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Bioactive extracts of fruiting bodies and cultured mycelia biomass of elm Oyster mushroom *Hypsizygus ulmarius* (Bull.:Fr.) alleviate alcohol-induced hepatic injury in Wistar rats

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Hepatic injury is a common presentation of chronic alcohol intake. Alcohol liver diseases lead to a spectrum of diseases, including fatty liver, hepatitis and cirrhosis. Currently, no ideal treatment is available for this condition. Natural products and their derivatives are considered promising hepatoprotective agents. Mushrooms are considered a delicacy and rich source of bioactive compounds. Hence, we examined the hepatoprotective activity of aqueous ethanolic extracts of an edible mushroom, *Hypsizygus ulmarius* and its cultured mycelia. Hepatic damages were induced by oral administration of ethanol (36%, 2 mL/ 100 g body weight) for 30 days. Silymarin (100 mg/Kg body weight) was used as a standard reference drug. Administration of ethanol significantly elevated serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, and alkaline phosphatase. Treatment with the bioactive extracts of fruiting bodies and cultured mycelia significantly down-regulated activities of liver injury maker enzymes and elevated activities of superoxide dismutase, catalase, reduced glutathione, glutathione-s-transferase, and reduced the level of malondialdehyde in hepatic tissue. Histopathological observations of hepatic tissue, such as reduction in centrilobular necrosis, fatty infiltration and lymphocytic infiltration, also supported the protective effect of the extracts. The results thus indicated that bioactive extracts of *H. ulmarius* and its mycelia possessed a significant protective effect against alcohol-induced liver toxicity in Wistar rats. The findings suggested the potential therapeutic use of this edible mushroom for ameliorating alcohol-induced liver injury.

Keywords: Alcohol, Hepatic injury, Hepatoprotection, *Hypsizygus ulmarius*, Liver injury markers, Medicinal mushroom

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Introduction

The liver is continuously exposed to environmental toxins, drugs and alcohol, which can eventually lead to various liver ailments. According to WHO, heavy drinking is related to several injury conditions resulting in 3.3 million deaths worldwide[1]. Alcoholic liver diseases are one of the current health problems and the second leading cause of death among all liver ailments. Alcohol consumption is associated with a large range of health problems. Alcohol liver diseases lead to a spectrum of diseases, including fatty liver, alcohol hepatitis and cirrhosis[2]. Currently, no ideal treatment is available for this condition. Oxidative stress is the key factor for alcoholic liver damage. The majority of the modern medicines to treat liver diseases have serious side effects. Hence, efforts have been made in recent years to develop potent hepatoprotective agents with little or no side effects. Natural products and their derivatives are considered promising candidates for developing hepatoprotective agents. Currently, a few natural product-based hepatoprotective drugs, such as Liv 52 and silymarin, are in clinical use.

One of the main strategies to prevent hepatic damage is reducing the effects of reactive oxygen species by antioxidants. Natural antioxidant sources such as mushrooms have been the subject of research as liver protectants[3]. Several medicinal mushrooms were examined in our laboratory for their hepatoprotective property. This includes *Ganoderma lucidum*, *Pleurotus ostreatus*, *Phellinus rimosus* and *Morchella esculanta*[4,5]. *Hypsizygus ulmarius*, an edible mushroom known as elm Oyster mushroom, is cultivated on a large scale for culinary purposes in India. The mushroom is demonstrated to possess significant anti-tumour (DLA cell line induced solid tumour model), anti-inflammatory (Carrageenan induced acute and Formalin induced chronic model) and antioxidant activities (DPPH, ABTS)[6]. However, adequate information is not available on the hepatoprotective activity of this mushroom. Hence,

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we examined the hepatoprotective activity of this mushroom against ethanol-induced hepatotoxicity in an animal model.

Materials and Methods

Animals

Wistar albino female rats were purchased from the Small Animal Breeding Centre, Kerala Agriculture University, Mannuthy, Thrissur. They were kept under environmentally controlled conditions such as temperature (22±2°C), humidity (50%), proper ventilation, caging, and free access to standard food and water. Rats weighing 200±20g were used for the experiment. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) (Approval No: ACRC/IAEC/15/04 -2) and approval of Institutional Animal Ethic Committee (IAEC).

Collection of Fruiting bodies

Fruiting bodies of *H. ulmarius* were collected from local mushroom growers in Kodungallur, Thrissur, during June, 2015.

Production of mushroom mycelium

The culture of *H. ulmarius* was developed from the fruiting bodies of the mushroom. The isolate was grown on a glucose peptone nutrient medium (10 g glucose, 3 g peptone, 1 g KH_2PO_4, 1 g K_2HPO_4, 0.25 g $MgSO_4$, 1 g yeast extract, 1000 mL distilled water). 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. After the incubation period, the cultures were filtered through several layers of muslin cloth, and the mycelia biomass was washed with tap water several times and then dried.

Preparation of extracts

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

Determination of hepatoprotective activity

Wistar albino female rats were divided into nine groups of six animals in each group. Group 1 was given saline alone and kept as a normal group. Group 2 was administered with ethanol (36%, 2 mL/100g body weight p.o) for 30 days. Group 3 was treated with the standard reference drug silymarin (100 mg/kg p.o). Aqueous ethanolic extract of fruiting bodies of *H. ulmarius* (250, 500, and 1000 mg/kg body weight) was administered to groups 4, 5 and 6, and the same concentrations of mycelia extract to groups 7, 8 and 9 orally. Groups 3, 4, 5, 6, 7, 8, and 9 were administered ethanol as in the case of group 2 for 30 days[6]. Twenty-four hours after the last dose of treatments, animals were sacrificed. Blood was collected from the heart. Serum separated for the determination of liver function enzymes. The liver of each animal was removed and then stored at -40°C[7].

Determination of activities of liver function enzymes

Serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), and Serum alkaline phosphatase (ALP) activities were determined by standard assay methods[8,9].

Determination of antioxidant status in the liver

The liver was excised and rinsed thoroughly in ice-cold saline to remove blood. 10% of the homogenate was prepared in 0.05 M phosphate buffer (pH 7) using a polytron homogeniser at 4°C. A part of this homogenate was used for the determination of reduced glutathione[10]. The rest of the homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was used for the estimation of superoxide dismutase (SOD)[11], catalase (CAT)[12] glutathione-S- transferase (GST)[13] and malondialdehyde (MDA)[14]. The protein content was estimated by the method of Bradford[15].

HPTLC analysis of bioactive extract

The samples were dissolved in methanol (10 mg/mL) and applied to a Silica gel 60 F_{254} TLC plate (E. Merck, Germany) (7 cm x 10 cm) using Linomat V sample applicator and the plate was then developed upto 80 mm in a twin trough glass chamber using the mobile phase chloroform-methanol-water 30:4:1, derivatised using vanillin sulphuric acid reagent, heated at 110°C for 10 min. The plate was then scanned by a densitometer at 580 nm using TLC Scanner 3 equipped with WinCats software.

Histopathological examination of liver tissue

A small portion of the liver was taken from each sample and placed in a bottle containing 10% formalin for histopathology observation. The tissue-

thin sections were cut on a microtome into 3–5 mm thick pieces and stained with 1% hematoxylin-eosin embedded in paraffin. Sections were observed for hepatocellular necrosis, fibrosis and other toxic manifestations.

Statistical analysis using SPSS

Experimental data were 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 injury marker enzymes

Ethanol administration elevated the levels of liver marker enzymes SGOT, SGPT, and ALP to 145.02±3.62, 56.533±2.85, and 234.58±4.51 IU/L, respectively. Treatment with aqueous ethanolic extract of fruiting bodies of *H. ulmarius* at doses of 1000, 500 and 250 mg/kg exhibited a significant reduction of SGOT, SGPT, and ALP levels to 94.34±4.34, 41.122±3.78, 191.903±3.344 IU/L, 107.035±7.77, 41.29±1.13, 192.905±1.10 IU/L, and 111.078±8.644, 47.51±3.16, 234.86±3.08 IU/L respectively. The mycelia extract at the same doses showed a reduction of SGOT, SGPT, and ALP levels to 95.74±3.90, 38.02±2.01, 180.53±3.06 IU/L, 105.49±4.35, 42.19±1.03, 192.90±3.39 IU/L, and 134.11±3.43, 55.43±1.34, 220.18±9.5 IU/L respectively. Administration of Silymarin also lowered the elevated marker enzyme level to 111.79±2.29, 35.366±6.68, and 133.45±8.89 IU/L (Fig. 1).

Antioxidant levels in liver tissue homogenate

The activity of catalase was observed to be decreased by ethanol intoxication to 40.63±3.25 U/mg compared to normal 69.23±4.03. The administration of fruiting bodies and mycelia extracts at concentrations 1000, 500 and 250 mg/kg enhanced the activity of catalase to 64.546±7.41, 58.76±1.99 and 46.14±2.25 and 66.09±7.46, 56.925±2.15 U/mg and 49.066±3.221 U/mg respectively. The treatment with silymarin also elevated the enzyme level to 65.285±3.11 U/mg (Table 1).

The SOD level in the normal animals was found to be 14.996±1.01 U/mg. Ethanol administration depleted SOD activities to 3.79±0.347 U/mg. The level of SOD after the ethanol treatment was restored by treatment with fruiting bodies and mycelia extracts at 1000, 500 and 250 mg/kg concentrations to 11.277±0.41, 10.186±0.31, 7.302±0.60 and 10.77±0.499, 7.908±0.337, 6.7±0.34 U/mg respectively. The SOD level was raised to 12.265±0.496 U/mg on administration with Silymarin (Table 1).

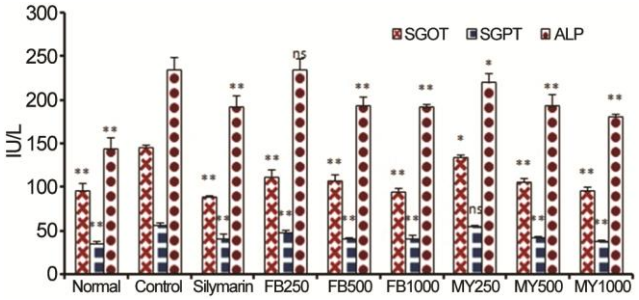

Fig. 1 — Effect of aqueous ethanolic extracts of fruiting bodies and mycelia of *H. ulmarius* on the levels of SGOT, SGPT, and ALP in rats exposed to ethanol.

Table 1 — Effect of aqueous ethanolic extracts of fruiting bodies and mycelia of *H. ulmarius* on the level of antioxidant enzymes in rat liver exposed to ethanol

Group	CATALASE (U/mg Protein)	GST (nmol CDNB Conjugate formed/min/mg protein)	SOD (U/mg Protein)	GSH (nmol DTNB/min/mg protein)
Normal Control	69.23±4.032**	918.89±36.95**	14.996±1.01**	8.56±0.408**
Control (Ethanol) 36% 2 mL/100g body weight	40.63±3.25	468.24±26.66	3.79±0.347	2.085±0.371
Silymarin (100 mg/Kg)+Ethanol	65.285±3.110**	700.30±6.66**	12.26±0.496**	6.61±0.416**
FB (1000 mg/Kg)+Ethanol	64.54±4.10**	837.87±32.63**	11.27±0.411**	6.36±0.300**
FB (500 mg/Kg)+Ethanol	58.76±1.99**	560.97±18.02 **	10.18±0.313**	4.788±0.134**
FB (250 mg/Kg)+Ethanol	46.14±2.25 ns	442.30±30.02 ns	7.302±0.66**	2.16±0.106 ns
MY (1000 mg/Kg)+Ethanol	66.09±7.46**	802.84±20.63**	10.77±0.499**	7.3±0.142**
MY (500 mg/Kg)+Ethanol	56.925±2.15**	771.92±3.79 **	7.9±0.33**	4.71±0.074**
MY (250 mg/Kg)+Ethanol	49.06±3.22 ns	454.43±8.39 ns	6.7±0.347**	2.3±0.114

FB – Fruiting Body extract, MY – Mycelium extract. All values are expressed as Mean±SD, n=6, **P <0.01 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.

GST level also declined in the ethanol-treated group of animals to 468.241±26.20 U/mg as compared to the normal 918.89±36.933 U/mg. The treatment with 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 837.87±32.63, 560.97±18.02 and 442.30±30.02 U/mg, and 802.84±20.63, 771.92±3.7 and 454.93±8.39 U/mg respectively. The administration of silymarin elevated the GST level to 700.30±6.662 U/mg (Table 1).

GSH level was decreased in the ethanol-intoxicated group to 2.085±0.371 nmol/mg compared to normal 8.56±0.408 nmol/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 6.36±0.330, 4.788±0.134 and 2.165±0.106 nmol/mg and 7.3±0.142, 4.72±0.074 and 2.3±0.114 nmol/mg respectively (Table 1).

Lipid peroxidation

A marked increase in the MDA level was found in the ethanol-administered group (0.86±0.013 nmol/mg) compared to the normal group of animals. MDA increases were found to be 0.319±0.002 nmol/mg. Treatment with fruiting body extract at different concentrations of 1000, 500, and 250 mg/kg reduced MDA level to 0.305±0.024, 0.319±0.004 and 0.402±0.014 nmol/mg. Whereas mycelia extract at the same concentrations decreased MDA level to 0.410±0.06, 0.552±0.0214 and 0.727±0.05 nmol/mg. Administration of Silymarin reduced the MDA level to 0.451±0.006 nmol/mg. (Fig. 2).

HPTLC analysis

The HPTLC analysis revealed that the aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H. ulmarius* contain many major and minor compounds. Nine major compounds were observed in both extracts but with more concentration in mycelia than fruiting bodies extract. The presence of different compounds with varying concentrations was detected in HPTLC analysis. Fig. 3 represents the HPTLC profile of the extracts using Chloroform-Methanol-Water (30:4:1) as a solvent system.

Histopathological examination

Histopathological observation of the liver tissue of animals intoxicated with alcohol showed severe toxic symptoms such as centrilobular necrosis, fatty infiltration and lymphocyte infiltration. The toxic manifestations were significantly decreased by the fruiting body and mycelia extracts of *H. ulmarius* and Silymarin treatment (Fig. 4).

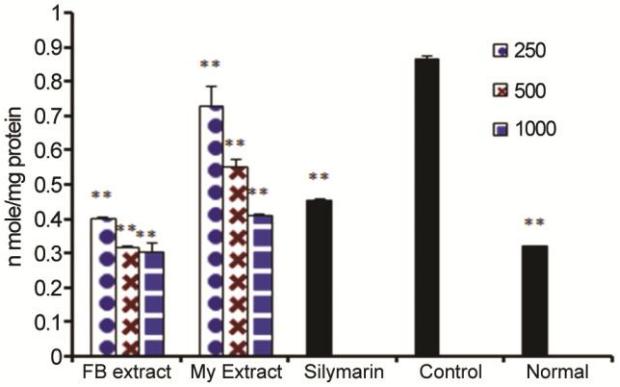

Fig. 2 — Effect of aqueous ethanolic extracts of fruiting bodies and mycelia of *H. ulmarius* on the level of MDA in rats exposed to ethanol.

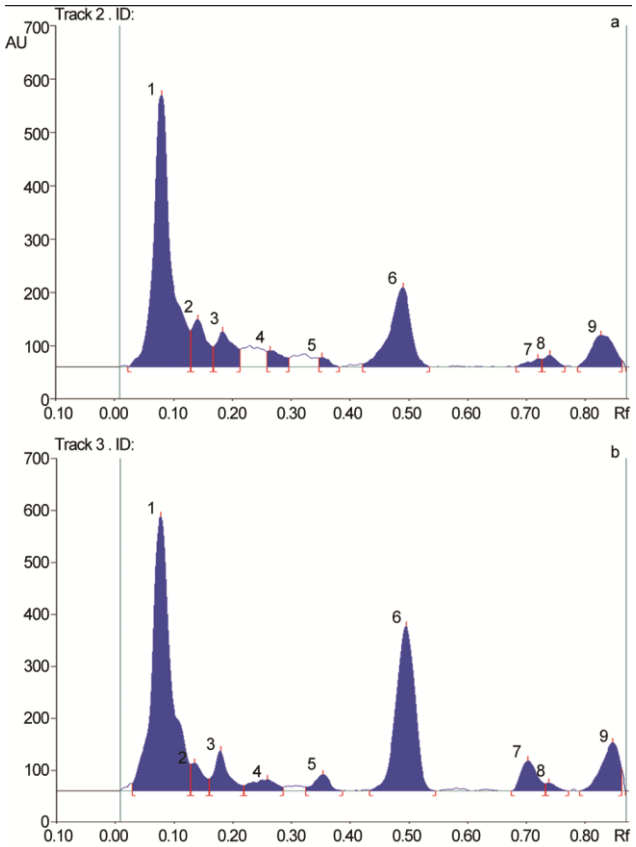

Fig. 3 — HPTLC finger print of aqueous ethanolic extracts of *Hypsizygus ulmarius*. Plates sprayed with vanillin- sulphuric acid and scanned with densitometer at 580 nm. a) FB: Fruiting body extract; and b) MY: Mycelium extract.

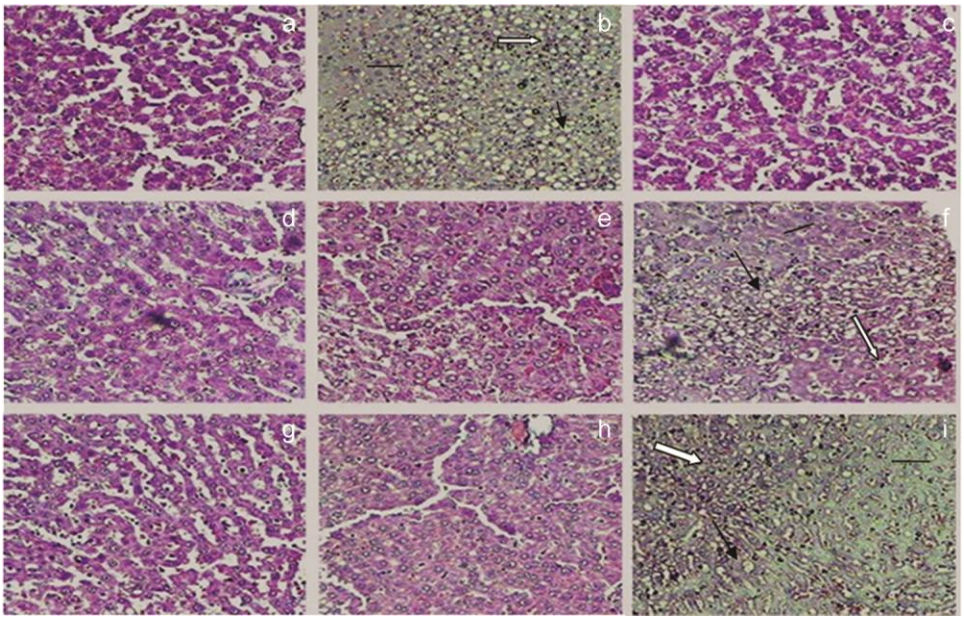

Fig. 4 — Histopathology of liver sections (Staining Hematoxylin- Eosin, 10×). Indicates Centrilobular Necrosis, Fatty infiltration Lymphocyte infiltration. a) Normal; b) Control (Ethanol); c) Standard (Silymarin,100 mg/kg); d) Fruiting body extract (1000 mg/kg + Ethanol); e) 500 mg/kg + Ethanol; f) 250 mg/Kg + Ethanol; g) Mycelium extract (1000 mg/Kg + Ethanol); h) 500 mg/kg + Ethanol; and i) 250 mg/kg + Ethanol.

Discussion

Liver diseases have been one of the major health concerns in recent years. The disease is an off-shoot of lifestyle changes. Ethanol causes hepatotoxic injury. Heavy alcohol drinkers consuming 50-60 g of ethanol daily are at high risk of developing alcoholic liver diseases (ALD_s)[16]. In the liver, alcohol is metabolised by alcohol dehydrogenase by xenobiotic metabolism through cytochrome P-450 pathways. Alcohol dehydrogenase and acetaldehyde dehydrogenase increase the production of acetaldehyde from alcohol, which is more toxic than alcohol as it reduces NAD to NADH. The change in the NAD/NADH ratio inhibits gluconeogenesis and fatty oxidation, causing liver fatty degeneration.

Similarly, when combined with cellular proteins, acetaldehyde becomes antigenic and enters the cycles of inflammation, leading to liver fibrosis and cirrhosis[17,18]. Acetaldehyde is further oxidised to acetate by Xanthine oxidase in the liver, leading to the production of reactive oxygen species (ROS) through CYP2E1 pathways, which result in hepatocyte necrosis, apoptosis and inflammation[19]. Alcohol consumption is known to cause changes in membrane fluidity due to increased lipid peroxidation, eventually resulting in loss of membrane structure and integrity[20]. The histopathological observations of liver samples of ethanol and bioactive extracts of *H. ulmarius* treated animals support this conclusion.

Our findings demonstrate that long-term administration of ethanol (36% v/v) causes severe hepatic damage, as evident by the significant elevation of aminotransferase enzyme activities, which are sensitive markers in the diagnosis of liver diseases. Ethanol administration for 30 days significantly increased all serum liver marker enzymes, whereas treatments with the bioactive extracts of *H. ulmarius* significantly reduced SGOT, SGPT and ALP, indicating the hepatoprotective effect[21]. Earlier studies suggest that the protective effect of the extracts could be the result of the stabilisation of the plasma membrane, preventing the structural integrity of liver cells and repairing hepatic tissue damage caused by ethanol and thus preventing enzyme leakage into blood circulation[22].

Mechanisms of alcohol-induced liver injury have been an area of intense investigation. Recent advances in this field mainly focus on alcohol-induced oxidative stress, inflammation, metabolic disorders, apoptosis, necrosis, gut-driven bacteria and endotoxin actions[1]. Oxidative stress and lipid accumulation play important roles in alcohol-induced liver injury. Alcohol administration in animals increases the formation of lipid peroxidation products, such as malondialdehyde (MDA), a marker of lipid

peroxidation[23]. Increased accumulation of lipid peroxidation products in cells can result in tissue damage and failure of antioxidant systems to prevent the formation of excessive ROS[24]. Near-normal levels of hepatic MDA maintained in extracts treated groups of animals provide additional evidence indicating their anti-lipid peroxidative effect and hepatoprotective activity[25]

Reactive Oxygen Species are important in the aetiology and progression of many diseases, including alcoholic liver diseases. Free radicals such as superoxide, hydroxyl radicals, and hydrogen peroxides generated during the metabolism of ethanol are the main causes of hepatic injury[26,27]. Earlier studies demonstrated that ROS formation by chronic alcohol challenge results in decreased antioxidant enzymes level, including CAT, SOD, and GST and causes an increase in lipid peroxidation of cellular membranes, protein and DNA damage, leading to hepatocyte injury[28]. Our studies show that the extracts of *H. ulmarius* caused a significant increase in the hepatic SOD and catalase activities, thus preventing free radical accumulation and protecting the liver from injury compared to control.

Glutathione s-transferase (GST) plays an important role in the liver by eliminating toxic acetaldehyde by conjugating it with glutathione[29]. GST activities were significantly decreased in the liver of rats exposed to ethanol. The decline in the activity may be due to its involvement in the detoxification process in the liver cells. Pre-administration of extracts of *H. ulmarius* to the alcohol-exposed rats normalised the activities of GST. Chronic ethanol-induced toxicity resulted in a significant decrease in GSH. The liver injury caused by ethanol is known to be related to low tissue levels of GSH. This is evident from the depleted GSH level in the ethanol-alone treated group, whereas *H. ulmarius* extracts treated groups of animals significantly restored its activity.

Mushrooms are functional foods and sources of valuable bioactive compounds that offer great therapeutic potential for preventing and managing several diseases. However, the therapeutic potentials of mushrooms are enormous, although the detailed mechanisms involved in eliciting most of these pharmacological activities remain elusive and thus require evidence–based insights[30].

Conclusion

Hypsizygus ulmaius is an excellently edible mushroom cultivated on a large scale for culinary purposes in India. Bioactive extracts of the fruiting bodies and mycelia of this mushroom are found to possess significant hepatoprotective activity against alcohol-induced liver injury. The results indicate the use of this mushroom as a useful dietary supplement for liver protection. The findings thus suggest the potential therapeutic use of *H. ulmarius* as a functional dietary supplement for ameliorating alcohol–induced liver injury. In conclusion, fruiting bodies and mycelia extracts of *H. ulmarius* possess significant hepatoprotective effects against ethanol-induced chronic liver injury in rats. The hepatoprotective activity of *H. ulmarius* can be attributed to the phytochemical constituents, which possessed significant antioxidant activity. Since *H. ulmarius* is an excellently edible mushroom, the findings have significant therapeutic use as a functional dietary supplement.

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Conflict of interest

The authors declare no conflict of interest.

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