



Original Research Article (Experimental)

Amelioration of anti-hepatotoxic effect by *Lichen rangiferinus* against alcohol induced liver damage in rats



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ABSTRACT

Background: Reindeer lichen, *Lichen rangiferinus* syn. or *Cladonia rangiferina* (L.) F. H. Wigg. (Cladonia-ceae) has been traditionally reported as a remedy to treat fever, colds, arthritis as well as convulsions, liver infections, coughs, constipation, and tuberculosis. The current study is aimed at rectification of alcohol induced liver damage by the use of *L. rangiferinus* extract.

Objectives: The aim of the study was to compare some biochemical markers for liver injury and hematological indices in normal untreated rats and treated rats.

Material and Methods: The study was performed using male Wistar rats. Animals were categorized into five groups, negative control group (normal diet only), treated groups (2 groups were lichen treated along with 10% ethanol & 1 group was only ethanol treated) and positive control group (Silymarin + 10% ethanol) of six animals in each group. Biochemical markers for liver injury and hematological indices of all animals were measured using standard diagnostic tools. The animals were then sacrificed and livers were sent to the pathology lab for histopathological analysis.

Results: Lichen extract showed a significant restoration of altered biochemical parameters towards normal in both *in vitro* and *in vivo* conditions. The total phenolic and flavonoid content of the LRE was found to be 21.78 µg PE/mg of extract and 5.13 µg RE/mg of extract respectively. The IC₅₀ values for atranorin and fumarprotocetraric acid were found to be 128.48 and 218.46 mg/mL respectively. Reducing power of the extract was found to be quite significant. After administration of lichen extract, endothelial cells were less injured around central vein and number of fat vacuoles was also lesser in hepatocytes.

Conclusion: Conclusively, treatment with lichen extract assuages alcohol-related damage and guards hepatic tissue from alcohol-induced toxicity.

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1. Introduction

Alcohol liver disease is a highly pervasive ailment among human beings which environs a range of hepatic disorders starting from simple steatosis (fatty liver) to cirrhosis/liver failure [1]. Ethanol consumption enhances the ratio of NADH/NAD⁺ in hepatocytes which causes disruption of β-oxidation of fatty acids in mitochondria leading to steatosis. Alcohol also increases the lipid transport to the liver from the small intestine leading to enhanced mobilization of fatty acids from adipose tissue which is taken up by the liver [2].

This causes damage to cell membrane of hepatocytes leading to augmented levels of transaminases (Alanine aminotransferase (ALT) and AST (Aspartate aminotransferase)) in blood stream. Alkaline phosphatase (ALP) is also present in hepatocytes which come into the circulation indicating hepatic damage. Gamma-glutamyl transferase (GGT) has a key role in preserving intracellular homeostasis of oxidative stress which protects cells against oxidative damage. It is present in cell membrane and is set free in circulation when cell membrane is damaged. Glutathione (GSH) is a powerful antioxidant in our body which prevents damage by oxidative stress which is lowered by alcohol. Alcohol intake causes accumulation of oxidative stress markers (Glutathione reductase (GR) and malondialdehyde (MDA) which causes further damage to liver [3].

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Traditionally *Lichen rangiferinus* (Family: Cladoniaceae, Genus: Cladonia) has been used to cure various ailments such as fever, diarrhoea, infections, skin diseases, epilepsy, convulsions, as purgative [4], against liver ailments amongst many other ailments [5,6]. It has great antimicrobial and antioxidant properties [7]. *L. rangiferinus* consists of a number of phytochemical constituents such as atranorin, fumarprotocetraric acid, communic acid, monbretol, acetylmbriatolonic acid, b resonicylic acid, obtuanhydride, sugiol, atranol, barbatic acid, homosekikaic acid, didymic acid, condidymic acid, imbricatolonic acid, junicedric acid, hana-gokenols A and 7 a -hydroxy-sandaracopimaric acid which have not yet been investigated for their medicinal significance [8]. Atranorin, a depside derivative is a pharmacologically active lichen secondary metabolite. Its anti-inflammatory and anti-nociceptive activities have already been reported and it is also widely used as atranorin-enriched lichen extract in traditional medicines [9]. Fumarprotocetraric a depsidone derivative, another important bioactive secondary metabolite produced by species of the genus Cladonia [10] is pharmacologically the most significant lichen substance. Hence, the aim of the current study was to expound the anti-hepatotoxic effect of the standardized extract of *L. rangiferinus* which has not been reported up till now in rat model of alcohol liver disease.

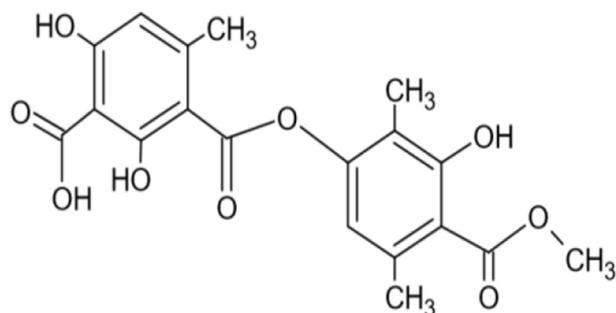
2. Materials and methods

2.1. Sample preparation

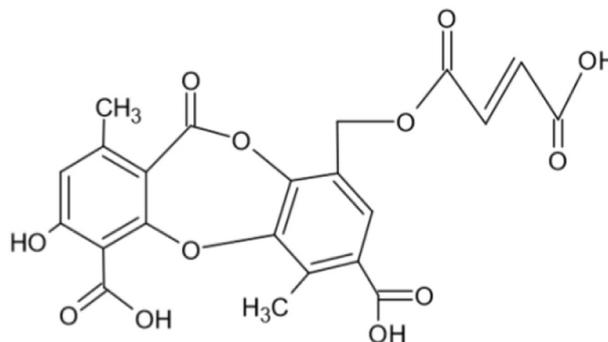
1000 g of thoroughly dried and finely powdered thalli of *L. rangiferinus* was extracted with 50% ethanol in a Soxhlet extractor. Lichen material was acquired from lichen laboratory, CSIR – National Botanical Research Institute, Lucknow, India. After solvent extraction, the extract was filtered and then concentrated over rota-vapour (IKARV10, Werke, Staufen, Germany). The *L. rangiferinus* extract (LRE) was stored at -18°C till it was used for experimentation. For the purpose of administration to the animals, extract was dissolved in dimethyl sulphoxide (DMSO) (5 mL/100 mL).

2.2. Chemicals

Analytical grade chemicals were used for the experimentation. Silymarin powder (brand name: Silybon (Silymarin 70 mg/140 mg tablets and 35 mg/5 mL suspension)) was obtained from Micro Labs



Atranorin



Fumarprotocetraric acid

Ltd., Lucknow, India. Standard kits for SGOT, SGPT and ALP etc. were obtained from Sigma–Aldrich Ltd., India.

2.3. Animals

Male Wistar rats weighing about 150–175 g were used, ethical approval having been obtained from Institutional Animal Ethics Committee. The animals were kept at the departmental animal house at $25 \pm 2^{\circ}\text{C}$ and relative humidity 45–56% and were weighed properly to maintain accuracy in dosing. Before initiating the experiment, they were observed for 5 days to eliminate the possibility of any intercurrent infection and then distributed accordingly into control and experimental groups. Their natural light–dark cycles were assured and were fed on standard pellet diet (Amrut, India), water *ad libitum*. All the animal studies were carried out in accordance with the guidelines for the care and use of laboratory animals, as adopted and promoted by the CPCSEA, India.

2.4. Experimental design

Animals were partitioned into five groups comprising six animals in each.

Group-1: Negative control (CTRL); administered equal volume of distilled water orally daily for four weeks.

Group-2: Alcohol Fed (AF); fed absolute ethanol (10%) in drinking water for four weeks.

Group-3: Test dose 1 (D1); given absolute ethanol (10%) and 50 mg/kg of lichen extract orally for four weeks.

Group-4: Test dose 2 (D2); given absolute ethanol (10%) and 100 mg/kg of lichen extract orally for four weeks.

Group-5: Positive control i.e standard (STD); administered Silymarin (25 mg/kg) with 10% ethanol for four weeks.

2.5. Determination of total phenolic and total flavonoid content of LRE

Total phenolic compounds in LRE were determined with Folin-Ciocalteu reagent as per the Slinkard and Singleton method [11] using pyrocatechol as standard. Dowd method [12] was used to calculate the total flavonoid content of the extract. LRE column yielded atranorin (19.8 mg/kg) and fumarprotocetraric acid (16 mg/kg). Both of them are colourless and crystalline in nature. Hence were recognized by their melting point and spectroscopic analysis.

2.6. Free radical scavenging assays

DPPH (1,1-diphenyl-2-picryl-hydrazil) was used for measuring the free radical scavenging activity of LRE [13]. Ascorbic acid was used as the positive control. IC₅₀ i.e., concentration at 50% inhibition was used to evaluate the radical scavenging activity. The reducing power of LRE was determined by the method of Oyaizu [14].

2.7. In vitro analysis

2.7.1. Isolation and culture of freshly isolated rat hepatocytes

Liver cells were isolated by a modified procedure of Seglen [15]. The isolated hepatocytes were cultured in Ham's F12 medium, supplemented with 10% fetal calf serum, antibiotics, 10⁻⁶ M dexamethasone, and 10⁻⁸ bovine insulin, and the cell suspension was incubated at 37 °C for 30 min in a humidified incubator under 5% CO₂ [16].

2.7.2. Alcohol induced in vitro hepatocytes injury

After an incubation of 24 h, the hepatocytes were exposed to the fresh medium containing alcohol (60–80 mM) along with/ without LRE or the medium alone (as normal). After 60 min of alcohol challenge, concentrations of ALT, AST, ALP and total protein in the medium were measured as an indication of hepatocytes necrosis by using diagnostic kits [17].

2.7.3. Hepatoprotective effect on HepG2 cell line

Tetrazolium assay was used to screen the anti-hepatotoxic activity against alcohol induced damage by estimating mitochondrial synthesis [18]. DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% newborn calf serum was used to routinely grow and subculture the HepG2 as monolayers. Cells taken for the experimentation were initially batch cultured for ten days. The cells were then exposed to toxicant (medium containing 60–80 mM alcohol) along with/without lichen extract or the medium alone (as normal). At the end of the period, cytotoxicity was evaluated using MTT reduction assay by determining the viability of HepG2 cells [19].

2.8. In vivo analysis

2.8.1. Acute toxicity study

An acute oral toxicity study was performed according to the OECD guidelines for the testing of chemicals, Test No. 420 (OECD, 2001; acute oral toxicity-fixed dose procedure). The animals were fasted overnight prior to the experiment and kept under standard laboratory conditions. The extract was administrated orally in

increasing dose up to 2000 mg/kg. The extract did not cause any mortality up to 2000 mg/kg dose level (LD₅₀ > 2000 mg/kg bw).

2.8.2. Blood and tissue sampling

At the end of the study blood samples were collected from the retro-orbital plexus. Blood samples were kept in a centrifuge tube and allowed to clot at room temperature for 40 min. After centrifugation at 3000 r.p.m. at 30 °C for 20 min sera were separated out for estimation of various biochemical and physiological parameters. After blood collection, animals were anesthetized using Pentobarbitone sodium (NEMBUTAL SODIUM-pentobarbital sodium injection, Oak Pharmaceuticals, Inc., Subsidiary of Akorn, Inc.) (50 mg/kg, i.p.) [20] for animal scarification. They were dissected and liver was excised and washed with saline. Excised liver was fixed in buffered formalin for 48 h, and then kept in 70% alcohol for histopathological examination. 0.75 g was homogenized in 6 mL 0.9% normal saline.

2.8.3. Biochemical analyses

Liver function biochemical markers such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total protein content was determined according to methods of Perry et al. [17], Lippi and Guidi [21], Malloy Evenyl [22] and Lowry et al. [23] respectively by using standard assay kits. γ -Glutamyltransferase (γ -GT) and glutathione S-transferase (GST) were estimated by the method of Szasz [24] and method of Habig et al. [25] respectively. Oxidative stress markers, glutathione (GSH), malondialdehyde (MDA) and glutathione reductase (GR) were determined according to the method of Ellman [26], Ohkawa et al. [27] and Mize and Langdon [28] respectively.

2.9. Histopathology

A portion of liver tissue from each group was fixed in 10% formalin (Formalin diluted to 10% with normal saline) and taken for histopathological evaluation. Fixed liver tissues were dehydrated and embedded in paraffin wax. Sections of 5 μ thickness were made, stained with Hematoxylin & Eosin (H and E) and examined under microscope.

2.10. Statistical analysis

The data were analyzed using the one way Analysis of Variance (ANOVA) followed by Scheffe's test to determine the statistical

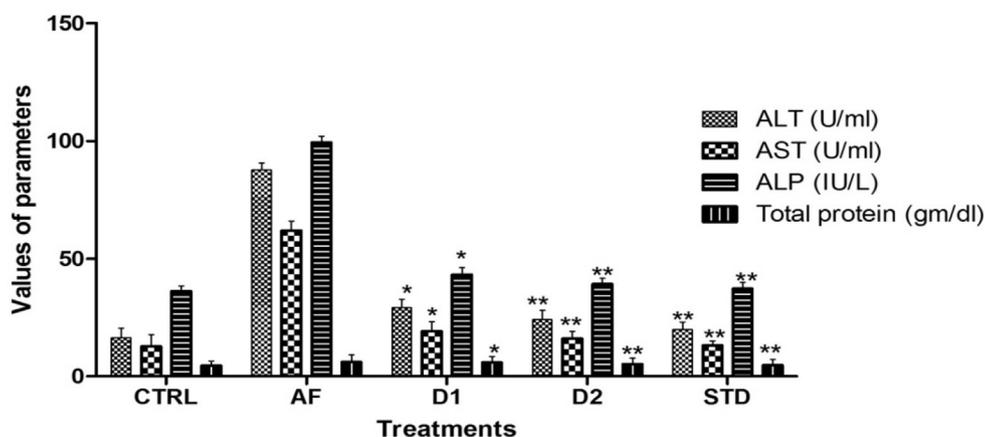


Fig. 1. Effect of LRE on liver function biochemical markers (ALT, AST, ALP and total protein) in freshly isolated rat hepatocytes. Significance: **p* < 0.05; ***p* < 0.01 as compared to alcohol treated group.

significance of difference in protein, enzymes and lipid levels between different groups. The level of significance was set at 0.05 and 0.01.

3. Results

3.1. Total phenolic and total flavonoid content

The total phenolic and flavonoid content of the LRE was found to be 21.78 µg PE/mg of extract and 5.13 µg RE/mg of extract respectively. Pyrocatechol equivalent (PE) was used to calculate the total phenolics and the total flavonoid content was determined as the

rutin equivalent (RE). LRE yielded atranorin (19.8 mg/kg) and fumarprotocetraric acid (16 mg/kg).

3.2. Reducing power and antioxidant activity of the extract

While reducing power of the extract was found to be significant, precisely 0.087 times of that of ascorbic acid, being used as standard, the reducing powers of isolated components were more significant than the extract itself. Reducing powers of atranorin and fumarprotocetraric acid were 0.1823 µg/mL and 0.986 µg/mL respectively as compared to the ascorbic acid. Higher absorbance indicates higher reducing power. The scavenging DPPH radicals of

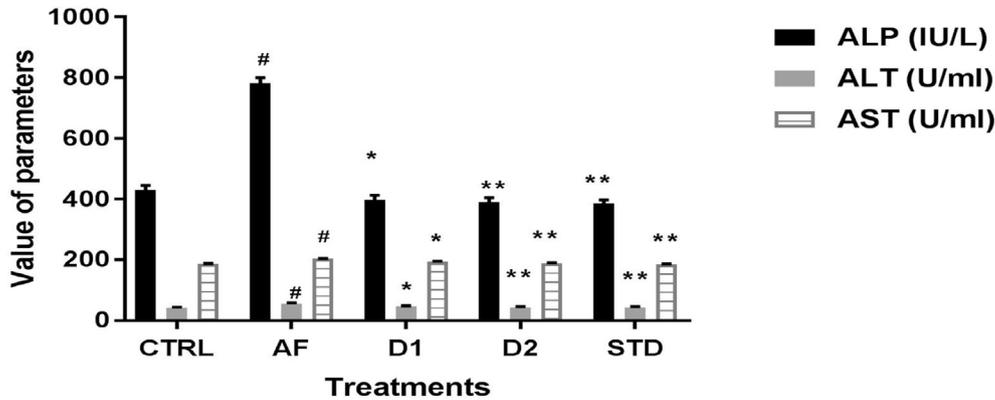


Fig. 2. LRE alleviated alcohol-induced fatty liver and liver injury. Data are mean ± SD (n = 6 for CTRL, AF, D1, D2, STD). CTRL: control, AF: alcohol-fed; D1: 50 mg/kg dose of lichen extract, D2: 100 mg/kg dose of lichen extract, STD: standard. Significance: *p < 0.05; **p < 0.01 as compared to alcohol treated group. #p < 0.01 compared with CTRL.

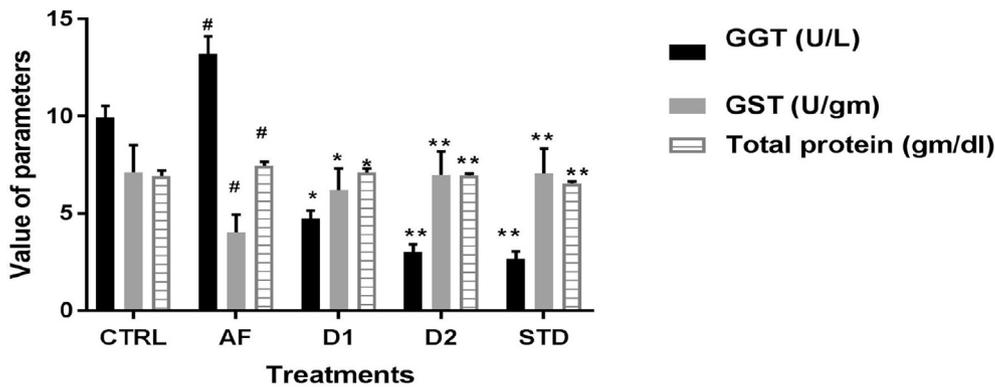


Fig. 3. LRE alleviated GGT, GST & total protein levels. Data are means ± SD (n = 6 for CTRL, AF, D1, D2, STD). CTRL: control, AF: alcohol-fed; D1: 50 mg/kg dose of lichen extract, D2: 100 mg/kg dose of lichen extract, STD: standard. Significance: *p < 0.05; **p < 0.01 as compared to alcohol treated group. #p < 0.01 compared with CTRL.

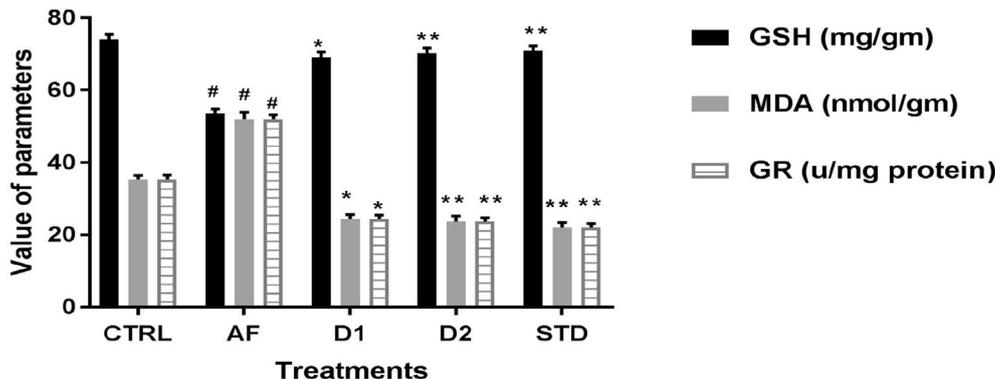


Fig. 4. Effect of LRE on MDA, GR and GSH levels. Data are means ± SD (n = 6 for CTRL, AF, D1, D2, STD). CTRL: control, AF: alcohol-fed; D1: 50 mg/kg dose of lichen extract, D2: 100 mg/kg dose of lichen extract, STD: standard. Significance: *p < 0.05; **p < 0.01 as compared to alcohol treated group. #p < 0.01 compared with CTRL.

LRE were found to be 978.16, IC_{50} μ g/mL. The isolated lichen components revealed very strong DPPH radical scavenging activity, more than that of extract itself. Atranorin showed stronger DPPH radical scavenging activity than fumarprotocetraric acid. The IC_{50} values for atranorin and fumarprotocetraric acid were found to be 128.48 and 218.46 mg/mL respectively.

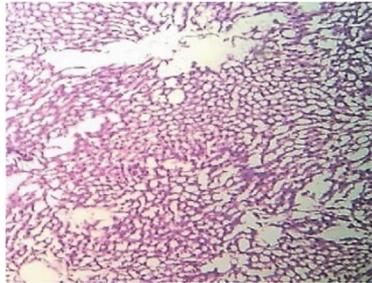
3.3. Hepatoprotective effects of LRE on freshly isolated rat hepatocytes

A significant increase in the levels of ALT, AST, ALP and total proteins ($p < 0.01$ and 0.05) were observed in hepatocytes exposed

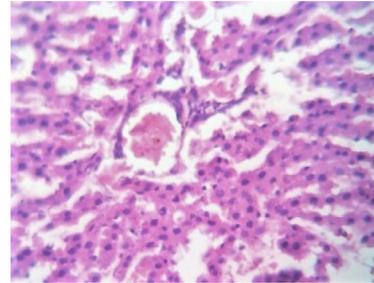
to alcohol when compared to normal rats. These cells, when treated along with the LRE showed a significant restoration of the altered biochemical parameters towards the normal. A similar result was obtained when alcohol-intoxicated hepatocytes were treated with the standard Silymarin (Fig. 1).

3.4. Hepatoprotective effects of LRE on the HepG2 cell line

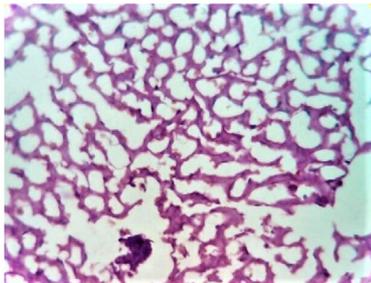
The alcohol-exposed HepG2 cells showed a percentage viability of 19%. These exposed cells, when treated with different concentrations of the LRE, showed a dose-dependent increase in



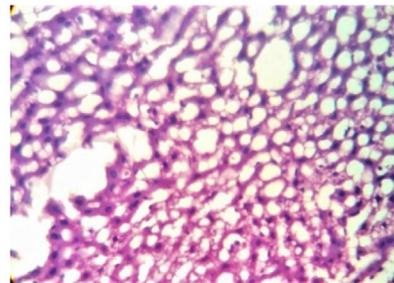
CTRL (A transverse section of a liver of normal control (distilled water) rat showing a central vein)



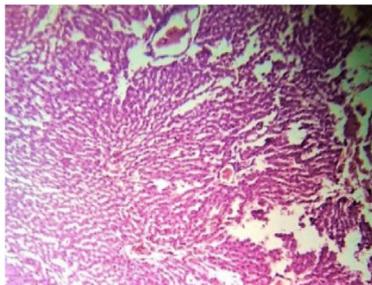
Fatty change was observed in diffuse manner all over the hepatocytes in ethyl alcohol treated group



Decrease in steatosis in group with D1(50 mg/kg) dosing



Further decrease in steatosis in group with D2 (100 mg/kg) dosing



Most of tissue recovered from fatty damage in silymarin administered group

Fig. 5. Histopathological changes in different groups during experimentation. D1, test dose 1(50 mg/kg) and D2, test dose 2 (100 mg/kg). Magnification: 10 \times .

percentage viability and the results were highly significant ($p < 0.01$, 0.05 when compared to alcohol intoxicated group).

3.5. Effect of lichen extract on ALT, AST and ALP

In alcohol treated group ALT, AST and ALP increased to 55.64 U/mL, 203.00 U/mL, and 782.41 IU/L while values for control group (negative control) were 41.50 U/mL, 186.73 U/mL, and 430.16 IU/L respectively. In contrast, groups treated with 50 and 100 mg/kg lichen extract, showed significant decrease in ALT, AST and ALP in a dose-dependent manner. Treatment with 100 mg/kg lichen extract showed remarkable activity which is almost comparable to the group treated with Silymarin, a potent hepatoprotective drug used as reference standard here (Fig. 2).

3.6. Effect of lichen extract on γ -GT & GST activities and protein levels

In alcohol fed group, γ -GT and protein levels were remarkably increased while GST activity was reduced as compared to negative control. Treatment with lichen extract shown appreciable decrease in γ -GT and protein levels while the decreased activity of GST was recovered. Results clearly showed that 100 mg/kg dose of lichen extract have similar effect as produced by Silymarin (Fig. 3).

3.7. Effect of lichen extract on GSH, MDA and GR levels

Alcohol administration drastically increased MDA production while on the other hand it has significantly reduced the levels of GSH and GR. Treatment with 50 and 100 mg/kg lichen extract produced vice versa results i.e. lowered the production of MDA, GSH and GR levels were recovered. The results were almost similar to the effects produced by Silymarin, a potent hepatoprotective marketed drug (Fig. 4).

3.8. Histopathological examination

The pathological changes in fatty liver and degeneration of liver cells can be classified as under (Fig. 5):

Group-1 (CTRL): Liver morphology was normal, hepatocytes were with central round nucleus and homogenous cytoplasm, and endothelial cells around central vein & sinusoid were intact.

Group-2 (AF): Necrosis and hepatocytic degeneration was seen around the central vein area. Diffuse intra-cytoplasmic vacuolar degeneration and endothelial lining of central vein exhibited more cell injury, as well as fat vacuoles increased in hepatocytes.

Group-3 (D1): Cell degeneration and necrosis (loss of nucleus) was observed around central vein, endothelial cells were less injured around central vein and number of fat vacuoles was reduced in hepatocytes.

Group-4 (D2): Lesser damage was observed as compared to the group 3.

Group-5 (STD): Liver morphology was comparable to the group 1.

4. Discussion

In the current study, we provided evidence about anti-hepatotoxic potential of *L. rangiferinus* extract against ethanol-induced hepatotoxicity in rats. The analyzed lichen extracts having higher content of phenols and flavonoids exerted strong radical scavenging effect, suggesting that phenolics are the main agents for their antioxidant activity. These results frequently agree with the literature, where we came across a number of reports for the

antioxidant activity of extracts with high content of phenolic contents [29]. In most lichens, phenols, including depsides, depsidones, and dibenzofurans, are important antioxidants because of their ability to scavenge free radicals such as singlet oxygen, superoxide, and hydroxyl radicals [30]. In our experiment, the components fumarprotocetraric acid and atranorin belonging phenols also displayed significantly strong antioxidant activity than extracts, which points to an important role of phenol in the antioxidant activity. However, some authors consider that the antioxidant activity of extracts may not be necessarily correlated with the content of polyphenolics [31], suggesting that the antioxidant activity of different lichens may also depend on other non-phenolic components.

The ability of an anti-hepatotoxic drug to diminish the deleterious effects or to maintain the normal liver physiology that has been disrupted by a hepatotoxin is defined as its index of protective effects. Ethanol intake directly or indirectly amplifies the effect of sterol regulatory element-binding protein 1c (SREBP-1c). Directly, it affects by boosting the transcription of SREBP-1c gene which is enhanced by acetaldehyde formed after alcohol metabolism and indirectly by stimulating factors which are responsible for expression of SREBP-1c protein. Steatosis is the foremost change that occurs in liver after alcohol consumption. It augments the oxidized form of nicotinamide adenine dinucleotide in the hepatocytes which leads to disruption of β -oxidation of fatty acids in mitochondria which is one of the reasons for fatty changes in liver [2]. These changes lead to hepatocellular necrosis which causes elevation of serum marker enzymes which are released by liver into the blood stream [32]. The increased levels of ALT, AST, ALP and serum total protein are conventional indicators of hepatic damage [33,34]. Administration of LRE at different doses (50 mg/kg and 100 mg/kg) assuaged the increased levels of the serum enzymes, caused by alcohol intake and led to a subsequent recovery towards normalization that was comparable to the control group animals. Hepatoprotective effect of LRE was further confirmed by histopathological examinations. LRE dose of 100 mg/kg produced more effective results, comparable to standard, Silymarin. Reduced activity of enzymatic antioxidants was rectified by administration of LRE. Regarding non-enzymatic antioxidants, GSH is a decisive determinant of tissue susceptibility to oxidative damage and depletion of GSH has been shown to be associated with an increased toxicity to chemicals, including alcohol [35]. Alcohol intake lowered the levels of GSH which were improved in LRE administered rats. This increase in hepatic GSH levels in LRE treated rats may be due to *de novo* GSH synthesis or GSH regeneration. Lipid peroxidation (MDA) is a measure of membrane damage and alteration in structure and function of cell membrane of liver cells. In current study, elevation of lipid peroxidation was observed in alcohol treated rats. Augmented levels of MDA points out towards increased lipid peroxidation leading to failure of antioxidant defence mechanism which prevents the formation of excessive free radicals [36]. These changes were reversed by administration of LRE.

5. Conclusion

Hence, *L. rangiferinus* can prove to be a breakthrough in the treatment of ALD, with drastically reduced side effects which cannot be achieved with other drugs being used in treatment of ALD.

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Conflict of interest

None.

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