

Original Research Article

The effect of *Vitex agnus-castus* hydroalcoholic extract on histopathological changes and *TGF-β* expression in cholestasis-induced liver damage in a bile duct obstruction model in rats

Haleh Piroozmand¹, Pejman Mortazavi^{2,*}, Bitā Vazir³, Reza Hosseiniara⁴

¹Faculty of Veterinary Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran

²Department of Pathology, Faculty of Specialized Veterinary, Science and Research Branch, Islamic Azad University, Tehran, Iran

³Division of Physiology, Department of Basic Sciences, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran

⁴Spiritual Health Research Center, Qom University of Medical Sciences, Qom, Iran

Article history:

Received: May 24, 2025

Received in revised form:

Sep 06, 2025

Accepted: Sep 17, 2025

Epub ahead of print

* Corresponding Author:

sp.mortazavi@gmail.com

sp.mortazavi@srbiau.ac.ir

Keywords:

Cholestasis

Liver damage

Liver enzymes

TGF-β

Vitex agnus-castus

Abstract

Objective: This study aimed to evaluate the effects of *Vitex agnus-castus* extract on histopathological alterations and Transforming growth factor-beta (*TGF-β*) expression in liver damage induced by cholestasis in a bile duct obstruction model in rats.

Materials and Methods: Forty-eight rats were randomly assigned to eight groups: a healthy control group; three healthy groups receiving 100, 200, and 400 mg/kg of *Vitex agnus-castus* hydroalcoholic extract (leaves, flowers, and immature fruits); a bile duct ligation (BDL) control group; and three BDL groups treated with the same doses. Treatment lasted four weeks, with daily gastric gavage. After euthanasia, liver enzymes and bilirubin were measured, and liver sections were assessed histopathologically and immunohistochemically for *TGF-β*.

Results: BDL-induced hepatic cholestasis resulted in significant elevations in serum levels of alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT), and bilirubin, as well as increased *TGF-β* expression (IHC) and histopathological damage in the liver compared to the healthy control groups. Treatment with *Vitex agnus-castus* extract at doses of 100, 200, and 400 mg/kg ameliorated these parameters and improved histopathological damage. The most pronounced effects were observed at the highest dose of 400 mg/kg which significantly influenced all measured outcomes.

Conclusion: *Vitex agnus-castus* significantly alleviates complications and injuries associated with liver cholestasis in a dose-dependent manner. Its antioxidant properties and bioactive compounds may help preserve liver tissue integrity in rats.

Please cite this paper as:

Piroozmand H, Mortazavi P, Vazir B, Hosseiniara R. The effect of *Vitex agnus-castus* hydroalcoholic extract on histopathological changes and *TGF-β* expression in cholestasis-induced liver damage in a bile duct obstruction model in rats. Avicenna J Phytomed, 2025. Epub ahead of print.

Introduction

Cholestasis is a pathological condition characterized by impaired bile flow which results in the obstruction of bile ducts and subsequent accumulation of bile constituents in the bloodstream. This condition can have both intrahepatic and extrahepatic origins. Intrahepatic causes include drug-induced liver injury, infectious diseases, pregnancy-related changes, systemic disorders such as sarcoidosis and amyloidosis, and autoimmune, metabolic, and genetic conditions. Conversely, extrahepatic causes often involve obstruction of the bile ducts due to gallstones, strictures associated with chronic pancreatitis, tumors in the pancreas or bile ducts, and gallbladder pathologies (Kullak-Ublick and Meier 2000; Sadiku et al. 2015).

If left untreated, cholestasis can progress to liver fibrosis and ultimately cirrhosis. Fibrosis represents a critical disorder in chronic liver diseases and is a leading cause of mortality among patients suffering from cholestasis. This condition is characterized by excessive accumulation of extracellular matrix proteins including collagen, within damaged tissues, leading to organ dysfunction (Biernacka et al. 2011). Hepatic stellate cells (HSCs), also known as Ito cells or perisinusoidal fat-storing cells, play a pivotal role in the development of fibrosis. Bile duct obstruction induces an increase in bile pressure, mild inflammatory responses, and the secretion of cytokines by biliary epithelial cells. These processes result in heightened expression of fibrogenic markers such as Transforming growth factor-beta (*TGF-β*), alpha-smooth muscle actin (α -SMA), and collagen type I, and elevated reactive oxygen species (ROS), and subsequent liver damage (Lu et al. 2023; Sufletel et al. 2020).

TGF-β is involved in many cellular processes and is an essential regulator of fibroblast phenotype and function. Three structurally related isoforms of *TGF-β* are found in mammals: *TGF-β1*, *TGF-β2*, and

TGF-β3. *TGF-β1* is largely responsible for the development of tissue fibrosis, even though all three isoforms are present in fibrotic tissues. *TGF-β* serves as a key pro-fibrotic mediator that stimulates collagen production and extracellular matrix synthesis. By activating HSCs during fibrosis, along with platelet-derived growth factor (*PDGF*), *TGF-β* promotes connective tissue synthesis and cell apoptosis, ultimately contributing to fibrosis. Consequently, *TGF-β*-mediated pathways present promising therapeutic targets for treating fibrotic conditions (Biernacka et al. 2011; Budi et al. 2021).

Liver function tests (LFTs) often reveal elevated levels of enzymes such as alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT), and bilirubin due to liver damage associated with cholestasis and fibrosis. Abnormal LFT results are crucial for laboratory evaluation to determine the underlying pattern of cholestasis. LFTs can be categorized into two patterns: hepatocellular injury and cholestasis. In cases of hepatocellular injury, there is a more significant increase in aminotransferases compared to ALP levels. Conversely, in cholestasis, ALP levels rise more prominently than aminotransferases. Serum bilirubin levels may increase in both hepatocellular injury and cholestasis (Kalas et al. 2021; Pieters et al. 2021).

Despite significant advances in our understanding of cholestasis and liver fibrosis, as well as the identification of potential therapeutic targets, the availability of antifibrotic medicines for therapeutic usage in individuals with advanced liver disease remains restricted (Guo and Lu 2020). Most patients do not respond adequately to conventional therapies, placing them at risk for disease progression to fibrosis and cirrhosis. Consequently, researchers have increasingly focused on the use of medicinal plants and their bioactive compounds as natural therapeutic alternatives. Previous studies have

suggested that complementary medicine may provide benefits in the management of cholestasis (Wang et al. 2020; Wei et al. 2022).

Vitex agnus-castus, a medicinal plant belonging to the Lamiaceae family, is indigenous to the Mediterranean region. Phytochemical analyses have identified a variety of compounds across different parts of this plant, including iridoid glycosides such as agnoside and aucubin; flavonoids such as castacin, kaempferol, quercetagenin, and vitxin; progestins like progesterone and hydroxyprogesterone; alkaloids such as viticin; and volatile compounds including linalool, terpinyl acetate, alpha-pinene, and beta-pinene. Additionally, essential fatty acids such as palmitic acid, linoleic acid, and stearic acid have been found in the plant. While numerous studies highlight the therapeutic potential of *Vitex agnus-castus*, the precise mechanisms underlying these effects require further investigation (Adamov et al. 2022; Das et al. 2022; Mendes et al. 2022).

The present study aimed to evaluate the effects of *Vitex agnus-castus* extract on liver enzyme levels, histopathological changes, and TGF- β expression in a rat model of cholestasis induced by bile duct obstruction. Bile duct ligation (BDL) was employed as a model for inducing cholestasis in laboratory animals. This method is known to induce cholestasis more effectively and with less tissue loss in rats compared to alternative techniques (Tag et al. 2015).

Materials and Methods

Time line

BDL or surgery was performed on Day 0, extract treatment began on Day 1, continued daily for 4 weeks, and liver tissues were collected at the end of Week 4 for histopathology and TGF- β protein evaluation using IHC.

Plant source and part used

The aerial parts (including leaves, flowers, and immature fruits) of *Vitex agnus-castus* (commonly known as chaste tree) were collected from an identified region in Golestan Province, Iran, during the late flowering season (August). The plant's identity was confirmed by a botanist at the Herbarium of the Forests and Rangelands Research Institute, where a voucher specimen (registration number: 9252) was deposited.

Extraction process

The plant material was washed, shade-dried at room temperature, and ground into a fine powder. A hydroalcoholic extract was prepared using the maceration method. Briefly, 100 g of the powdered plant material was soaked in 1 L of a methanol-water solvent mixture (70:30, v/v) for 72 hours at room temperature with periodic stirring. The mixture was then filtered through Whatman No. 1 filter paper, and the residue was re-macerated with an additional 200 mL of the same solvent. The combined filtrates were concentrated under reduced pressure using a rotary evaporator at 40°C to obtain a crude extract. Finally, to remove any residual solvent, the concentrated extract was placed in a vacuum oven at 40°C for 48 hours, yielding a dry solid extract. The extraction yield was calculated and reported as a percentage.

Standardization and preparation for administration

The dry extract was accurately weighed and suspended in distilled water immediately prior to administration. The desired doses (100, 200, and 400 mg per kg of body weight) were prepared fresh daily from this suspension and administered to the rats via oral gavage. To ensure quality and relative consistency of the active constituents, the produced extract was assessed for its total phenolic content using the Folin-Ciocalteu method and total flavonoid content using the aluminum chloride colorimetric method.

BDL surgery and animal treatment

BDL surgery was performed following the established protocol by Uchinami et al. (Uchinami et al. 2006). After the two-week acclimatization period, the rats were anesthetized using a combination of ketamine (100 mg/kg body weight) and xylazine (20 mg/kg body weight). The BDL procedure involved suturing and occluding the common bile duct at two locations: distal to the intrahepatic bile duct and proximal to the pancreatic duct, utilizing silk suture thread size 0-4. After resection of the intervening portion, 1 ml of 10% normal saline solution was injected into the abdominal cavity. Size 0–4 absorbable vicryl sutures were used to close the muscle layers, while size 0–3 non-absorbable nylon sutures were used to close the skin. An oxytetracycline spray was used to keep the rats from gnawing on the sutures.

Following surgery, the rats received daily doses of *Vitex agnus-castus* extract via gavage for a total duration of four weeks. At the conclusion of this treatment period, the rats were euthanized under deep anesthesia induced by intraperitoneal injection of ketamine (80–100 mg/kg) and xylazine (5–10 mg/kg), followed by exsanguination via cardiac blood collection as a secondary method to ensure a humane and confirmed death, according to standard laboratory animal care protocols, and blood samples were obtained directly from the heart using a 5 ml syringe. Additionally, a portion of liver tissue was fixed in a 10% formalin buffer solution for subsequent immunohistochemical analysis. All rats survived throughout the experimental duration. Successful BDL was confirmed by observing yellowing in the urine and ears of the rats approximately 2-3 days post-surgery.

Serum biochemical analyses

To assess serum activity levels of ALP, ALT, AST, GGT, and total bilirubin,

blood samples were allowed to coagulate at room temperature for 30 min. Subsequently, serum was separated via centrifugation at 1000 rpm for 10 min. The biochemistry enzymes levels were quantified according to the guidelines established by the international federation of clinical chemistry and laboratory medicine (IFCC), utilizing a kit from Pars Azmoun Company (Iran) and a BT1500 autoanalyzer (Italy).

Histopathological evaluation

Histopathological examination involved two staining techniques: hematoxylin and eosin, and Masson's trichrome staining. Liver tissue samples were excised and fixed in 10% buffered formalin. Following the preparation of paraffin blocks, sections of 4 micrometers in thickness were cut and stained. Tissue alterations were assessed, and cell counts were performed. The histopathological evaluation of liver tissue was systematically categorized into four primary parameters: inflammation, bile duct hyperplasia, necrosis, and fibrosis. Each parameter was assessed using a standardized grading scale ranging from 0 to 3, with 0 indicating absence of pathological changes and 3 representing extensive or severe alterations. The scoring methodology quantified the extent of tissue involvement, specifically measuring the percentage of liver tissue or lobules affected, which allowed for a precise and objective assessment of hepatic pathology. This comprehensive approach enabled a detailed characterization of liver tissue changes, facilitating accurate diagnostic and research insights into potential hepatic disorders and their progression.

Immunohistochemical evaluation

Immunohistochemistry was employed to detect the presence and localization of proteins within tissue sections, facilitating the observation of biological processes in intact tissues. Following tissue

stabilization, preparation was conducted using an autotechnicon device. Tissue slides were prepared using standard methods and stained with a TGF- β antibody (abcam, USA). Evaluation was performed under light microscopy. The expression of TGF- β was quantified by converting qualitative data into quantitative metrics suitable for statistical analysis.

TGF- β scoring was performed manually by two independent pathologists in a blinded manner. Ten random fields were evaluated per section, and discrepancies were resolved by consensus.

For grading each sample, ten random fields were examined and scored as follows:

Grade 0: Negative; no TGF- β expression in hepatocyte nuclei

Grade 1: Low; TGF- β expression in less than 25% of hepatocyte nuclei

Grade 2: Moderate; TGF- β expression in 25-50% of hepatocyte nuclei

Grade 3: Severe; TGF- β expression in 50-75% of hepatocyte nuclei

Grade 4: Very severe; TGF- β expression in more than 75% of hepatocyte nuclei

Statistical analysis

Statistical analyses were conducted using two-way ANOVA and Kruskal-Wallis tests, employing the Prism statistical software. Results are presented as mean $\pm$ SEM, with statistical significance determined at $p < 0.05$.

Results

ALP assessment in serum

Figure 1A illustrates that liver cholestasis, as observed in the BDL group, resulted in a significant increase in ALP levels. In contrast, the administration of *Vitex agnus castus* extract at doses of 200 and 400 mg/kg led to a notable reduction in ALP levels. The most pronounced decrease in ALP was observed in the group treated

with a dose of 400 mg/kg of *Vitex agnus castus* compared to the BDL group.

ALT assessment in serum

Figure 1B demonstrates that liver cholestasis resulted in a significant elevation of ALT levels. However, treatment with *Vitex agnus castus* extract at all administered doses resulted in a significant reduction in ALT levels. The most substantial decrease was recorded in the group receiving 400 mg/kg of *Vitex agnus castus* compared to the BDL group.

AST assessment in serum

Figure 1C indicates a significant increase in AST levels due to liver cholestasis. Nevertheless, treatment with *Vitex agnus castus* extract at all doses resulted in a significant decrease in AST levels. The most considerable reduction was observed in the group treated with *Vitex agnus castus* at a dose of 400 mg/kg when compared to the BDL group.

GGT assessment in serum

Figure 1D shows that liver cholestasis led to a significant rise in GGT levels. However, administration of *Vitex agnus castus* extract at doses of 200 and 400 mg/kg resulted in significant decreases in GGT levels. The most noteworthy reduction was noted in the group receiving *Vitex agnus castus* at a dose of 400 mg/kg compared to the BDL group.

Total bilirubin assessment in serum

Figure 1E demonstrates that liver cholestasis leads to a significant elevation in total bilirubin levels. In contrast, treatment with *Vitex agnus castus* extract at doses of 200 and 400 mg/kg resulted in a marked reduction in total bilirubin levels. The most pronounced decrease was observed in the group administered 400 mg/kg of *Vitex agnus castus* compared to the BDL group.

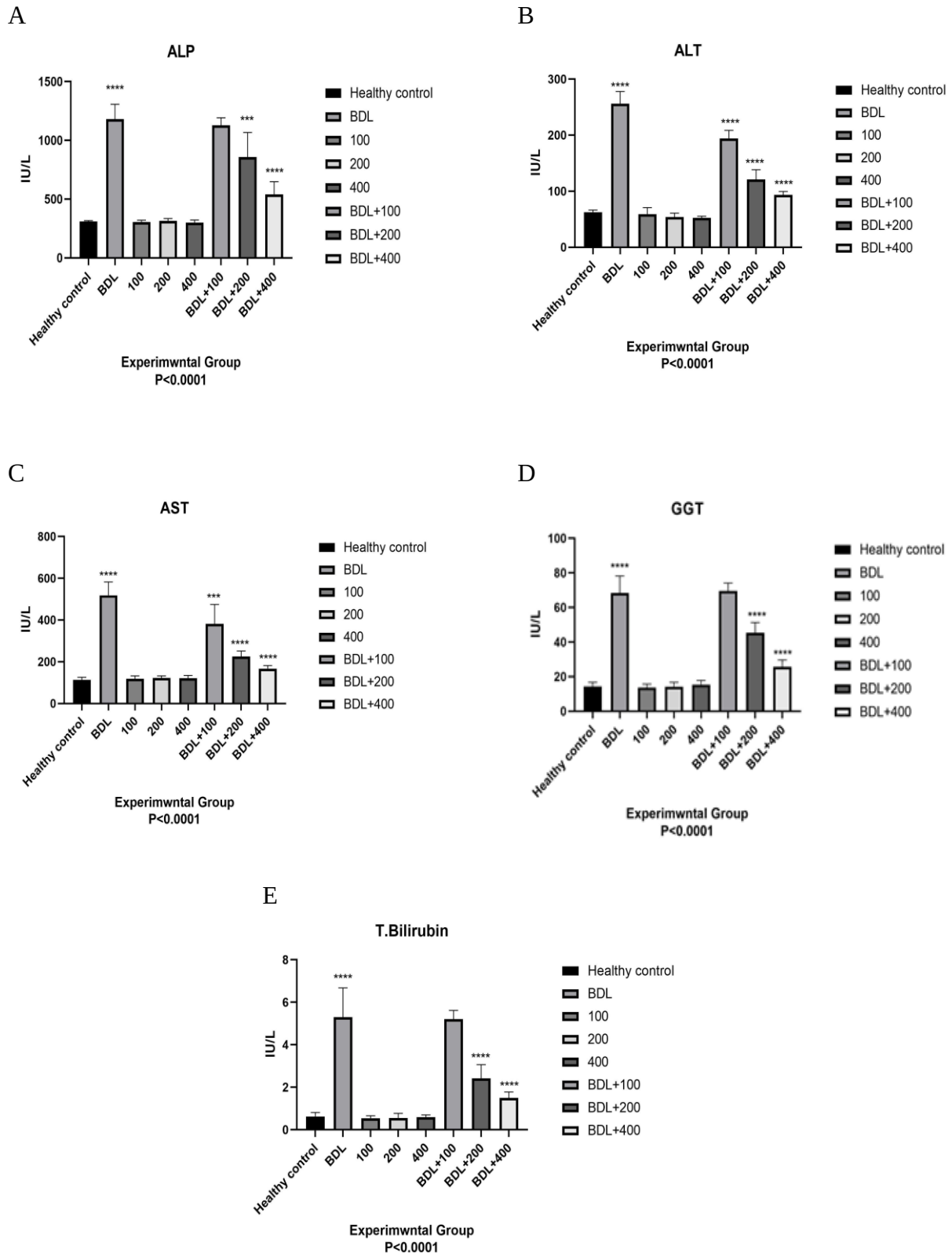


Figure 1. Serum enzyme and bilirubin changes across the studied groups. A. alkaline phosphatase (ALP), B. alanine transaminase (ALT), C. aspartate aminotransferase (AST), D. gamma-glutamyl transferase (GGT), E. Total Bilirubin. As compared to***p<0.001(BDL=Bile Duct Ligation, 100,200 400= healthy experimental groups receiving doses of 100, 200, and 400 mg/kg of Vitex agnus-castus hydroalcoholic extract, BDL+100, BDL+200, BDL+400= BDL groups treated with the 100, 200, and 400 mg/kg of Vitex agnus-castus hydroalcoholic extract)

Histopathology

Hepatocyte necrosis, lymphocyte-type inflammatory cell infiltration, bile duct hyperplasia, and the development of fake bile ducts were among the histopathological damage seen in the cholestasis group. Significant connective tissue infiltration between necrotic and degenerated hepatocytes also occurred, leading to the

development of incomplete hepatic lobules in all groups, varied in severity (Figure 2I).

As illustrated in Figure 2(A-H), liver cholestasis induced notable histopathological injuries. However, treatment with *Vitex agnus castus* extract at all doses significantly mitigated these injuries. The most substantial therapeutic effect was observed with the administration of 400 mg/kg of *Vitex agnus castus* extract.

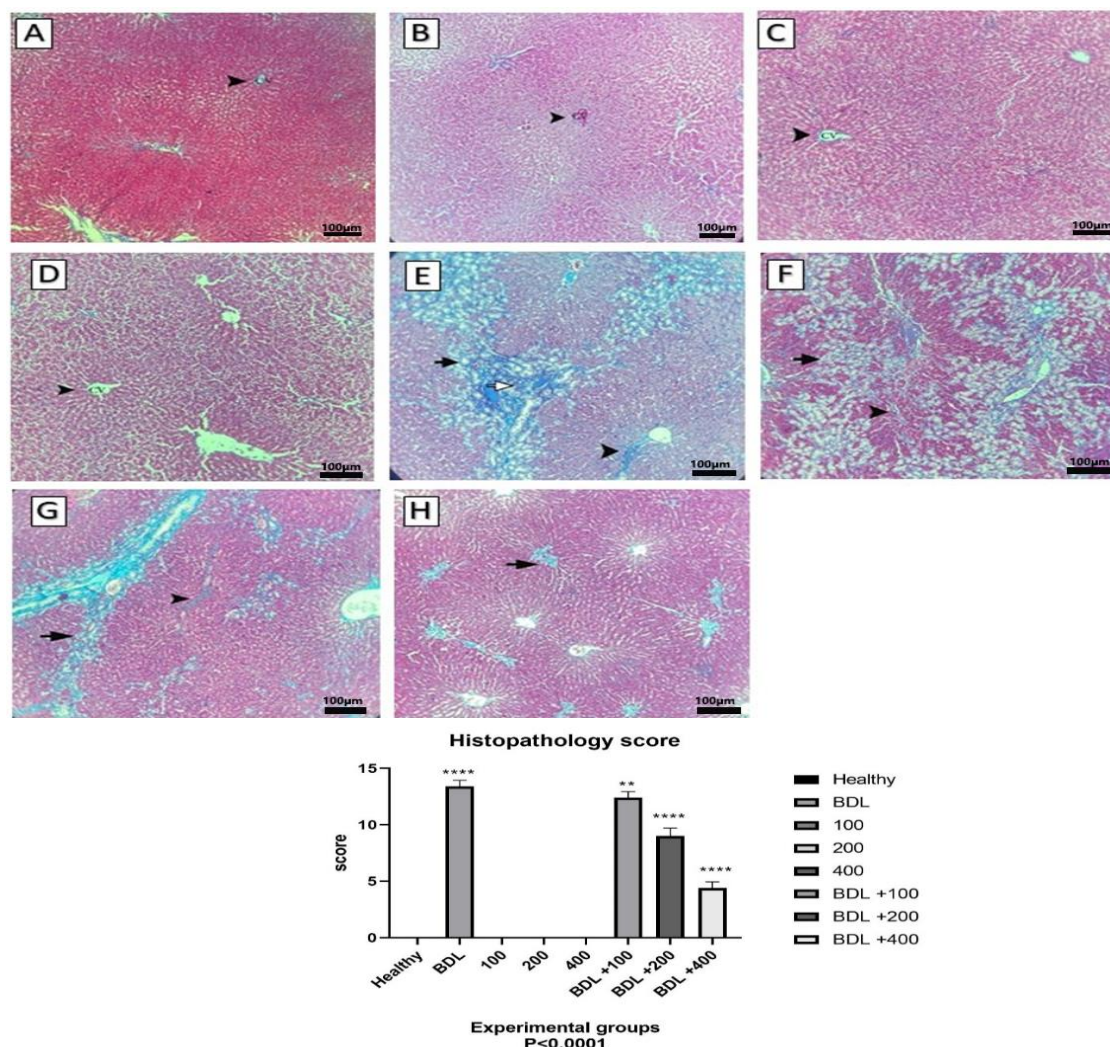


Figure 2. A–H): Histopathology of liver tissues in experimental groups. A. Healthy control group showing central vein (CV) with normal hepatocytes (arrowhead) (Trichrome, 100X). B. Healthy experimental group (100 mg/kg) with central vein (CV) and healthy hepatocytes (arrowhead). C. Healthy experimental group (200 mg/kg) with central vein (CV) and healthy hepatocytes (arrowhead). D. Healthy experimental group (400 mg/kg) with central vein (CV) and healthy hepatocytes (arrowhead). E. Cholestasis control group showing connective tissue infiltration forming complete walls (arrowhead), bile duct hyperplasia (black arrow), and intense inflammatory infiltration (white arrow). F. Cholestasis + 100 mg/kg showing severe bile duct hyperplasia (arrow) and connective tissue infiltration (arrowhead). G. Cholestasis + 200 mg/kg showing connective tissue penetration (arrowhead) and bile duct hyperplasia (arrow). H. Cholestasis + 400 mg/kg showing slight bile duct hyperplasia (arrow). I. Severity of histopathological injuries observed in different study groups. As compared to * $p < 0.01$ and *** $p < 0.0001$. (BDL=Bile Duct Ligation, 100,200 400= healthy experimental groups receiving doses of 100, 200, and 400 mg/kg of *Vitex agnus-castus* hydroalcoholic extract, BDL+100, BDL+200, BDL+400= BDL groups treated with the 100, 200, and 400 mg/kg of *Vitex agnus-castus* hydroalcoholic extract)

Immunohistochemistry and $TGF-\beta$ expression

The expression of $TGF-\beta$ was assessed in various regions of liver tissue slides using immunohistochemical techniques, followed by microscopic examination. Figure 3I illustrates the variations in $TGF-\beta$ expression across different experimental

groups. The findings indicate that liver cholestasis significantly elevated the expression of the $TGF-\beta$. Conversely, administration of *Vitex agnus castus* extract at doses of 200 and 400 mg/kg resulted in a notable reduction in $TGF-\beta$ expression, with the most substantial effect observed at the higher dose of 400 mg/kg.

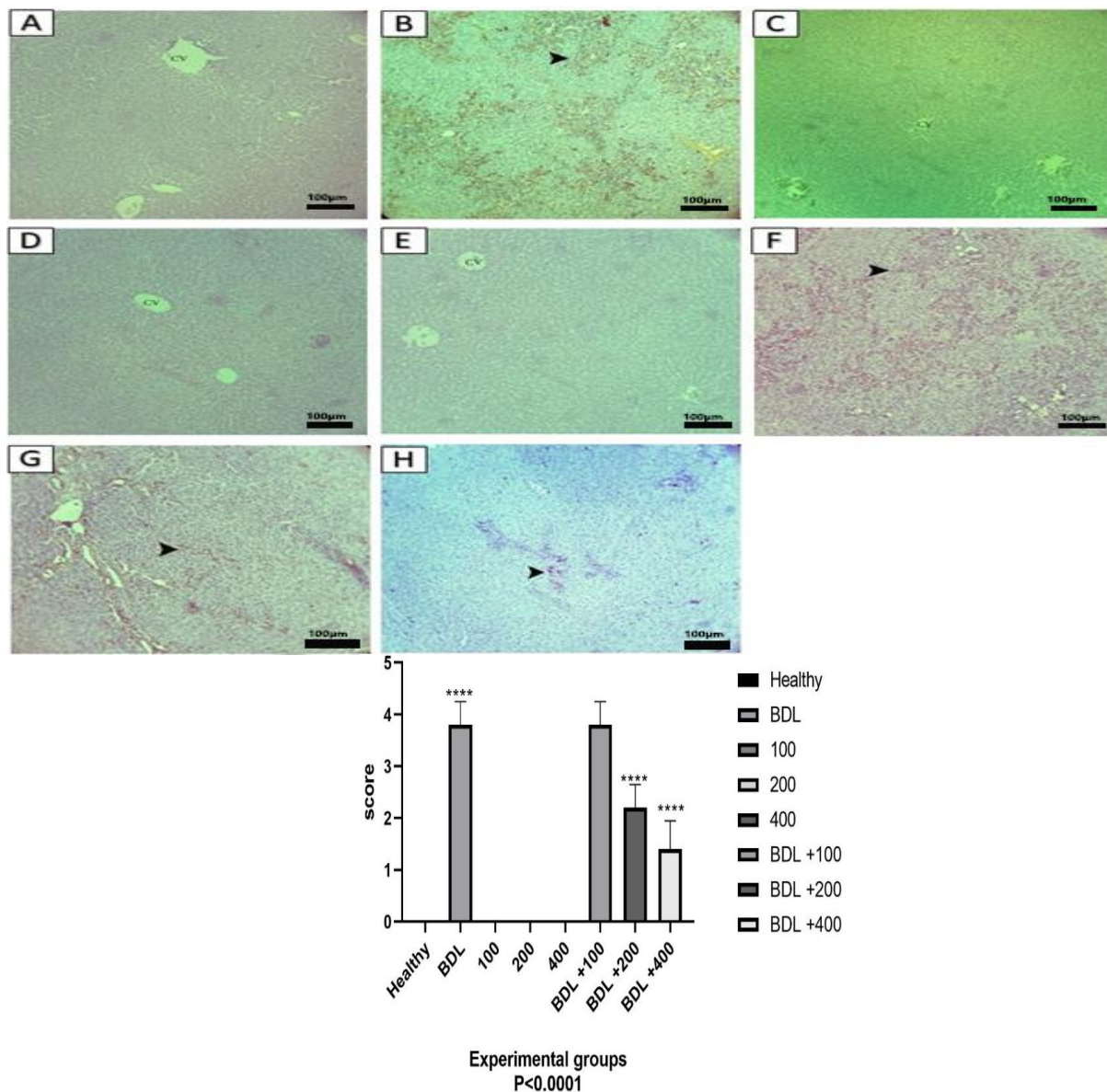


Figure 3. (A–H): Immunohistochemical Analysis of Liver Tissues and Transforming growth factor-beta ($TGF-\beta$) expression. A. Healthy control: absence of $TGF-\beta$ expression in hepatocytes around central vein (CV). B. Cholestasis group: intense $TGF-\beta$ expression in hepatocytes and bile duct cells (arrowhead). C. Healthy experimental (100 mg/kg): no $TGF-\beta$ expression. D. Healthy experimental (200 mg/kg): no $TGF-\beta$ expression. E. Healthy experimental (400 mg/kg): no $TGF-\beta$ expression. F. Cholestasis + 100 mg/kg: strong $TGF-\beta$ expression in hepatocytes and bile duct cells (arrowhead). G. Cholestasis + 200 mg/kg: significant $TGF-\beta$ expression in hepatocytes and bile duct cells (arrowhead). H. Cholestasis + 400 mg/kg: low $TGF-\beta$ expression in bile duct cells (arrowhead). I. Transforming growth factor-beta ($TGF-\beta$) expression levels across the studied groups. As compared to $p < 0.0001$. (BDL=Bile Duct Ligation, 100,200 400= healthy experimental groups receiving doses of 100, 200, and 400 mg/kg of *Vitex agnus-castus* hydroalcoholic extract, BDL+100, BDL+200, BDL+400= BDL groups treated with the 100, 200, and 400 mg/kg of *Vitex agnus-castus* hydroalcoholic extract)

Figure 3(A-H) presents the immunohistochemical analysis of TGF- β expression in liver tissues. In the healthy control and experimental groups treated with *Vitex agnus castus* extract at doses of 100, 200, and 400 mg/kg, TGF- β expression in hepatocytes surrounding the central vein was absent. A positive criterion for TGF- β expression is defined as the browning of the cytoplasm in at least 50% of hepatocytes, which was not observed in any of the healthy groups treated with *Vitex agnus castus* extract. Thus, there was no significant difference in TGF- β expression among these groups. However, BDL significantly increased TGF- β expression in the cholestasis group. In this cohort, TGF- β expression was markedly positive around the central vein and fibrotic areas, with extensive cytoplasmic browning noted in hepatocytes within fibrotic regions and false bile ducts. In the groups treated with doses of 100, 200, and 400 mg/kg, TGF- β expression was categorized as mild, moderate, and severe, respectively.

Discussion

This study employed BDL surgery to induce cholestasis and subsequent liver fibrosis in rats, aiming to evaluate the effects of *Vitex agnus castus* extract on histopathological changes and TGF- β expression associated with liver damage. The serum levels of LFTs across the various experimental groups indicated that hepatic cholestasis significantly elevated the activities of enzymes such as ALT, AST, ALP, and GGT, and bilirubin when compared to the healthy control group. These results align with previous studies that reported increased serum levels of functional liver enzymes in cholestasis models (Giménez-Garzó et al. 2015; Moloudi et al. 2014; Nabavizadeh et al. 2010).

Pathological examinations revealed distinct changes in the liver of the cholestasis group compared to the other groups, confirming the successful induction

of the model in rats. The observed injuries included hepatocyte necrosis, infiltration of lymphocytic inflammatory cells, hyperplasia of bile ducts, formation of false bile ducts, infiltration of connective tissue between necrotic and degenerated hepatocytes, and the development of incomplete liver lobules.

The production of ROS and increased expression of fibrogenic markers are the main ways that BDL causes liver fibrosis. One of the main causes of liver injury in cholestasis is the retention and buildup of hydrophobic bile acids within hepatocytes. Because of their detergent qualities, bile acids can damage cell membranes, causing the generation of ROS that oxidatively alter proteins, lipids, and nucleic acids, ultimately leading to hepatocyte death. Furthermore, these bile acids have the ability to activate Kupffer cells, which increases the production of ROS and aids in the death of hepatocytes. The pathophysiology of cholestatic liver diseases is largely dependent on the structural and functional damage that hydrophobic bile acids cause to hepatocyte membranes through the production of ROS. Hydrophobic bile acids can damage organelle membranes when their concentration exceeds the hepatocytes' cytosolic proteins' ability to bind. As a result, oxidative damage is more severe in fibrotic cells than in normal cells (Conde de la Rosa et al. 2022; Ezhilarasan 2022; Jafaripour et al. 2023). Serum liver enzyme levels are raised as a result of this membrane damage; in our investigation, the BDL group showed a marked rise in serum liver enzyme levels four weeks after the experiment started.

Treatment of rats in the BDL group with various doses of *Vitex agnus castus* extract resulted in a significant, dose-dependent reduction in serum levels of liver enzymes, indicating a mitigation of complications associated with cholestasis. This effect may be attributed to the antioxidant compounds present in the *Vitex agnus castus* plant. Medicinal plants known for their

antioxidant properties are recognized for their ability to prevent oxidative stress and regulate liver enzyme levels (Ghadermazi et al. 2018; Hofmeister 2022; Ranjbar et al. 2014).

Latoui et al. conducted a study on the leaves, roots, and seeds of *Vitex agnus castus*, demonstrating that this plant possesses high antioxidant capacity due to its rich content of phenolic compounds, total polyphenols, diphenols, flavonoids, and anthocyanins (Latoui et al. 2012). Antioxidants are substances that have the ability to postpone, stop, or eradicate oxidative damage to certain molecules (Namdar et al. 2023). The antioxidant properties of *Vitex agnus castus* may have contributed to maintaining the stability of hepatocyte membranes, improving histochemical integrity, and reducing serum levels of liver enzymes. This antioxidant effect is likely due to the flavonoid compounds found in the plant. Previous phytochemical analyses of *Vitex agnus castus* have identified various glycosides such as vitexin and vitexinin, flavonoids including castesin, orientin, and isovitexin, as well as iridoid glycosides like okubin and agnoside estrozoside, and steroids such as A 3-4 ketoster. Beyond their antioxidant properties, these isolated compounds exhibit several protective functions, including the correction of endothelial defects, inhibition of smooth muscle cell proliferation and migration, suppression of macrophage-derived foam cell formation, and reduction of inflammatory responses (Adamov et al. 2022; Das et al. 2022; Mendes et al. 2022). Given that antioxidants can significantly protect the liver by reducing lipid peroxidation and hepatocyte necrosis, it is plausible that the antioxidants in *Vitex agnus castus* extract play a crucial role in alleviating injuries caused by cholestasis (Jawad et al. 2022).

In this study, BDL surgery led to an increase in *TGF-β* expression in liver tissue from the BDL group, attributed to the accumulation of bile acids and the production of ROS. Previous research has

similarly reported a significant elevation in *TGF-β* expression in liver tissue following BDL (Ezhilarasan 2022; Jiménez-Urbe et al. 2021; Karimi et al. 2022). HSCs, also known as Ito cells, are the primary source of extracellular matrix components; their activation triggers collagen deposition during fibrosis through the release of ROS, cytokines, and growth factors such as *PDGF*, *TGF-β*, connective tissue growth factor (CTGF), interleukin-6, and interleukin-13. Among these factors, *TGF-β* is recognized as the most potent pro-fibrotic cytokine. Its expression encourages fibroblasts to proliferate and differentiate into myofibroblasts, which results in an overabundance of extracellular matrix components being produced and deposited, ultimately causing fibrosis. *TGF-β* can activate signaling pathways via Smad-dependent or Smad-independent mechanisms and is involved in the generation of intracellular ROS, which further contributes to fibrosis (Budi et al. 2021; Lu et al. 2023; Sufletel et al. 2020).

Treatment of BDL rats with *Vitex agnus castus* at doses of 200 and 400 mg/kg resulted in a significant reduction in the expression of *TGF-β*. The plant appears to exert its effects by suppressing pro-inflammatory cytokines such as *TNF-α*, thereby preventing hepatocyte apoptosis and the activation of intracellular signaling cascades associated with inflammation (Ezhilarasan 2022; Jiménez-Urbe et al. 2021). By inhibiting *TGF-β* expression and reducing fibroblast activity, *Vitex agnus castus* effectively decreased collagen production and deposition in the liver, leading to an improvement in fibrosis. Conversely, the 100 mg/kg dose did not produce any significant effects, while both the 200 and 400 mg/kg doses markedly reduced *TGF-β* expression and mitigated liver fibrosis in BDL rats.

These findings illustrate the dose-dependent anti-fibrotic effects of *Vitex agnus castus* extract in a cholestatic liver model. While previous studies have not specifically assessed the impact of this

plant on cholestatic liver conditions, related research involving other medicinal plants, such as *Epimedium grandiflorum* (Karimi et al. 2022) and *Berberis vulgaris* (Mirazi et al. 2015), has yielded results that align with those observed in this study.

This study has strengths including dose-dependent effects and combined biochemical and histological evaluation, weaknesses such as a relatively small sample size and lack of mechanistic molecular assays, and limitations including short-term duration and absence of long-term outcomes. Furthermore, higher-resolution images would improve precision in future studies.

The findings from this study suggest that *Vitex agnus castus* administration can effectively alleviate complications and injuries associated with liver cholestasis in rats. The therapeutic effects were dose-dependent, indicating that the antioxidant and other bioactive compounds present in this plant play a significant role in preserving liver tissue integrity. Further investigation is warranted to evaluate the potential of *Vitex agnus castus* as a medicinal agent with anti-fibrotic properties for individuals suffering from hepatic cholestasis.

Conflicts of interest

The authors declare that they have no competing interests.

Funding

This research did not receive any specific grants from public, commercial, or non-profit funding agencies.

Ethical considerations

This study adhered to the ethical principles outlined in the Helsinki Declaration regarding the use of animals in research. The ethics code for this study was IR.IAU.SRB.REC.1401.219.

Code of ethics

IR.IAU.SRB.REC.1401.219.

Authors' contributions

H.P. designed the study; H.P. conducted the experiments; H.P., P.M., and B.V. processed the data; P.M. analyzed and interpreted the pathological results; H.P. drafted the manuscript; R.H. edited the article; P.M. supervised the study.

Ethics approval and consent to participate

The ethical committee of Azad University of Science and Technology, Tehran, Iran, approved this study under approval number IR.IAU.SRB.REC.1401.219.

Availability of data and materials

All data analyzed during this study are included in this published article and its supporting information.

Consent for publication

By submitting this document, the authors consent to the publication of the final accepted version of the manuscript.

References

- Adamov GV, Rendyuk TD, Saybel OL, Dargaeva TD, Tsitsilin AN, Bokov DO (2022) *Vitex agnus-castus*: Botanical features and area, chemical composition of fruit, pharmacological properties, and medicinal uses. *Journal of Applied Pharmaceutical Science* 12(3):034-44 doi:10.7324/JAPS.2022.120305
- Biernacka A, Dobaczewski M, Frangogiannis NG (2011) TGF- β signaling in fibrosis. *Growth factors* 29(5):196-202 doi:10.3109/08977194.2011.595714
- Budi EH, Schaub JR, Decaris M, Turner S, Derynck R (2021) TGF- β as a driver of fibrosis: physiological roles and therapeutic opportunities. *J Pathol* 254(4):358-373 doi:10.1002/path.5687
- Conde de la Rosa L, Goicoechea L, Torres S, Garcia-Ruiz C, Fernandez-Checa JC (2022) Role of oxidative stress in liver disorders. *Livers* 2(4):283-314 doi:10.3390/livers2040022

- Das N, Salgueiro AC, Choudhury DR, et al. (2022) Traditional uses, phytochemistry, and pharmacology of genus *Vitex* (Lamiaceae). *Phytotherapy Research* 36(2):571-671 doi:10.1002/ptr.7334
- Ezhilarasan D (2022) Hepatic stellate cells in the injured liver: Perspectives beyond hepatic fibrosis. *J Cell Physiol* 237(1):436-449 doi:10.1002/jcp.30582
- Ghadermazi R, Khoshjou F, Hossini Zijoud SM, Behrooj H, Kheiripour N, Ganji M (2018) Hepatoprotective effect of tempol on oxidative toxic stress in STZ-induced diabetic rats. *Toxin Reviews* 37(1):82-86 doi:10.1080/15569543.2017.1326494
- Giménez-Garzó C, Salhi D, Urios A, Ruíz-Sauri A, Carda C, Montoliu C (2015) Rats with mild bile duct ligation show hepatic encephalopathy with cognitive and motor impairment in the absence of cirrhosis: effects of alcohol ingestion. *Neurochem Res* 40:230-240 doi:10.1007/s11064-014-1389-9
- Guo YC, Lu LG (2020) Antihepatic fibrosis drugs in clinical trials. *Journal of Clinical and Translational Hepatology* 8(3):304 doi:10.14218/JCTH.2020.00014
- Hofmeister M (2022) Kratom consumption can be addictive and have adverse health effects. *Novel Clin Med* 1(4):168-172 doi:10.22034/ncm.2022.350622.1050
- Jafaripour L, Sohrabi Zadeh B, Jafaripour E, Ahmadvand H, Asadi-Shekaari M (2023) Gallic acid improves liver cirrhosis by reducing oxidative stress and fibrogenesis in the liver of rats induced by bile duct ligation. *Scandinavian Journal of Gastroenterology* 58(12):1474-1483 doi:10.1080/00365521.2023.2261447
- Jawad M, Al-Ameer A, Salih S, Musa M (2022) The Effect of An Aqueous Extract of *Vitex Agnus-Castus* Leaves on the Liver and Kidney in Female Rabbits. *Basrah Journal of Veterinary Research* 21(S1):84-96
- Jiménez-Urbe AP, Gomez-Sierra T, Aparicio-Trejo OE, Orozco-Ibarra M, Pedraza-Chaverri J (2021) Backstage players of fibrosis: NOX4, mTOR, HDAC, and S1P; companions of TGF- β . *Cellular Signaling* 87:110123 doi:10.1016/j.cellsig.2021.110123
- Kalas MA, Chavez L, Leon M, Taweessdt PT, Surani S (2021) Abnormal liver enzymes: A review for clinicians. *World J Hepatol* 13(11):1688 doi:10.4254/wjh.v13.i11.1688
- Karimi F, Mortazavi P, Asghari A (2022) Evaluation of Enzymatic changes in cholestasis index and TGF- β expression in cholestatic rat's liver were treated with ethanolic extract of *Epimedium*. *Journal of Comparative Pathobiology* 18(2):3565-3574
- Kullak-Ublick GA, Meier PJ (2000) Mechanisms of cholestasis. *Clinics in liver disease* 4(2):357-385 doi:10.1016/S1089-3261(05)70117-4
- Latoui M, Aliakbarian N, Casazza A, Seffen M, Converti A, Perego P (2012) Extraction of phenolic compounds from *Vitex agnus-castus* L. *Food and Bioproducts Processing* 90(4):748-754 doi:10.1016/j.fbp.2012.01.004
- Lu JL, Yu CX, Song LJ (2023) Programmed cell death in hepatic fibrosis: current and perspectives. *Cell Death Discovery* 9(1):449 doi:10.1038/s41420-023-01738-x
- Mendes C, Fonseca AM, Alves MS, et al. (2022) Narrative review of *Vitex agnus-castus* in symptoms in Gynecology. *Revista da Associação Médica Brasileira* 68:716-719 doi:10.1590/1806-9282.20210832
- Mirazi N, Tohidi F, Hosseini A (2015) Effects of berberis vulgaris hydroethanolic extract on serum bilirubin level and liver enzymes in male cholestatic rats. *Journal of Knowledge & Health* 10(3):59-67
- Moloudi MR, Hassanzadeh K, Rouhani S, Zandi F, Ahmadi A, Khalwatian P (2014) Effect of chloroformic extract of *Cichorium intybus* on liver function tests and serum level of TNF in obstructive cholestasis in rat. *Scientific Journal of Kurdistan University of Medical Sciences (SJKUMS)* 19:10-19
- Nabavizadeh F, Moloudi R, Dehpour AR, Nahrevanian H, Shahvaisi K, Salimi E (2010) The effects of cholestasis and cirrhosis on gastric acid and pepsin secretions in rat: Involvement of nitric oxide. *Iran J Basic Med Sci* 13:207-12 doi:10.22038/IJBMS.2010.5170
- Namdar F, Shahyad S, Mohammadi MT (2023) Fullerene C60 nanoparticles potentiate the antioxidant defense system of the brain and liver by increasing catalase activity in normal rats. *Novel Clin Med* 2(1):32-38 doi:10.22034/ncm.2023.360397.1056
- Pieters A, Gijbels E, Cogliati B, Annaert P, Devisscher L, Vinken M (2021) Biomarkers of cholestasis. *Biomarkers in*

- Medicine 15(6):437-454
doi:10.2217/bmm-2020-0747
- Ranjbar A, Mohsenzadeh F, Chehregani A, Khajavi F, Zijoud SM, Ghasemi H (2014) Ameliorative effect of *Matricaria chamomilla* .L on paraquat: Induced oxidative damage in lung rats. *Pharmacognosy Res* 6(3):199-203 doi:10.4103/0974-8490.132595
- Sadiku E, Taci S, Dibra A, Nela E, Babameto A (2015) The differential diagnosis of intra and extra-hepatic cholestasis: Causes and diagnosis of intrahepatic cholestatic disorders. *Alban Med J*:72-82
- Sufleţel RT, Melincovici CS, Gheban BA, Toader Z, Miha CM (2020) Hepatic stellate cells—from past till present: morphology, human markers, human cell lines, behavior in normal and liver pathology. *Romanian Journal of Morphology and Embryology* 61(3):615 doi:10.47162/RJME.61.3.14
- Tag CG, Weiskirchen S, Hittatiya K, Tacke F, Tolba RH, Weiskirchen R (2015) Induction of experimental obstructive cholestasis in mice. *Lab Anim* 49(1 Suppl):70-80 doi:10.1177/0023677214567748
- Uchinami H, Seki E, Brenner DA, D'Armiento J (2006) Loss of MMP 13 attenuates murine hepatic injury and fibrosis during cholestasis. *Hepatology* 44:420-429 doi:10.1002/hep.21315
- Wang R, Cheng N, Peng R, Yu Z, Nan M, Cao H (2020) Oral herbal medicine for women with intrahepatic cholestasis in pregnancy: a systematic review of randomized controlled trials. *BMC complementary medicine and therapies* 20(1):1-24 doi:10.1186/s12906-020-03089-x
- Wei C, Qiu J, Wu Y, et al. (2022) Promising traditional Chinese medicine for the treatment of cholestatic liver disease process (cholestasis, hepatitis, liver fibrosis, liver cirrhosis). *J Ethnopharmacol*:115550 doi:10.1016/j.jep.2022.115550