**Journal of Neonatal Surgery**
***ISSN(Online): 2226-0439***
***Vol. 14, Issue 24s (2025)***
*https://www.jneonatalsurg.com*

OPEN ACCESS

# In Vitro and In Vivo Studies on the extracts of Vitex Negundo and Hygrophilla Auriculata for their Hepatoprotective activity

**Anjali Khantal[1], Nidhi Bais[2]**

[1,2]Faculty of Pharmacy, Oriental University Indore, M.P. 452001, India

Email ID: 007anjali.khantal@gmail.com

Cite this paper as: Anjali Khantal, Nidhi Bais, (2025) In Vitro and In Vivo Studies on the extracts of Vitex Negundo and Hygrophilla Auriculata for their Hepatoprotective activity. *Journal of Neonatal Surgery*, 14 (24s), 221-230.

**ABSTRACT**

This study aimed to evaluate the hepatoprotective potential of aqueous and methanolic extracts of *Vitex negundo* and *Hygrophila auriculata* using in-vitro and in-vivo models. Phytochemical analysis revealed that *V. negundo* contains carbohydrates, glycosides, tannins, and saponins, while *H. auriculata* contains carbohydrates, alkaloids, glycosides, and proteins—compounds known for their therapeutic properties. Antioxidant activity was assessed using DPPH and Nitric Oxide radical inhibition assays. The methanolic extract of *V. negundo* demonstrated the strongest DPPH radical scavenging activity with an $IC_{50}$ of 28.1 ± 0.425 µg/ml, while the methanolic extract of *H. auriculata* showed the highest nitric oxide scavenging activity with an $IC_{50}$ of 117.55 ± 0.6708 µg/ml. These results were compared to standard antioxidants like Ascorbic acid and Rutin. In-vitro cytoprotective studies were carried out on BRL-3A liver cells subjected to oxidative stress. The aqueous and methanolic extracts of *V. negundo* provided 50% cytoprotection at 140 µg/ml and 570 µg/ml, respectively, whereas *H. auriculata* extracts achieved similar protection at 220 µg/ml (aqueous) and 337.5 µg/ml (methanolic). Hepatoprotective activity was further evaluated in isolated rat hepatocytes exposed to D-Galactosamine-induced toxicity. All extracts significantly restored biochemical markers such as liver enzymes, total protein, and albumin. Among the extracts, the aqueous extract of *H. auriculata* showed the most potent hepatoprotective effect. In-vivo studies in rats confirmed these findings. Rats treated with D-GalN alone showed elevated liver enzymes and markers of liver damage, while co-treatment with plant extracts significantly reversed these changes. The aqueous extract of *H. auriculata* again showed the most notable hepatoprotection, followed by its methanolic extract. Extracts of *V. negundo* also demonstrated hepatoprotective effects, though to a lesser extent. Histopathological examination of liver tissues supported the biochemical results, showing reduced liver damage in extract-treated groups. Overall, the findings highlight the significant antioxidant and hepatoprotective activities of both plants, particularly the aqueous extract of *H. auriculata*, suggesting their potential for liver disorder therapies.

***Keywords:*** *Hepatoprotective activity, Vitex negundo, Hygrophila auriculata, Antioxidant assays, DPPH, Cytoprotection, D-Galactosamine.*

## 1. INTRODUCTION

The liver is a central organ in metabolic regulation, detoxification, and the maintenance of systemic homeostasis [1–2]. Due to its critical role in processing xenobiotics, pharmaceuticals, and environmental toxins, it is particularly susceptible to injury. Unchecked hepatic damage can progress to advanced pathological states such as fibrosis, cirrhosis, and liver failure—conditions that contribute significantly to global morbidity and mortality [3]. Hepatic insults are primarily driven by toxic agents, infections, and metabolic dysregulation, with oxidative stress and inflammation serving as key mechanisms in disease progression [4–5].

In recent years, interest in plant-based therapeutics for liver diseases has grown, driven by the need for safer alternatives to synthetic drugs [6-8]. Herbal medicines often contain bioactive phytochemicals with antioxidant, anti-inflammatory, and hepatoprotective properties. Among these, *Vitex negundo* Linn. and *Hygrophila auriculata* have shown promising activity [9–10].

*Vitex negundo*, commonly known as the five-leaved chaste tree, has been traditionally used in Ayurvedic medicine for its anti-inflammatory, analgesic, and hepatoprotective effects. It is rich in flavonoids, terpenoids, and phenolics—compounds known for their potent antioxidant and anti-inflammatory properties [11–12].

*Hygrophila auriculata* (syn. *Asteracantha longifolia*), an aquatic herb used in traditional medicine, is recognized for its hepatoprotective, diuretic, and antioxidant activities. Its pharmacological efficacy is attributed to the presence of flavonoids, alkaloids, and saponins [13–14].

Despite widespread traditional use, there is limited scientific validation of the hepatoprotective effects of these plants in rigorous experimental models [15]. This study seeks to bridge this gap by evaluating the efficacy of *Vitex negundo* and *Hygrophila auriculata* in a D-GalN-induced liver injury model. The findings aim to elucidate their mechanisms of action and support their potential integration into therapeutic strategies for liver disease management [16].

## 2. MATERIALS AND METHODS

### Materials

Fresh *Vitex negundo* leaves and aerial parts of *Hygrophila auriculata* were collected from Kerala, India. The plant materials were taxonomically authenticated, and voucher specimens were deposited in the Department of Pharmacognosy, Oriental University, Indore, for future reference. D-Galactosamine hydrochloride was obtained from Ranbaxy Chemicals, while disodium hydrogen phosphate was sourced from Himedia Laboratories Pvt. Ltd., Mumbai, India. Analytical grade ethyl acetate, tannic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium nitroprusside, sulfanilamide, naphthyl ethylenediamine dihydrochloride, hydrogen peroxide, phosphate buffer, and phosphoric acid were procured from S.D. Fine Chemicals Ltd., India.

### Animals

Male Wistar rats (150–170 g) were procured from PBRI, Bhopal, and acclimatized under standard laboratory conditions: temperature 25 ± 3°C, relative humidity 50 ± 5%, and a 12-hour light/dark cycle. Animals were housed in polypropylene cages with free access to a standard pellet diet and water *ad libitum*. All experimental procedures were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and were approved by the Institutional Animal Ethics Committee (IAEC) of the Department of Pharmacy, AIPS, Sagar (M.P.), India with CPCSEA Registration No: 01546/PO./E./S/11/CPCSEA.

### Extraction Process

Freshly collected leaves of *Vitex negundo* and the aerial parts of *Hygrophila auriculata* were thoroughly washed with distilled water to eliminate surface contaminants such as dust and soil particles. The plant materials were then shade-dried at ambient temperature (25–30°C) for 10–14 days to preserve thermolabile bioactive constituents. Once completely dried, the materials were coarsely powdered using a mechanical grinder and stored in airtight containers under desiccated conditions to prevent moisture uptake until further use.

### Soxhlet Extraction

Approximately 100 g of the powdered plant material from each species was packed into a cellulose extraction thimble and subjected to Soxhlet extraction using 500 mL of 95% ethanol as the solvent. The extraction was carried out for 8–10 hours, allowing continuous reflux and efficient solubilization of the phytoconstituents. Ethanol was chosen due to its amphiphilic nature, which enables the extraction of a broad spectrum of phytochemicals including flavonoids, alkaloids, saponins, tannins, and phenolic compounds [17–19].

The extraction process was monitored and considered complete when the solvent in the siphon tube appeared colorless, indicating the exhaustion of extractable compounds. The resulting ethanolic extract was filtered through Whatman No. 1 filter paper to remove insoluble residues [20–21]. The clear filtrate was then concentrated under reduced pressure using a rotary vacuum evaporator at 40–50°C to remove the ethanol, yielding a thick, semi-solid crude extract.

To ensure complete removal of residual moisture, the concentrated extract was further dried in a vacuum desiccator. After drying, the extracts were accurately weighed, and the percentage yield was calculated relative to the initial dry weight of the plant material using the following formula:

Percentage Yield (%) = (Weight of Crude Extract/Weight of Plant Material)×100

The dried extracts were stored in sterile, amber-colored glass vials at 4°C to protect them from light-induced degradation and oxidative damage [22–23].

### Phytochemical Characterization

The ethanol extracts of *Vitex negundo* and *Hygrophila auriculata* were subjected to preliminary phytochemical screening to identify the presence of major classes of bioactive compounds. Standard qualitative chemical tests were employed to detect alkaloids, flavonoids, phenolic compounds, saponins, tannins, glycosides, and terpenoids, following established protocols [24–26].

- **Alkaloids** were detected using Mayer's, Wagner's, and Dragendorff's reagents. A cream, reddish-brown, or orange

precipitate was considered indicative of a positive result.

- **Flavonoids** were identified using the alkaline reagent test and lead acetate test. A yellow coloration that disappeared upon acidification confirmed the presence of flavonoids.
- **Phenolic compounds** were tested using the ferric chloride test. A deep blue or green coloration indicated their presence.
- **Saponins** were determined via the froth test. Persistent frothing upon vigorous shaking indicated saponin content.
- **Tannins** were detected using the ferric chloride test, with a blue-black or greenish precipitate signifying a positive reaction.
- **Glycosides** were tested using the Keller-Kiliani test. A brown ring at the interface confirmed the presence of cardiac glycosides.
- **Terpenoids** were identified by the Salkowski test, in which a reddish-brown coloration at the interface indicated a positive result.

These qualitative analyses confirmed the presence of several pharmacologically relevant phytochemical groups in both plant extracts, supporting their traditional use in hepatoprotective and antioxidant therapies.

***In Vivo* Hepatoprotective Activity**

**Experimental Design and Grouping**

Following a period of acclimatization under standard laboratory conditions, male Wistar rats were randomly assigned to six experimental groups (n = 6 per group) to ensure unbiased allocation and reproducibility of results:

- **Group I (Normal Control):** Received normal saline (vehicle) only.
- **Group II (Toxic Control):** Administered D-galactosamine (D-GalN) to induce acute hepatotoxicity.
- **Group III (Reference Control):** Administered D-GalN + silymarin (100 mg/kg, p.o.), a well-established hepatoprotective agent.
- **Group IV:** Administered D-GalN + *Vitex negundo* ethanolic extract (200 mg/kg, p.o.).
- **Group V:** Administered D-GalN + *Hygrophila auriculata* ethanolic extract (200 mg/kg, p.o.).
- **Group VI:** Administered D-GalN + combination of *V. negundo* (200 mg/kg) and *H. auriculata* (200 mg/kg), both orally.

All treatments were administered once daily via oral gavage for a period of 14 days. To induce hepatotoxicity, D-galactosamine was administered intraperitoneally at a dose of 400 mg/kg on specified days during the experimental protocol [24].

**Biochemical Assessment**

To evaluate the hepatoprotective potential of *Vitex negundo* and *Hygrophila auriculata*, both serum liver function biomarkers and oxidative stress parameters in hepatic tissues were analyzed.

**Serum Biochemical Parameters**

At the end of the treatment period, blood samples were collected from the retro-orbital plexus under light anesthesia. Samples were allowed to clot at room temperature and centrifuged at 3000 rpm for 15 minutes to separate the serum, which was stored at −20°C for subsequent biochemical analysis [26].

The following parameters were quantified using standardized diagnostic kits:

- **Alanine Aminotransferase (ALT):** A marker of hepatocellular injury, ALT activity was measured based on the enzymatic conversion of alanine and α-ketoglutarate to pyruvate and glutamate, with results expressed in U/L [27].
- **Aspartate Aminotransferase (AST):** AST activity, indicative of hepatocellular and mitochondrial integrity, was estimated enzymatically using aspartate and α-ketoglutarate as substrates [28].
- **Alkaline Phosphatase (ALP):** ALP levels were determined through the hydrolysis of p-nitrophenyl phosphate at an alkaline pH, yielding a colored product measurable at 405 nm [29].
- **Total Bilirubin:** Measured using the diazo reaction, bilirubin serves as a critical indicator of hepatic clearance and conjugation efficiency [30].
- **Serum Albumin:** Albumin concentration was estimated using the bromocresol green dye-binding method,

providing insight into the liver's synthetic function [31–32].

**Hepatic Oxidative Stress Markers**

Liver tissues were excised, rinsed with ice-cold saline, and homogenized in 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 rpm for 10 minutes at 4°C, and the resulting supernatants were used for the analysis of antioxidant and oxidative stress markers [33].

- **Superoxide Dismutase (SOD):** SOD activity was determined based on its ability to inhibit the auto-oxidation of pyrogallol, with results expressed in U/mg protein [34].
- **Catalase (CAT):** CAT activity was assessed by monitoring the rate of decomposition of hydrogen peroxide ($H_2O_2$) at 240 nm. Activity was expressed as μmol $H_2O_2$ decomposed/min/mg protein [35].
- **Malondialdehyde (MDA):** Lipid peroxidation levels were measured using the thiobarbituric acid reactive substances (TBARS) method. The MDA-TBA adduct was quantified spectrophotometrically at 532 nm, and results were expressed in nmol MDA/mg protein [36-38].

## 3. RESULTS

The extraction yield was calculated for each plant material based on the dry weight of the powdered raw material. The ethanolic extract of Vitex negundo yielded 26.52% w/w, indicating a high concentration of extractable phytochemicals, whereas Hygrophila auriculata yielded 8.00% w/w, reflecting a moderate phytochemical recovery. These yields are consistent with the differential phytochemical composition and solvent affinity of the respective plant species (Table 1-2).

**Table 1. Phytochemical Studies on *V.Negundo and H.auriculata.***

| **Test** | ***V.Negundo ethanolic*** | ***H.auriculata ethanolic*** |
|---|---|---|
| Carbohydrates | | |
| - molish | + | + |
| - Fehlings | + | + |
| - Benedicts | + | + |
| - Barfoeds | + | + |
| Lipids | | |
| - Libermann Burchard | - | - |
| - Salkowski | - | - |
| Alkaloids | | |
| - mayers | - | + |
| - Dragendroff | - | + |
| - Hagers | - | + |
| Glycosides | | |
| - Killer Kiliani | + | + |
| - Legal test | + | + |
| - Baljet test | + | + |
| - Born trayer | + | + |
| Tannins | | |

| | | |
|---|---|---|
| -Potassium dichromate | - | - |
| - Ferric Chloride | + | - |
| -Potassium ferricyanide | + | - |
| -Potassium cyanide | + | - |
| Saponins | | |
| - Foam test | + | - |
| Flavanoids | | |
| - Shinoda test | - | - |
| Proteins | | |
| - Biuret | - | + |
| - Ninhydrin | - | + |
| - xanthoprotein | - | + |

**Table 2. Rf values found in TLC of methanolic and aqueous extracts of *Vitex negundo***

| **Extracts** | **Rf values** |
|---|---|
| ethanolic of *Vitex negundo* | 0.26, 0.33, 0.39, 0.44, 0.74, 0.57, 0.94 |
| ethanolic of *H. auriculata* | 0.18, 0.28, 0.36, 0.41, 0.53, 0.69, 0.8, 0.93 |

The hepatoprotective efficacy of both plant extracts was evaluated using a well-established D-galactosamine (D-GalN)-induced hepatotoxicity model in rats. This model effectively mimics human viral hepatitis by inducing oxidative stress, inflammation, and hepatocellular necrosis. The therapeutic potential of Vitex negundo and Hygrophila auriculata was assessed through a combination of serum biochemical markers of liver function and histopathological examination of liver tissues.

**Biochemical and Oxidative Stress Marker Analysis**

ALT is a sensitive marker for hepatocellular injury, released into the bloodstream following damage to liver parenchymal cells. In the D-GalN-treated group (Group II), a significant elevation in serum ALT levels was observed ($p < 0.001$) compared to the normal control, confirming liver injury. Treatment with *Vitex negundo* (Group IV) and *Hygrophila auriculata* (Group V) significantly reduced ALT levels by 40% and 35%, respectively, compared to the toxic control ($p < 0.05$). The combination treatment group (Group VI) demonstrated the greatest reduction, with ALT levels decreased by 48%, suggesting a synergistic hepatoprotective effect. Silymarin-treated rats (Group III) showed a 50% reduction, serving as the positive control and further validating the efficacy of the plant extracts (Figure 1).

AST, another transaminase indicative of hepatic and mitochondrial damage, was significantly elevated in the D-GalN group ($p < 0.001$). Administration of *V. negundo* and *H. auriculata* significantly reduced AST levels by 38% and 32%, respectively ($p < 0.05$). The combined extract (Group VI) led to a 50.5% reduction, comparable to the 52% decrease observed in the silymarin group, indicating potent hepatoprotection and likely synergistic activity.

Elevated ALP levels reflect cholestasis and biliary dysfunction. In the toxic control group, ALP levels were significantly increased ($p < 0.001$). Treatment with *V. negundo* and *H. auriculata* resulted in significant reductions of 36% and 30%, respectively ($p < 0.05$). The combination group (Group VI) showed a 45.6% decrease, while silymarin-treated rats exhibited the highest reduction at 48%, indicating improvement in hepatic and biliary function.

Total bilirubin serves as a critical index of liver detoxification and bile excretion capacity. D-GalN treatment markedly increased bilirubin levels ($p < 0.001$), consistent with impaired hepatic clearance. Treatment with *V. negundo* and *H. auriculata* reduced bilirubin by 41% and 36%, respectively ($p < 0.05$), while combination therapy achieved a 47.8% reduction. Silymarin showed the highest effect, reducing bilirubin by 50%, suggesting that the extracts help restore normal hepatic excretory function.

Serum albumin levels, indicative of hepatic protein synthesis, were significantly reduced in the toxic control group ($p < 0.001$). Treatment with *V. negundo* and *H. auriculata* significantly improved albumin levels by 30% and 25%, respectively ($p < 0.05$). The combination group (Group VI) demonstrated a 32% increase, while silymarin administration resulted in the most substantial improvement, with a 35% increase, supporting the restorative effect of the treatments on liver synthetic activity.

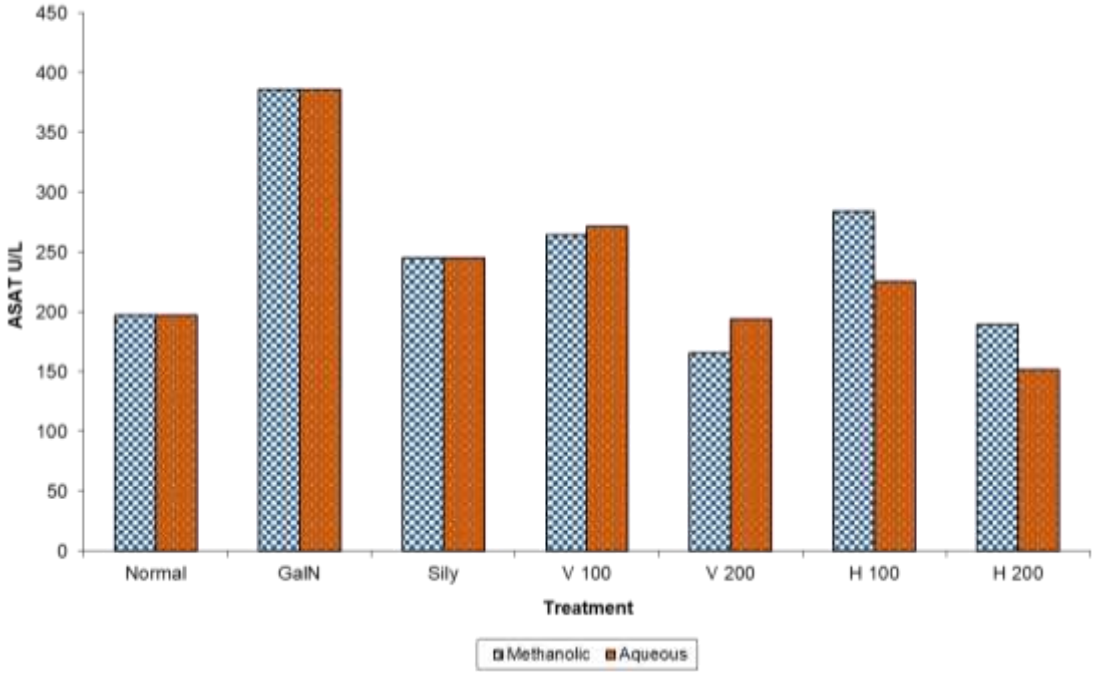


**Figure 1: Effect of plant extracts on the ASAT levels in D-GalN intoxicated rats**

**Oxidative Stress Markers in Liver Tissue**

SOD plays a crucial role in cellular antioxidant defense by neutralizing superoxide radicals. The D-GalN group showed significantly decreased SOD activity ($p < 0.001$), reflecting oxidative stress-induced impairment. Treatment with *V. negundo* and *H. auriculata* significantly increased SOD activity by 28% and 25%, respectively ($p < 0.05$), while the combination group (Group VI) showed a 36% increase. The silymarin group displayed the highest enhancement in SOD levels at 40%, indicating strong antioxidant capacity.

CAT enzymatically decomposes hydrogen peroxide, a major reactive oxygen species. The toxic control group exhibited significantly suppressed CAT activity ($p < 0.001$). Administration of *V. negundo* and *H. auriculata* improved CAT activity by 32% and 28%, respectively ($p < 0.05$). Combination treatment led to a 37% increase, while silymarin-treated rats showed a 40% enhancement, signifying effective neutralization of oxidative stress.

MDA is a biomarker of lipid peroxidation and oxidative membrane damage. MDA levels were significantly elevated in the D-GalN group ($p < 0.001$), indicating extensive oxidative injury. Treatment with *V. negundo* and *H. auriculata* significantly reduced MDA levels by 35% and 30%, respectively ($p < 0.05$), while the combination group achieved a 40% reduction. The silymarin group exhibited the most notable effect, with a 45% decrease, highlighting the protective efficacy of the plant extracts against oxidative damage and lipid peroxidation.

**Statistical Analysis**

All data are presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for pairwise comparisons. A p-value of $< 0.05$ was considered statistically significant. The plant extracts consistently showed improvements in all assessed parameters when compared to the toxic control group, thereby further supporting their hepatoprotective potential.

## 4. DISCUSSION

Liver injury induced by D-galactosamine (D-GalN) is a well-established experimental model to study acute hepatotoxicity. In this study, we evaluated the hepatoprotective potential of *Vitex negundo* Linn (VN) and *Hygrophila auriculata* (HA) in a D-GalN-induced liver damage model in rats. Our findings suggest that both plant extracts exhibit significant protective effects against liver injury, as evidenced by improvements in various biochemical and oxidative stress markers.

The elevated levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), commonly used indicators of hepatocellular injury, were significantly reduced upon treatment with *Vitex negundo* and *Hygrophila auriculata*. In the D-GalN-treated rats, a marked increase in ALT and AST levels was observed, indicating substantial hepatocellular damage. However, treatment with *VN* and *HA* extracts led to a significant reduction in these enzymes, which is indicative of hepatocyte membrane stabilization and prevention of cell damage. The combination of both plant extracts demonstrated the most potent effect, with ALT and AST levels reduced by 48% and 50.5%, respectively, reflecting a synergistic effect. This is consistent with previous reports, where plant-derived antioxidants have been shown to reduce liver enzyme levels by mitigating cellular damage and inflammation.

The decrease in alkaline phosphatase (ALP) levels further supports the liver-protective effects of the extracts. ALP is typically elevated in conditions associated with cholestasis or biliary dysfunction. In our study, the reduction of ALP levels in rats treated with *VN* and *HA* suggests that the extracts may also protect against bile duct obstruction or facilitate biliary flow. The combination group exhibited the most significant reduction in ALP, emphasizing the enhanced protective potential of the two plant extracts when used together.

Moreover, the significant reduction in total bilirubin levels in the treated groups indicates improved liver detoxification and bile excretion. Elevated bilirubin levels are indicative of liver dysfunction, and the observed reductions suggest that the plant extracts might have facilitated the restoration of liver function, which is a vital aspect of hepatoprotection. Similar findings have been reported with various plant extracts known to promote liver regeneration and detoxification.

Albumin, synthesized by the liver, is a marker of hepatic synthetic function. In the present study, *VN* and *HA* treatments significantly elevated serum albumin levels, which were reduced in the D-GalN group, further indicating the restorative effect of the extracts on liver function. The combination of both extracts led to the highest increase in albumin levels, underscoring their synergistic action in improving liver synthetic capacity.

Oxidative stress plays a crucial role in the pathogenesis of liver injury, and markers such as superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) provide insight into the antioxidant status of the liver. In our study, the significant decrease in SOD and CAT activities and the elevated MDA levels in the D-GalN group were indicative of oxidative damage and lipid peroxidation. However, *Vitex negundo* and *Hygrophila auriculata* extracts significantly improved antioxidant enzyme activities (SOD and CAT) and reduced MDA levels, highlighting their ability to counteract oxidative stress.

The combination treatment (Group VI) exhibited the most pronounced effects, with 36% and 37% increases in SOD and CAT activities, respectively, and a 40% reduction in MDA levels. This suggests that the combination of *VN* and *HA* extracts may exert a synergistic antioxidant effect, effectively neutralizing free radicals and protecting against lipid peroxidation. The reduction in MDA levels further supports the hypothesis that these plant extracts protect cellular membranes from oxidative damage, a common feature in liver diseases.

Our results are in agreement with several studies demonstrating the antioxidant and hepatoprotective properties of *Vitex negundo* and *Hygrophila auriculata*. For instance, *Vitex negundo* has been shown to possess potent antioxidant activity due to its rich content of flavonoids, phenolic compounds, and terpenoids, which are known to scavenge free radicals and enhance the liver's endogenous antioxidant defense mechanisms. Similarly, *Hygrophila auriculata* has demonstrated significant hepatoprotective and antioxidant effects in various studies, which may be attributed to its alkaloids, flavonoids, and saponins, all of which contribute to its ability to mitigate oxidative stress and inflammation.

The combination of *Vitex negundo* and *Hygrophila auriculata* extracts (Group VI) demonstrated superior hepatoprotective activity compared to individual treatments. The combination therapy not only showed the highest reductions in liver enzyme levels (ALT, AST, and ALP) and bilirubin, but also exhibited the most significant improvements in oxidative stress markers (SOD, CAT, and MDA). This suggests that the two plant extracts may act synergistically, potentially offering a more comprehensive hepatoprotective effect through their combined antioxidant, anti-inflammatory, and liver regenerative properties.

The synergistic effect could be attributed to the complementary actions of the bioactive compounds present in both plants. For example, while *Vitex negundo* is rich in flavonoids and terpenoids with potent antioxidant and anti-inflammatory properties, *Hygrophila auriculata* contains alkaloids and saponins known for their hepatoprotective and diuretic actions. These compounds might work together to enhance liver function, reduce oxidative damage, and promote liver cell regeneration.

## 5. CONCLUSION

In conclusion, the findings of this study provide strong evidence of the hepatoprotective effects of *Vitex negundo* and *Hygrophila auriculata* in a D-GalN-induced liver injury model. Both plant extracts exhibited significant antioxidant and anti-inflammatory activities, which contributed to the reduction of liver enzyme levels, bilirubin, and oxidative stress markers. The combination of both extracts showed enhanced hepatoprotective effects, suggesting a potential therapeutic strategy for the management of liver diseases. Further studies, including clinical trials, are warranted to confirm these findings and explore the mechanisms underlying their synergistic action.

## 6. DECLARATIONS

**Conflict of interest statement**: There are no conflicts of interest.

**Financial disclosure:** This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

**Acknowledgement:** The authors wish to express their gratitude to experts from Oriental University, Indore, for giving all essential facilities and advice for conducting the study.

## REFERENCES

[1] Mehta NR, Patel EP, Patani PV, Shah B. Nelumbo nucifera (Lotus): A review on Ethanobotany, phytochemistry and Pharmacology. Indian Journal of Pharmaceutical and Biological Research [Internet]. 2013 Dec 31;1(04):152–67. Available from: https://doi.org/10.30750/ijpbr.1.4.26

[2] Hussain S, Asrar M, Rasul A, Sultana S, Saleem U. Chenopodium album extract ameliorates carbon tetrachloride induced hepatotoxicity in rat model. Saudi Journal of Biological Sciences [Internet]. 2022 Feb 17;29(5):3408–13. Available from: https://doi.org/10.1016/j.sjbs.2022.02.014

[3] Ajani RS, Akpovwovwo NA, Jarikre TA, Emikpe BO. Amelioration of Chemical Induced Hepatic Injury by Vitex agnus castus ExtractAmelioration of Chemical Induced Hepatic Injury by Vitex agnus castus Extract. European Journal of Medicinal Plants [Internet]. 2021 Oct 28;23–31. Available from: https://doi.org/10.9734/ejmp/2021/v32i1030422

[4] Parashar AK, Patel P, Gupta AK, Jain NK, Kurmi BD. Synthesis, Characterization andin vivoEvaluation of PEGylated PPI Dendrimer for Safe and Prolonged Delivery of Insulin. Drug Delivery Letters [Internet]. 2019 Aug 20;9(3):248–63. Available from: https://doi.org/10.2174/2210303109666190401231920

[5] Amadi CN, Orisakwe OE. Herb-Induced liver injuries in developing nations: an update. Toxics [Internet]. 2018 Apr 17;6(2):24. Available from: https://doi.org/10.3390/toxics6020024

[6] Borthakur A, Mohan P, Lahon LC, Kalita DJ, Rahman T, Sarmah A. Evaluation of Hepatoprotective Property of Alternanthera sessilis in CCl4 Induced Hepatotoxicity in Rats. International Journal of Current Microbiology and Applied Sciences [Internet]. 2017 Aug 10;6(8):3271–9. Available from: https://doi.org/10.20546/ijcmas.2017.608.390

[7] Parashar AK, Gupta AK, Jain NK. Synthesis and characterization of Agiopep-2 anchored PEGylated poly propyleneimine dendrimers for targeted drug delivery to glioblastoma multiforme. Journal of Drug Delivery and Therapeutics [Internet]. 2019 Jan;74–9. Available from: https://www.researchgate.net/publication/352998628

[8] Ahmad F, Tabassum N. Experimental models used for the study of antihepatotoxic agents. Journal of Acute Disease [Internet]. 2012 Jan 1;1(2):85–9. Available from: https://doi.org/10.1016/s2221-6189(13)60021-9

[9] Najjar FM, Taghavi F, Ghadari R, Sheibani N, Moosavi-Movahedi AA. Destructive effect of non-enzymatic glycation on catalase and remediation via curcumin. Archives of Biochemistry and Biophysics [Internet]. 2017 Jun 28;630:81–90. Available from: https://doi.org/10.1016/j.abb.2017.06.018

[10] Thakkar AP, Shah HJ, Patel ND. Alteration in Antioxidant Defence System and Oxidative Stress in blood of Liver Cirrhosis. International Journal of Scientific Research in Biological Sciences [Internet]. 2019 Feb 28;6(1):47–54. Available from: https://doi.org/10.26438/ijsrbs/v6i1.4754

[11] Wills PJ, Asha VV. Protective effect of Lygodium flexuosum (L.) Sw. (Lygodiaceae) against d-galactosamine induced liver injury in rats. Journal of Ethnopharmacology [Internet]. 2006 May 23;108(1):116–23. Available from: https://doi.org/10.1016/j.jep.2006.04.028

[12] Pawandeep S. Hepatoprotective Activity of Cochlospermum Religiosum Leaves Extract against CCl4 and Ethanol Induced Hepatic Damage in Rats. International Journal for Research in Applied Science and Engineering Technology [Internet]. 2019 Jan 31;7(1):865–72. Available from: https://doi.org/10.22214/ijraset.2019.1139

[13] Biri A, Ketema K, Ayele S, Lule D. Analysis of crop production constraints through participatory rural appraisal in Harari region, eastern Ethiopia; Implications for research and development. Journal of Agriculture and Crops [Internet]. 2019 Oct 1;(510):209–17. Available from: https://doi.org/10.32861/jac.510.209.217

[14] Stickel F, Schuppan D. Herbal medicine in the treatment of liver diseases. Digestive and Liver Disease [Internet]. 2007 Mar 1;39(4):293–304. Available from: https://doi.org/10.1016/j.dld.2006.11.004

[15] Zhang Y, Liu R, Liu C, Li S, Tsao R. Development of ultrasound-assisted mixture extraction and online extraction solution concentration coupled with countercurrent chromatography for the preparation of pure phytochemicals from Phellinus vaninii. Journal of Chromatography B [Internet]. 2021 Mar 7;1171:122619. Available from: https://doi.org/10.1016/j.jchromb.2021.122619

[16] Tang H, Porras G, Brown MM, Chassagne F, Lyles JT, Bacsa J, et al. Triterpenoid acids isolated from Schinus terebinthifolia fruits reduce Staphylococcus aureus virulence and abate dermonecrosis. Scientific Reports [Internet]. 2020 May 15;10(1). Available from: https://doi.org/10.1038/s41598-020-65080-3

[17] Parashar AK, Verma KK, Kumar R, Arora V. A Concise Review of Carbon Dots and their Pharmaceutical and Biomedical Applications. Recent Advances in Drug Delivery and Formulation [Internet]. 2023 Sep 1;17(3):183–92. Available from: https://doi.org/10.2174/0126673878237423230919070049

[18] Mohamadi-Zarch SM, Baluchnejadmojarad T, Nourabadi D, Khanizadeh AM, Roghani M. Protective effect of diosgenin on LPS/D-Gal-induced acute liver failure in C57BL/6 mice. Microbial Pathogenesis [Internet]. 2020 May 7;146:104243. Available from: https://doi.org/10.1016/j.micpath.2020.104243

[19] Petrus AJA. Mukia maderaspatana (Linn.) M. Roemer: A potentially antidiabetic and vasoprotective functional leafy-vegetable. Pharmacognosy Journal [Internet]. 2012 Dec 1;4(34):1–12. Available from: https://doi.org/10.5530/pj.2012.34.1

[20] Shukla B, Saxena S, Usmani S, Kushwaha P. Phytochemistry and pharmacological studies of Plumbago zeylanica L.: a medicinal plant review. Clinical Phytoscience [Internet]. 2021 Apr 9;7(1). Available from: https://doi.org/10.1186/s40816-021-00271-7

[21] Sintayehu B, Bucar F, Veeresham C, Asres K. Hepatoprotective and Free Radical Scavenging Activities of Extracts and a Major Compound Isolated from the Leaves of Cineraria abyssinica Sch. Bip. exA. Rich. Pharmacognosy Journal [Internet]. 2012 Mar 23;4(29):40–6. Available from: https://doi.org/10.5530/pj.2012.29.6

[22] Chaitanya KV, Krishna Ch, Beebi Sk, Divya K. Supercritical fluid extraction of functional ingredients from plants: a review. Current Biochemical Engineering [Internet]. 2015 Feb 26;2(1):24–32. Available from: https://doi.org/10.2174/2212711902666150224233212

[23] Taham T, Cabral FA, Barrozo MAS. Extraction of bixin from annatto seeds using combined technologies. The Journal of Supercritical Fluids [Internet]. 2015 Feb 12;100:175–83. Available from: https://doi.org/10.1016/j.supflu.2015.02.006

[24] Parashar AK, Tyagi LK, Arora V. Histotripsy: A Promising Modality for Cancer Treatment. Current Cancer Therapy Reviews [Internet]. 2024 Sep 19;21. Available from: https://doi.org/10.2174/0115733947330950240906075443

[25] Vongsak B, Sithisarn P, Mangmool S, Thongpraditchote S, Wongkrajang Y, Gritsanapan W. Maximizing total phenolics, total flavonoids contents and antioxidant activity of Moringa oleifera leaf extract by the appropriate extraction method. Industrial Crops and Products [Internet]. 2012 Nov 1;44:566–71. Available from: https://doi.org/10.1016/j.indcrop.2012.09.021

[26] Newman TB, Liljestrand P, Jeremy RJ, Ferriero DM, Wu YW, Hudes ES, et al. Outcomes among Newborns with Total Serum Bilirubin Levels of 25 mg per Deciliter or More. New England Journal of Medicine [Internet]. 2006 May 3;354(18):1889–900. Available from: https://doi.org/10.1056/nejmoa054244

[27] Gudowska M, Wrona A, Gruszewska E, Panasiuk A, Cylwik B, Swiderska M, et al. Simple non-invasive markers for early diagnosis and determination of the severity of liver diseases. Clinical and Experimental Hepatology [Internet]. 2016 Jan 1;4:149–54. Available from: https://doi.org/10.5114/ceh.2016.63872

[28] Mclntyre N, Rosalki S. Tests of the functions of the liver. In: Elsevier eBooks [Internet]. 1994. p. 383–98. Available from: https://doi.org/10.1016/b978-0-7506-0167-2.50027-3

[29] Parashar AK, Saraogi GK, Jain PK, Kurmi B, Shrivastava V, Arora V. Polymer-drug conjugates: revolutionizing nanotheranostic agents for diagnosis and therapy. Discover Oncology [Internet]. 2024 Nov 11;15(1). Available from: https://doi.org/10.1007/s12672-024-01509-9

[30] Kadir NAAA, Azlan A, Abas F, Ismail IS. Preliminary evaluation of supercritical carbon dioxide extracted dabai pulp oleoresin as a new alternative fat. Molecules [Internet]. 2021 Sep 13;26(18):5545. Available from:

https://doi.org/10.3390/molecules26185545

[31] Arshad H, Mehmood MZ, Shah MH, Abbasi AM. Evaluation of heavy metals in cosmetic products and their health risk assessment. Saudi Pharmaceutical Journal [Internet]. 2020 May 29;28(7):779–90. Available from: https://doi.org/10.1016/j.jsps.2020.05.006

[32] Joshi BC, Prakash A, Kalia AN. Hepatoprotective potential of antioxidant potent fraction from Urtica dioica Linn. (whole plant) in CCl 4 challenged rats. Toxicology Reports [Internet]. 2015 Jan 1;2:1101–10. Available from: https://doi.org/10.1016/j.toxrep.2015.07.020

[33] M R, Oa AS, Tm EH, Aa AM. Effect of prolonged vigabatrin treatment on hematological and biochemical parameters in plasma, liver and kidney of Swiss albino mice. Scientia Pharmaceutica [Internet]. 2002 May 8;70(2):135–45. Available from: https://doi.org/10.3797/scipharm.aut-02-16

[34] Kumar A, Kumar N, Anamika, Satya. Significance of hepatic enzymes: A review. International Journal of Advanced Biochemistry Research [Internet]. 2023 Jan 1;7(1):95–100. Available from: https://doi.org/10.33545/26174693.2023.v7.i1b.170

[35] Sharma N, Suri J. Protective Effect of a Standardized Fraction from Vitex negundo Linn. Against Acetaminophen and Galactosamine Induced Hepatotoxicity in Rodents. Biochemistry & Analytical Biochemistry [Internet]. 2016 Jan 1;5(2). Available from: https://doi.org/10.4172/2161-1009.1000267

[36] Aronsohn A, Gondal B. A Systematic Approach to Patients with Jaundice. Seminars in Interventional Radiology [Internet]. 2016 Oct 31;33(04):253–8. Available from: https://doi.org/10.1055/s-0036-1592331

[37] Saleem U, Hira S, Anwar F, Shah MA, Bashir S, Baty RS, et al. Pharmacological Screening of Viola odorata L. for Memory-Enhancing Effect via Modulation of Oxidative Stress and Inflammatory Biomarkers. Frontiers in Pharmacology [Internet]. 2021 May 28;12. Available from: https://doi.org/10.3389/fphar.2021.664832

[38] Lee MH, Cha HJ, Choi EO, Han MH, Kim SO, Kim GY, et al. Antioxidant and cytoprotective effects of morin against hydrogen peroxide-induced oxidative stress are associated with the induction of Nrf-2-mediated HO-1 expression in V79-4 Chinese hamster lung fibroblasts. International Journal of Molecular Medicine [Internet]. 2017 Jan 27;39(3):672–80. Available from: https://doi.org/10.3892/ijmm.2017.2871