

Elm Oyster mushroom *Hypsizygus ulmarius* (Bull.:Fr.) attenuates carbon tetrachloride induced hepatic injury in Wistar rats

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Liver is a vital organ of the body that manages metabolic disposition of drugs and other foreign substances. Liver damage is one of the major causes of human mortality. Natural products including mushrooms have gained researchers' attention as the source of for drugs to treat liver diseases. In this context, here, we examined hepatoprotective activity of ethanolic extracts of the fruiting bodies and mycelia of the Elm oyster *Hypsizygus ulmarius*. Carbon tetrachloride was used to induce hepatic injury and silymarin served as standard drug. Hepatoprotection was evaluated by determining the activities of liver function enzymes, antioxidant status and the histopathological changes in liver tissue of experimental animals. Treatment with the extracts decreased elevated liver function enzymes, such as serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase and alkaline phosphatase and also enhanced depleted antioxidant levels in liver tissue. The histopathological observations supported these findings. The results suggested the potential use of elm oyster mushroom to prevent liver disorders.

Keywords: Antioxidants, CCl₄, Elm Oyster Mushroom, Oxidative stress, *Pleurotus ulmarius*

Liver diseases caused by viral infection, bacterial invasion, alcoholism, toxic chemicals, drugs, obesity, autoimmune disorder are one of the major causes of human mortality. However, drug induced liver injury is considered serious as liver is central to the metabolic disposition of all drugs and foreign substances¹⁻³. Liver damage is commonly associated with cellular necrosis, fat deposition, and increase in tissue lipid peroxidation, oxidative damages and depletion or elevation of many biochemical markers such as liver marker enzymes, triglycerides, cholesterol, bilirubin and alkaline phosphatase⁴.

The efficacy of treatments of liver diseases by modern medicines is limited and associated with serious side effects. Hence, natural products have gained acceptability as effective hepatoprotective agents. Mushrooms are largely unexplored natural source for bioactive compounds for drug discovery although many of them have been used as home remedy to treat various ailments such as rheumatoid arthritis, diabetes, cardiovascular diseases, liver diseases and cancer⁵. A number of mushrooms have been demonstrated to possess significant hepatoprotective effects.

Edible mushrooms are not only considered as food, but also are rich in bioactive compounds of high medicinal value⁶. The elm oyster mushroom, *Hypsizygus ulmarius* (Bull.:Fr.) Redhead (Lyophyllaceae, Agaricomycetes), that grows in clusters on elm trees is one among them. It is widely distributed in the temperate forests of North America, Europe and Japan. *H. ulmarius* is edible and now cultivated on large scale for culinary purposes. Investigations in our laboratory have shown that *H. ulmarius* possessed profound anti-inflammatory, antioxidant and antitumor activities⁷. In this study, we examined the hepatoprotective effect of fruiting bodies and mycelia of *H. ulmarius*.

Materials and Methods

Animals

Male Wistar rats were purchased from Animal Breeding Centre, Kerala Agriculture University, Mannuthy, Thrissur. They were kept under environmentally controlled conditions with free access to standard food and water. Rats weighing 200±20 g were used for the experiment. Experiment was carried out according to the guidelines and approval of Institutional Animal Ethic Committee (IAEC) under the regulation of CPCSEA (Approval No: ACRC/IAEC/15/04 -2).

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Collection of Fruiting bodies

Fruiting bodies were collected from Indian institute of Horticultural Research, Bangalore.

Production of mushroom mycelium

Mycelia of *H. ulmarius* were grown on glucose peptone nutrient medium. The medium was inoculated with 10-day old cultures of *H. ulmarius* and incubated at 25-27°C for 20 days as a stationary culture.

Preparation of mushroom extracts

Fruiting bodies and mycelium were dried at 40-50°C, powdered and extracted with 70% (v/v) aqueous ethanol for 8-10 h using a Soxhlet apparatus. The extract was collected and filtered through Whatman no. 1 filter paper. The solvent was completely evaporated at 40°C using a rotary vacuum evaporator and finally lyophilized, yield of extracts from fruiting bodies and mycelium was 11% (w/w) and 8% (w/w), respectively.

Experimental design

Carbon tetrachloride induced hepatotoxicity

Wistar rats were divided into nine groups of six animals each. Group 1 was given saline alone and kept as normal group. Group 2 was administered with CCl₄ in paraffin oil (1:5, v/v, 3.75 mL/kg body wt., i.p.). Group 3 was treated with standard reference drug silymarin (100 mg/kg p.o.) Aqueous ethanolic extract of fruiting bodies of *H. ulmarius* (250, 500, and 1000 mg/kg, respectively) was administered to groups 4, 5 and 6 and the same concentrations of mycelia extract to groups 7, 8 and 9 orally. Groups 3-9 were administered CCl₄ as in case of group 2. 72 h after the CCl₄ injection animals were sacrificed. Blood was collected from heart. Serum separated for the determination of liver function enzymes. Liver of each animal was removed and then stored at -40°C⁸.

Biochemical analysis

Activities of liver function enzymes

Serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) activities⁹ and Serum alkaline phosphatase (ALP)¹⁰ were determined by standard assay methods.

Determination of antioxidant status in the liver

Liver was excised and rinsed thoroughly in ice cold saline to remove blood. About 10% of the homogenate was prepared in 0.05M phosphate buffer (pH 7) using a polytron homogeniser at 4°C. A part of this homogenate was used for determination of reduced glutathione. Rest of the homogenate was centrifuged at

10000 rpm for 20 min. The supernatant was used for the estimation of superoxide dismutase¹¹, catalase¹², glutathione-S-transferase¹³ and malondialdehyde¹⁴. The protein content was estimated by the method of Bradford¹⁵.

Phytochemical analysis

The aqueous ethanolic extract of *H. ulmarius* was analyzed for the presence of major chemical components using standard methods¹⁶.

Histopathological examination

A small portion of the liver was taken from each sample and placed in a bottle containing 10% formalin and embedded in paraffin, cut into 4-5 µM thick sections and stained with hematoxylin-eosin. Sections were observed for the hepatocellular necrosis, fibrosis and other toxic manifestations.

Statistical analysis

Experimental data are expressed as means ± SD. One-way analysis of variance followed by Dunnet's test was applied for expressing the significance. $P < 0.05$ was considered significant.

Results

Liver function enzymes

CCl₄ administration elevated the level of liver marker enzymes SGOT, SGPT, ALP to 153.12±18.9, 61.65±1.55, 59.18±2.4 IU/L, respectively. Treatment with aqueous ethanolic extracts of fruiting bodies and mycelia of *H. ulmarius* at doses of 1000, 500 and 250 mg/kg exhibited a significant reduction of SGOT, SGPT, ALP levels to 127.4±5.99, 148.23±4.9, 151.41±14.49 IU/L and 40.60±3.01, 51.16±1.13, 58.19±17.43 IU/L and 36.14±26.444, 50.26±13.85, 57.33±1.37 IU/L, respectively. Administration of silymarin also brought down the elevated level of marker enzymes to 111.79±2.29, 35.366±6.68, and 33.45±8.89 IU/L (Fig. 1).

Antioxidant levels in liver

The activity of Catalase was observed to be decreased by CCl₄ intoxication to 38.25±3.0 U/mg compared to normal 110.03±9.80 U/mg. The treatment with Silymarin elevated the enzyme level to 96.44±2.04 U/mg. However the administration of fruiting bodies and mycelia extracts at concentrations 1000, 500 and 250 mg/kg enhanced the activity of catalase to 94.058±7.41, 89.098±8.390 and 84.335±3.64 and 93.87±17.62 U/mg, 77.60±9.49 U/mg and 56.513±3.0 U/mg, respectively (Fig. 1).

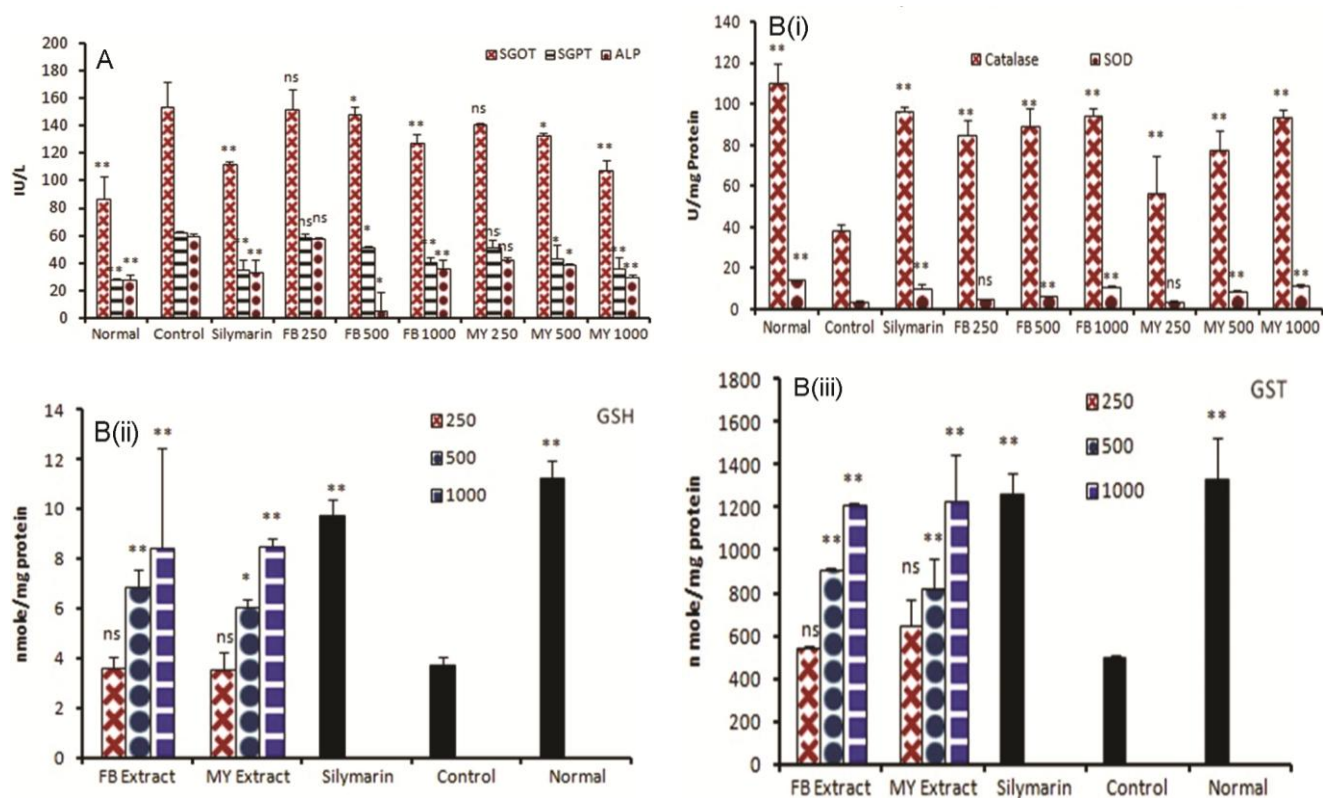


Fig. 1—(A) Effect of aqueous ethanolic extracts of fruiting body and mycelia of *H. ulmarius* on the levels of liver marker enzymes SGOT, SGPT and ALP; and (B) Effect of aqueous ethanolic extracts of fruiting body and mycelia of *H. ulmarius* on the level of various antioxidant enzymes in rat liver in rats exposed to CCl₄. [FB: Fruiting bodies, MY: Mycelia, SGOT: Serum Glutamate Oxaloacetate Transaminase, SGPT: Serum Glutamate Pyruvate Transaminase, ALP: Alkaline Phosphatase. SOD: Superoxide dismutase, GSH: Reduced glutathione, GST: Glutathione-S-transferase. All values are expressed as Mean \pm SD, n=6, ** P < 0.01; * P < 0.05 as compared to control considered as significant and ns P > 0.05 (one way ANOVA followed by Dunnett's test). The P value < 0.001 is considered extremely significant]

The SOD level in the normal animals was found to be 14.09 ± 0.75 U/mg. CCl₄ intoxication depleted SOD activity to 3.08 ± 1.10 U/mg. The SOD level was raised to 13.08 ± 2.29 U/mg on administration with silymarin. The reduced level of SOD after the CCl₄ treatment was restored by treatment with fruiting bodies and mycelia extracts at 1000, 500 and 250 mg/kg concentrations to 11.277 ± 0.41 U/mg, 10.186 ± 0.31 U/mg, 7.302 ± 0.60 U/mg and 11.12 ± 0.76 U/mg, 8.23 ± 1.02 U/mg, 3.07 ± 0.91 U/mg, respectively (Fig. 1).

GST level was also reduced in the CCl₄ treated group of animals to 493.23 ± 13.17 U/mg in comparison with the normal 1334.56 ± 189.46 U/mg. The administration of silymarin elevated the GST level to 1259.65 ± 102.56 U/mg. The treatment with the various concentrations of fruiting bodies and mycelia extracts (1000, 500 and 250 mg/kg) also increased the GST level in a dose dependent manner to 1213.53 ± 7.27 U/mg, 912.101 ± 7.311 U/mg and 548.19 ± 4.38 U/mg and 1231.80 ± 213.89 U/mg, 822.55 ± 143.04 U/mg and 645.70 ± 123.30 U/mg, respectively. (Fig. 1)

Table 1 — Phytochemical screening of aqueous ethanolic extracts of fruiting body and mycelia of *Hypsizygus ulmarius*

Compound	Fruiting body extract	Mycelia extract
Phenolics	++	++
Alkaloids	+++	+++
Saponins	-	-
Flavonoids	-	-
Coumarins	-	-
Tannins	++	++
Quinone	-	-
Steroids/terpenoids	++	++
Polysaccharides	+++	+++
Proteins	+++	+++

GSH level was decreased in CCl₄ intoxicated group to 3.75 ± 0.31 n mol/mg when compared with normal 11.24 ± 0.69 n mol/mg. The treatment with the extracts of fruiting bodies and mycelia at different concentrations 1000, 500 and 250 mg/kg elevated the GSH level in a dose dependent manner. The increase in GSH levels was 8.45 ± 4.033 , 6.88 ± 0.694 and 3.61 ± 0.475 and 8.49 ± 0.33 , 6.05 ± 0.33 and 3.58 ± 0.68 n mol/mg, respectively. (Fig. 1)

Lipid peroxidation

A marked increase in the level of MDA was found in CCl_4 intoxicated group (0.57 ± 0.06 n mol/mg) as compared to normal group which was found to be 0.27 ± 0.10 n mol/mg. Administration of silymarin reduced the MDA level to 0.28 ± 0.04 n mol/mg. Treatment with fruiting body extract at different concentrations 1000, 500 and 250 mg/kg reduced MDA level to 0.234 ± 0.047 , 0.315 ± 0.026 and 0.4321 ± 0.014 n mol/mg. Whereas mycelia extract at same concentrations decreased MDA level to 0.30 ± 0.06 n mol/mg, 0.36 ± 0.021 n mol/mg and 0.43 ± 0.05 n mol/mg. (Fig. 2)

Phytochemical analysis

Phytochemical screening of the extracts was carried out by standard procedures. The major chemical components in the fruiting bodies and its mycelia were alkaloids, polysaccharides and proteins. Other chemical components were phenolics, tannins and terpenoids. (Table 1)

Histopathological examination

Histopathological observation of the hepatic tissue of CCl_4 challenged group showed severe areas of necrosis and plenty of inflammatory cells. About 80% of the hepatocytes showed cytoplasm vacuolation. The necrosis and vacuolation in hepatocytes were significantly reduced by silymarin and extracts treatment. (Fig. 3)

Discussion

Mushrooms represent a major untapped source of bioactives for the development of pharmaceutical products. Studies carried out in our laboratory showed that several mushrooms such as *Ganoderma lucidum*, *Phellinus rimosus*, *Pleurotus* species have significant antioxidant, antitumor and hepatoprotective activities¹⁷. The current experimental studies reveal that aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H. ulmarius* possessed profound hepatoprotective activity against CCl_4 induced hepatotoxicity in Wistar rats. Extracts of *H. ulmarius* were capable to effectively reduce the elevated levels of liver markers such as serum ALT, AST, ALP and lipid peroxidation in CCl_4 challenged rats. The extracts also restored the hepatic antioxidant status after CCl_4 challenge. The histopathological examination of liver tissue revealed that extracts significantly ameliorated the CCl_4 induced liver damage by stimulating liver function or regenerating hepatocytes. These results

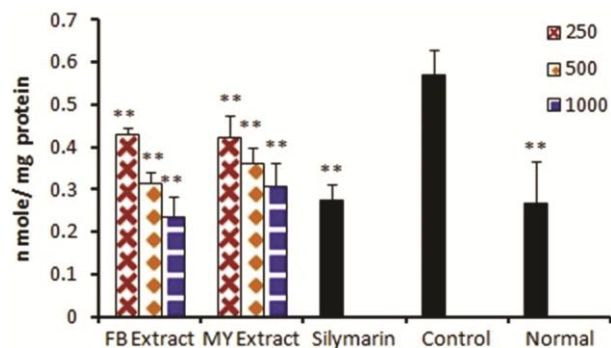


Fig. 2—Effect of aqueous ethanolic extracts of fruiting body and mycelia of *H. ulmarius* on the level of MDA in rats exposed to CCl_4 . [MDA: Malondialdehyde; All values are expressed as Mean \pm SD, n=6, ** $P < 0.01$ as compared to control considered as significant (one way ANOVA followed by Dunnett's test). The p value < 0.001 was considered extremely significant]

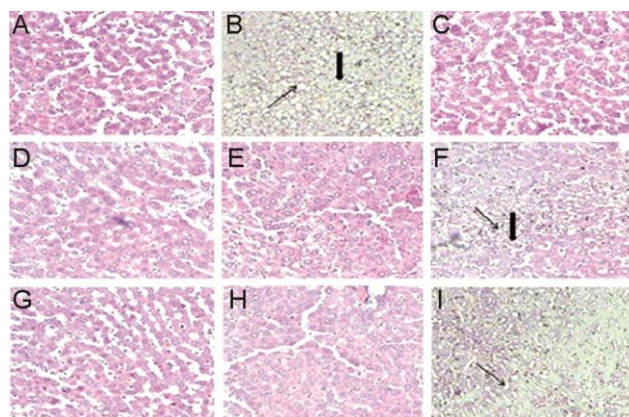


Fig. 3—Histopathology of liver sections (Staining Hematoxylin-Eosin, 10 \times). Black thin arrows: cell necrosis and vacuolation, Black thick arrows: Infiltration of lymphocytes. (A) Normal; (B) Control (CCl_4); (C) Standard (silymarin, 100 mg/kg); (D) Fruiting body extract (1000 mg/kg + CCl_4); (E) 500 mg/kg + CCl_4 ; (F) 250 mg/kg + CCl_4 ; (G) Mycelia extract (1000 mg/kg + CCl_4); (H) 500 mg/kg + CCl_4 ; and (I) 250 mg/kg + CCl_4]

demonstrate the hepatoprotective effect of *H. ulmarius* and its cultured mycelia.

Chemical toxins such as carbon tetrachloride induce acute hepatocyte injury and single exposure can rapidly lead to severe centrilobular necrosis, and steatosis. In the initial phase, CCl_4 is metabolically activated by Cytochrome P450-dependent oxidases in the endoplasmic reticulum and mitochondria to form highly reactive trichloromethyl and trichloromethyl peroxy free radicals which covalently bind to cellular macromolecules, to induce lipid peroxidation, resulting in the loss of membrane integrity and leakage of microsomal enzymes. Reactive aldehydes, such as MDA are released as byproducts of lipid peroxidation that can form protein and DNA adducts leading to

hepatotoxicity¹⁸. The second phase involves the activation of Kupffer cells, which is accompanied by the production of pro-inflammatory mediators. Recent reports have demonstrated that induced nitric oxide overproduction occurs in the liver of rats by CCl₄-induced acute liver injury. This suggested that iNOS might act as a mediator in the pathogenesis of hepatotoxicity in rats which upregulates the inflammatory response through specific signaling mechanisms¹⁹.

Serum transaminases and alkaline phosphatase have long been considered as sensitive indicator of hepatic injury²⁰. Injury to the hepatocytes alters their transport function and membrane permeability, leading to leakage of enzymes from liver cells to the blood²¹. This leakage causes a decrease in levels of GPT, GOT and ALP in hepatic cells but increase in level of serum GOT, GPT and ALP²². This explains the increase in level of SGOT, SGPT and ALP in the control group in the present study. Extracts significantly preserved the structural integrity of the hepatocellular membrane and reduced CCl₄-induced hepatic injury in a dose dependent manner. The observation is also supported by histological evidences. In this study, CCl₄ caused a variety of histological changes to the liver, including necrosis and inflammation. These changes were significantly attenuated by the extracts of fruiting bodies and mycelia of *H. ulmarius*

Glutathione is a major, non-protein thiol in living organisms which performs a key role in co-ordinating innate antioxidant defence mechanisms. It is involved in the maintenance of normal structure and function of cells, probably by its redox and detoxification reactions²³. Reduced glutathione (GSH) plays a key role in the detoxification of the reactive toxic metabolites of CCl₄. Liver necrosis is initiated when reserves of GSH are markedly depleted^{24,25}. Thus, the reduced level of GSH was observed in the present investigation in CCl₄ alone treated group is consistent with the results of earlier studies. Interestingly, in the present study, group of animals administered with fruiting bodies and mycelia extracts at concentrations 1000, 500 and 250 mg/kg showed significant elevation in GSH level. Earlier studies have reported that when the lipid peroxidation rate is very high, GSH gets depleted because of high rate of scavenging²⁶. Hence, the observed low levels of GSH in the CCl₄ alone treated group indicates high rate of lipid peroxidation.

MDA (Malondialdehyde) a major reactive aldehyde is an indicator of tissue damage involving a series of

chain reactions. Lipid peroxidation has been implicated in the pathogenesis of increased membrane rigidity, osmotic fragility, reduced erythrocyte survival and perturbations in lipid fluidity. It has been hypothesized that one of the principal causes of CCl₄ induced hepatotoxicity is lipid peroxidation of hepatocyte membranes by free radical derivatives of CCl₄²⁷. The increase in MDA levels in liver of CCl₄ alone treated group suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals²⁸. Maintenance of normal levels of hepatic MDA in treated group is of great interest since it provides additional evidence to support the hepatoprotective effect of *H. ulmarius*.

Free radicals such as superoxide radical, hydroxyl radical (.OH), hydrogen peroxide (H₂O₂), and lipid peroxide radicals have been implicated in liver diseases²⁹. These reactive oxygen species (ROS) are produced as a consequence of biochemical processes in the body as a result of increased exposure to xenobiotics³⁰. In general, antioxidative enzymes such as SOD and CAT are easily inactivated by peroxy radical (O²⁻) or ROS which results in decreased activities of these enzymes in CCl₄ toxicity. This explains the significant reduction in the activities of CAT and SOD observed in rats administered with CCl₄. The activity of these enzymes was brought almost near to normal level in the extracts treated group of animals.

Preliminary phytochemical analysis showed the presence of polysaccharides, tannins, alkaloids, steroids/terpenoids in both extracts (Table 1). Recent studies demonstrated that phenolics, triterpenes, alkaloids, polysaccharides and peptides are the classes of compounds which might be responsible for the hepatoprotective activity of the mushroom extracts³¹. However, the current experimental results indicate that polysaccharides might be the major bioactive compound responsible for the hepatoprotective activity of *H. ulmarius*.

Conclusion

The present experimental findings reveal that the aqueous ethanolic extracts of *HypsizyGus ulmarius* and its cultured mycelia possessed profound hepatoprotective activity as evident from their effect to decrease elevated liver function enzymes and to up regulate the depleted antioxidant status in liver tissue. Being an edible mushroom, hepatoprotective effect of this mushroom has significant use in the development of

dietary supplements or nutraceuticals, for therapeutic use.

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Conflict of interest

The authors declare that they have no conflict of interest.

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