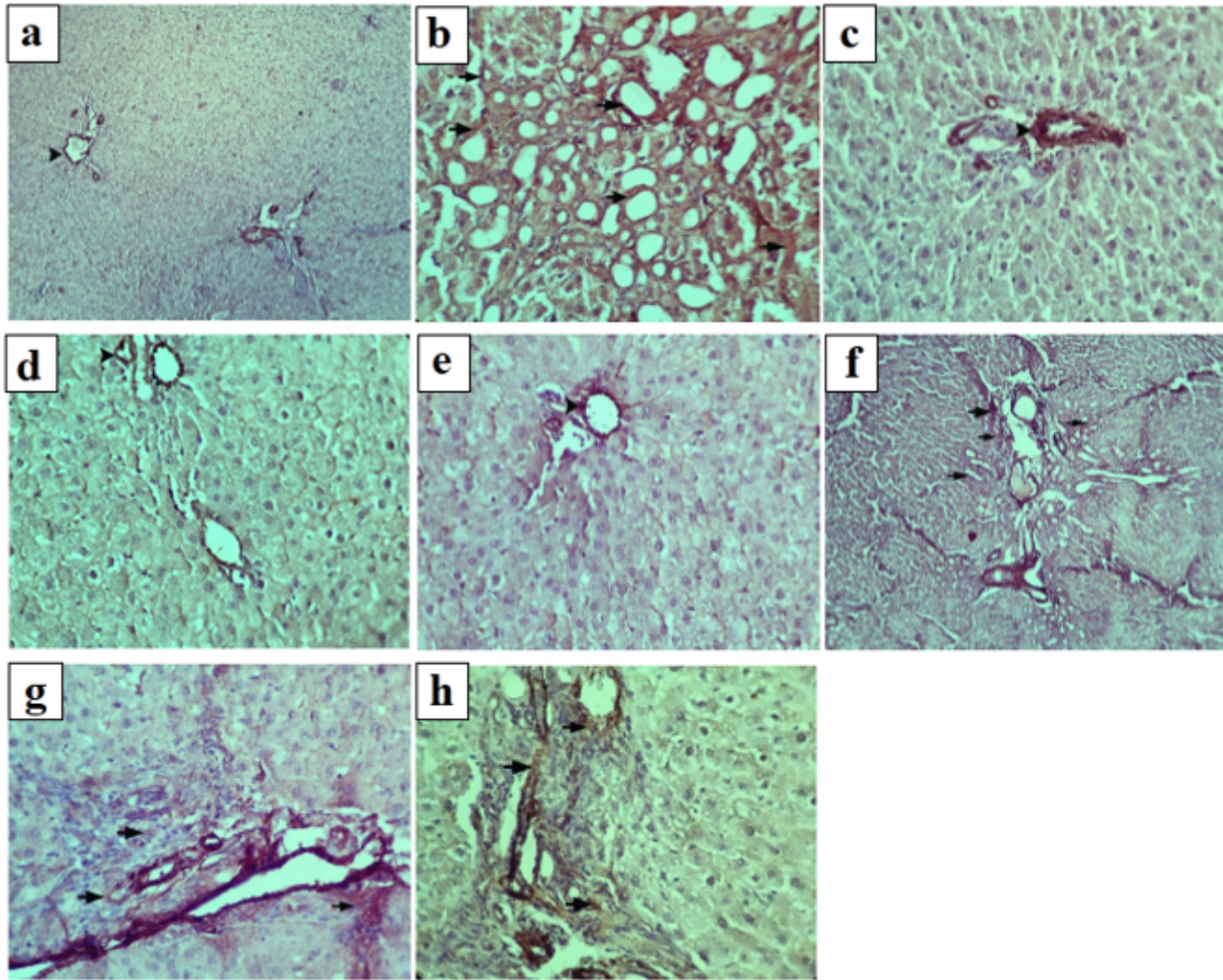


Figure 3

Expression of α -SMA in liver tissue sections in different study groups (immunohistochemical staining (IHC). Control healthy group that α -SMA expression was negative; arteries (arrowhead) (a), BDL group severe expression of α -SMA; In hepatocytes of false bile ducts (arrow) (b), *Plantago ovata* treatment at doses of 100, 200 and 400mg/kg; Slight expression around the arteries (arrowhead) (c, d, and e), BDL group with *Plantago ovata* treatment at dose of 100 mg/kg; Severe expression of α -SMA In fibrous areas around the false bile ducts (arrow) (f), 200 mg/kg; Significant expression of α -SMA in fibrous areas and around false bile ducts (arrow) (g), and 400 mg/kg; Significant expression of α -SMA in fibrous areas around false bile ducts (arrow) (g). (Magnification X400).