



Original article

Hepatoprotective activity of depsidone enriched *Cladonia rangiferina* extract against alcohol-induced hepatotoxicity targeting cytochrome P450 2E1 induced oxidative damageIla Shukla^a, Lubna Azmi^b, Ch. V. Rao^a, Talha Jawaid^c, Mehnaz Kamal^{d,*}, Amani S. Awaad^e, Saleh I. Alqasoumi^f, Osama A. Alkhamees^c, Saud M. Alsanad^c^a Department of Pharmacology, CSIR-National Botanical Research Institute, Lucknow, Uttar Pradesh, India^b Department of Chemistry, University of Lucknow, Lucknow, India^c Department of Pharmacology, College of Medicine, Al Imam Mohammad Ibn Saud Islamic University (IMSIU), Riyadh 13317, Saudi Arabia^d Department of Pharmaceutical Chemistry, College of Pharmacy, Prince Sattam Bin Abdulaziz University, P.O. Box No. 173, Al Kharj 11942, Saudi Arabia^e Gateway to United Kingdom Education Ltd., Bradford, United Kingdom^f Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia

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ABSTRACT

Alcoholic liver disease (ALD) is a broad-spectrum disorder, covering fatty liver, cirrhosis, alcoholic hepatitis and in extreme untreated condition hepatocellular carcinoma (HCC) may also develop. *Cladonia rangiferina* (CR) is a class of lichen having a broad spectrum of pharmacological activity. It is used like traditional natural sources in ancient times in India, China, Sri Lanka, etc. Folkloric record about CR has reported their use as an antimicrobial, antitumor, antioxidant, anti-inflammatory activities, etc. Hence, the present study was requested to ascertain the effect of the ethanolic extract of *Cladonia rangiferina* (CRE) on alcohol-induced hepatotoxicity. The animals were evaluated for the estimation of the liver *in vivo* biochemical antioxidant parameters. The liver tissues were further evaluated histopathologically and western blotting examination for localization of apoptotic gene expression that plays a pivotal role in hepatotoxicity. The results of this study reveal that CRE proves to be helpful in the treatment of alcohol-induced hepatotoxicity and oxidative stress. Results of different markers have shown that among all, CRE has demonstrated the best hepatoprotective activity. These observations say about the importance of the components of the extract. The ameliorative action of CRE in alcoholic liver damage may exist due to antioxidant, anti-inflammatory, and anti-apoptotic activities.

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Abbreviations: ALD, Alcoholic liver disease; CR, *Cladonia rangiferina*; CRE, *Cladonia rangiferina* extract; CMC, Carboxymethylcellulose; ROS, Reactive oxygen species; MDA, Malondialdehyde; GGT, γ -glutamyl transferase; GSH, Glutathione; RT-PCR, Reverse Transcription Polymerase Chain Reaction; DAB, 3,3'-diaminobenzidine tetrahydrochloride; WHO, World Health organization; CTRL, Control.

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1. Introduction

Alcohol is one of the most widely used psychoactive substances after caffeine. Long-term consumption of alcohol has been the key cause of major health issues. WHO report, 2009 determines alcohol consumption has more detrimental effects than tobacco use, high cholesterol levels, or hypertension. The liver is the first organ involved in the metabolism of alcohol consumed (Shanmugam et al., 2010). Alcohol damages the liver by creating oxidative stress, which leads to metabolic disturbances. Changes by which alcohol causes oxidative stress are the formation of acetaldehyde, damage to the cell membrane and mitochondria, hypoxia, disturbed immune system and cytokine production, CYP2E1 induction and mobilization of iron (Baskaran et al., 2010). Stages of alcoholic liver disease (ALD) are mainly divided into fatty liver/steatosis, alcoholic

hepatitis and liver cirrhosis. Previously reported pieces of evidence indicated that intermediates formed from the reduction of oxygen may be responsible for the occurrence of ALD. A steep amplification in the levels of free radicals (in human's hepatocytes) is seen after alcohol consumption because ethanol or its metabolites act either as pro-oxidant or lessens the level of antioxidants in the body. This is the reason behind the progression of a huge range of chronic liver diseases. Reactive oxygen species (ROS) are very harmful and may cause considerable damage to lipids, protein, and DNA (Saalu et al., 2012). *Cladonia rangiferina* is a class of lichen having a broad spectrum of pharmacological activity. It is used in ancient times in India, China, Sri Lanka, etc. as traditional natural sources. The previous phytochemical investigation of these lichens has proved that they have a variety of secondary metabolites like depsides, depsidones, etc. Folkloric record about this lichens has reported their use as an antimicrobial, an antitumor agent, antioxidant, anti-inflammatory activities, etc. (Boustie and Grube, 2005). This evidence suggests that CR was known for their various medicinal values, but to the best of our knowledge, these lichens were not yet, thoroughly explored for their hepatoprotective activity.

2. Materials and methods

2.1. Extraction

The lichen *Cladonia rangiferina* (Fam. Cladoniaceae) was acquired from the Department of Lichenology, CSIR-NBRI (National Botanical Research Institute), Lucknow, India. (Accession code of *Cladonia rangiferina*: 4/63/006521). 500 g of powdered material of CR was extracted three times by ethanol (50% v/v) by carrying out cold percolation at room temperature. Rotary evaporator (Buchi, USA) was employed to concentrate the extract at reduced temperature (5 °C). After that, it was freeze-dried (FreeZone 4.5, Labconco, USA) over high vacuum and at a reduced temperature of 133×10^{-3} mBar and -35 ± 2 °C respectively. Pharmacological analysis of CRE was carried out by suspending the dried extract in double distilled water containing a surfactant, carboxymethylcellulose (CMC, 1% w/v).

2.2. Chemicals

Ethanol, carboxymethylcellulose, trichloroacetic acid, thiobarbituric acid, L-gamma-glutamyl-3-carboxy-4-nitroanilide, glycylglycine, sodium phosphate buffer, 5,5'-dithiobis-(2-nitrobenzoic acid), phosphate buffered saline, Triton X-100, sodium deoxycholate, sodium dodecyl sulfate, tris-buffered saline, Tween 20, hydrogen peroxide, ethanol, and 3,3-diaminobenzidine tetrahydrochloride was purchased from the Sigma Aldrich, (Mumbai, India). The assay kits were also purchased from Sigma Aldrich (Mumbai, India). Trizol reagent, DNA ladder extraction kit, and antibodies were acquired from Sigma Aldrich (Bangalore, India). All other chemicals and solvents were of the analytical grade.

2.3. Preparation and administration of ethanol

A dose of ethanol (30% v/v solution of ethanol) used here for causing liver damage is 7 g/kg body weight. A volume of 6.2 ml of the prepared solution was administered to the rats for four weeks (Rahman et al., 2006).

2.4. Animal

Thirty male Wistar rats were purchased and used in this study. They were acquired from the animal house of Central Drug Research Institute, Lucknow, India and were kept in the departmental animal

house. Animals weighed about 140–170 g were kept in departmental animal house. All the methods followed here were conducted according to the guidelines provided by CPCSEA for animal experimentation (Reg. No. 1732/GO/Re/S/13/CPCSEA).

2.5. Preparation of animal model for ethanol-induced hepatic injury

Thirty rats were randomly divided into six groups (five animals in each group).

Group 1: Received 1% CMC-Na (200 mg/kg) by gastric gavage for 28 days, once daily. This group has been abbreviated as CTRL

Group 2: Received Ethanol (7 g/kg) by gastric gavage for 28 days, once daily. This group has been abbreviated as AF

Group 3: Received ethanol (7 g/kg) orally, O.D., 60 mins. Later CRE 50 mg/kg was administered. abbreviated as CRE50

Group 4: Received ethanol (7 g/kg) orally, O.D., 60 mins. Later CRE 100 mg/kg was administered. Abbreviated as CRE100

Group 5: Received ethanol (7 g/kg) orally, O.D., 60 mins. Later CRE 200 mg/kg was administered. Abbreviated as CRE200

Group 6: Received ethanol (7 g/kg) orally O.D., 60 mins. Later Liv. 52 (The Himalaya Drug Company) in a dose of 0.216 ml/kg was administered (Sapakal et al., 2008). Abbreviated as Liv 52.

2.6. Estimation of in vivo oxidative stress markers

For the estimation of oxidative stress markers, rat livers were homogenized in an ice-cold Tris-EDTA buffer (pH 7.4) and tissue homogenate obtained was used in further analysis.

2.6.1. Estimation of malondialdehyde (MDA)

The extent of lipid peroxidation and oxidative stress in tissue is directly proportional to the level of MDA. For this assay, firstly homogenate was properly mixed with trichloroacetic acid (30%) and thiobarbituric acid (2%). After that, the mixture was boiled in a water bath at a temperature of 90 °C for 15 min. This complete mixture was centrifuged at 1500g, for 10 min. and absorbance of the supernatant (pink color, 532 nm) was recorded using ELISA plate reader (BioTek). The concentration of malondialdehyde was expressed as nmol protein (Rahman et al., 2006).

2.6.2. γ -glutamyl transferase (GGT)

In serum, GGT interacts with L-gamma-glutamyl-3-carboxy-4-nitroanilide and glycylglycine, which results in the formation of L-gamma-glutamyl-glycylglycine and 5-amino-2-nitrobenzoate. The rate of reaction is recorded per minute for 3 min. at 405 nm, distilled water was taken as blank. Reagents reconstituted for the analysis are, tris buffer (182 mM, pH 8.25) and L-gamma-glutamyl-3-carboxy-4-nitroanilide (2.97 mM) having glycylglycine (85 mM), were taken as the working reagent (1 ml) were thoroughly mixed with 0.1 ml of serum. After 1 min., again the variations in absorbance were recorded per minute for 3 min at 405 nm. Here distilled water was taken as blank (Szasz, 1969).

2.6.3. Estimation of reduced glutathione (GSH) content

For assessment of GSH, the homogenate was thoroughly mixed with 0.1 M sodium phosphate buffer (pH 8.0) and 6 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). After that, it was incubated for 10 mins at room temperature leading to the formation of the deep yellow colored product. Absorbance for this product was recorded at 412 nm by using ELISA plate reader (BioTek). The concentration of GSH (as μ M of GSH/ μ g protein) was counted by preparing the standard curve with GSH (Ellman, 1959).

2.6.4. Estimation of inflammatory mediators

2.6.4.1. Estimation of tumor necrosis factor- α (TNF- α) in serum and liver tissues. Rat TNF- α ELISA kit was used for assessing the levels of TNF- α levels. The microtitre plate was thoroughly cleaned four times by using a diluted wash buffer. Standard solution of TNF- α and samples to be analyzed were mixed in quantities as given in the manufacturer's protocol. The plates were incubated and kept shaking for 2 h followed by exclusion of the solution and washing for 4 times as instructed. The detection antibody was properly affixed for one hour. Again incubated the plate, this time for 30 mins and after that added avidin-HRPD with steady shaking. This was again followed by the washing of plates in the same manner. Once again the plate was properly washed and the substrate solution was specified and a stop solution was added after 15 mins. The absorbance was recorded at 450 nm within 30 mins (Petrovas et al., 1999).

2.6.4.2. Estimation of interleukins levels (IL-1 β , IL-6, and IL-10) in liver tissues. For estimation of levels of interleukins, rat specific ELISA kits (Sigma Aldrich) was used. All the procedures followed were in accordance with the protocol given by the manufacturer.

2.7. Caspase-3 and caspase-8 activities

For measuring the caspases activities, instructions provided with kits were strictly followed. Quickly, the mixture of detection buffer (80 μ L), samples (10 μ L), and Ac-IETD-pNA (10 μ L) was incubated at 37 °C for 60 mins and after that OD₄₀₅ was recorded. Their activities were calculated with the help of a standard curve (Casciola-Rosen et al., 1996).

2.8. TUNEL assay

Tunel staining was done to detect apoptosis by using a kit for *in situ* apoptosis detection (Sigma Aldrich, Bangalore, India). Liver tissues processed for this assay were embedded in paraffin. The images were taken by fluorescence microscopy (Olympus, Lucknow, India) (Kyrylkova et al., 2012).

2.9. DNA ladder

For this analysis samples of DNA were extracted by employing the kit along with the spin column. After that, samples were separated by electrophoresis in 1% agarose gel and ethidium bromide was employed for staining. Agarose gel was carefully visualized and photographs were taken under UV light with the help of the BioSpectrum Gel Imaging System (Saadat et al., 2015).

2.10. Western blot analysis

For this analysis, livers were thoroughly washed two times with cold PBS and protein extracted. After that, they were lysed by using an appropriate amount of lysis buffer (cold) consisting of 1 mM PMSF lysates. After that, they were centrifuged at 12,000g, at a temperature of 4 °C for 15 mins. Total protein obtained was verified by coomassie brilliant blue G. For carrying out the western blot assay, protein (5 mg/mL) was carefully denatured by thoroughly mixing it with an equivalent volume of 2 \times buffer for loading sample. This step was followed by the boiling of this mixture at 100 °C for a duration of 5 mins (Saadat et al., 2015). An equivalent amount of protein was loaded on the SDS gel. Protein was separated by the help of electrophoresis and then it was transferred to the PVDF membrane. Electrophoresis was carried out by using 10% polyacrylamide gel. FasL, Fas, and NF- κ B p65 were 30, 35, 45, and 50 mins respectively. First of all, the PVDF membrane was carefully incubated in 10 mM TBS along with 1% Tween 20 and then treated with

5% dehydrated skimmed milk for blocking non-specific protein binding. The membrane was properly incubated with primary antibodies overnight at a temperature of 4 °C, either with rabbit anti-Fas (1:200 dilution), anti-FasL (1:500 dilution), rabbit anti-NF- κ B p65 (1: 600 dilution), or mouse anti-GAPDH (1: 2000 dilution). After that blots were incubated in horseradish conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated goat anti-mouse IgG for 2 h at a dilution of 1:2000 (at room temperature). Detection was carried out by an enhanced chemiluminescence method and photographs were taken by Biospectrum Gel Imaging System. The data were normalized with the help of GAPDH (objective protein IOD vs GAPDH protein IOD) (Kurien and Scofield, 2006).

2.11. RT-PCR (Reverse transcription Polymerase Chain Reaction) analysis

Trizol reagent was employed for the preparation of RNA from the cells. For RT-PCR analysis here, a total of 500 ng RNA was required every time. RT-PCR was carried out in accordance with the protocol given with the RT-PCR kit (Thermofischer, Mumbai, India). PCR system (Bio-Rad Laboratories India Private Limited) was employed for the amplification. First of all, RNA samples were reverse transcribed and after that, without any delay, these were amplified by the help of PCR. For performing amplification first step is denaturation (94 °C, 1 min.), followed by annealing (60 °C) and after that extension at 72 °C for 1 min. Extra fifty cycles were used for amplification. Biospectrum Gel Imaging System was employed for analyzing the IOD values of the electrophoresis bands (Kurien and Scofield, 2006).

2.12. Histology and immunohistochemistry

For observing the liver damage, sections of 5 μ m thickness were made, stained by hematoxylin-eosin (H & E) and examined under light microscopy (40x, Olympus BX50). For analyzing immunohistochemistry, additional sections were employed for further two-step IHC detection. The activity of endogenous peroxidase was carefully blocked for 10 mins by using 3% H₂O₂. The non-specific protein binding was blocked for 30 mins by using normal goat serum. The part was incubated at a temperature of 4 °C with rabbit anti-Bcl-2 and anti-p53 antibody (1: 100, dilution). After that antigen repair was microwaved and left for complete night. This process was followed by the incubation at a temperature of 37 °C in PV6001 for 30 mins. These were visualized by using 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate and were counter-stained with hematoxylin. Images were taken with the help of inverted digital image light microscopy. The tissues which acquired brown stain were considered to be damaged and the IOD values were examined by using for evaluating the protein expressions (de Araujo et al., 2016).

3. Results and discussion

3.1. Estimation of *in vivo* oxidative stress markers

CRE treated group showed a significant increase in GST, GSH, and GR concerning the AF group. As compared to the control group AF group showed a marked reduction in GST, GSH, and GR. The increase in GST, GSH, and GR in CRE treated group was comparable to the Liv. 52 group. Liv. 52 is a product marketed by Himalaya Drug Company, Bengaluru, Karnataka, India. It is an Ayurvedic medicine and hence its ingredients are made of rare herbs which have been scientifically proven to cure liver ailments. The percentage change recorded for AF intoxicated group was GST 56.8

($P < 0.001$), GSH 30.5 ($P < 0.001$), and GR 37.8 ($P < 0.001$). The values of percentage protection for 50, 100 and 200 mg/kg, Liv. 52 respectively were, GST 131.7 ($P < 0.05$), 148.4 ($P < 0.01$), 141.2 ($P < 0.001$), 149.1 ($P < 0.001$); GSH 211.7 ($P < 0.05$), 272.1 ($P < 0.01$), 209.4 ($P < 0.001$), 276.4 ($P < 0.01$) and GR 190.3 ($P < 0.05$), 238.12 ($P < 0.001$), 212.26 ($P < 0.01$), 241.7 ($P < 0.01$). Among different dosing groups of CRE, 100 mg/kg has shown the highest protection. Levels of percentage protection for MDA and GGT in rats of AF group were 726.4 ($P < 0.001$) and 1262.6 ($P < 0.01$), in comparison to the control group. Undertreatment with CRE at different dose levels of 50, 100, 200 mg/kg and effect of Liv. 52, percentage protection values for MDA were 72.4 ($P < 0.001$), 35.7 ($P < 0.001$), 40.3 ($P < 0.01$), 37.4 ($P < 0.01$) and for GGT were 83.1 ($P < 0.001$), 36.7 ($P < 0.01$) and 42.5 ($P < 0.05$), 34.58 ($P < 0.01$) respectively (Fig. 3a, b). These values show CRE (100 mg/kg) and Liv. 52 significantly lowered the MDA and GGT values to near normal (Fig. 1).

3.2. Effect of CRE treatment upon inflammatory mediators

Levels of TNF-alpha ($P < 0.01$) and IL-1 β ($P < 0.001$) were elevated in the AF group, but the concentration of IL-10 ($P < 0.01$) in samples was decreased, in comparison to the control group. CRE

treatment reversed the alcohol-induced effects. The remarkable decline in levels of IL-1 β and TNF-alpha were observed in the CRE (100 mg/kg) group, an effect opposite to as observed in the AF group. Moreover, in all three groups of CRE (50 mg/kg, 100 mg/kg, and 200 mg/kg) levels of IL-10 were elevated as compared to the AF group ($P < 0.001$). CRE (100 mg/kg) group was found to be most effective among all treatment groups and its effect was almost comparable to the Liv. 52 group (Fig. 2).

3.3. Caspase-3 and caspase-8 activities

The caspases were significantly augmented in the AF group. CRE treatment at three different doses considerably reduced their activities (Fig. 3). Caspase-3 and caspase-8 activities in 100 mg/kg dose of CRE were decreased by 58.08% and 48.77% in comparison to the AF group and the values were found almost similar to the Liv. 52 group 61.24% and 50.18%.

3.4. TUNEL assay and DNA ladder

For assessing the apoptosis in liver tissues and *in situ* detection kit for cell apoptosis was employed. The TUNEL-positive apoptotic nuclei increased drastically in the AF group, and very

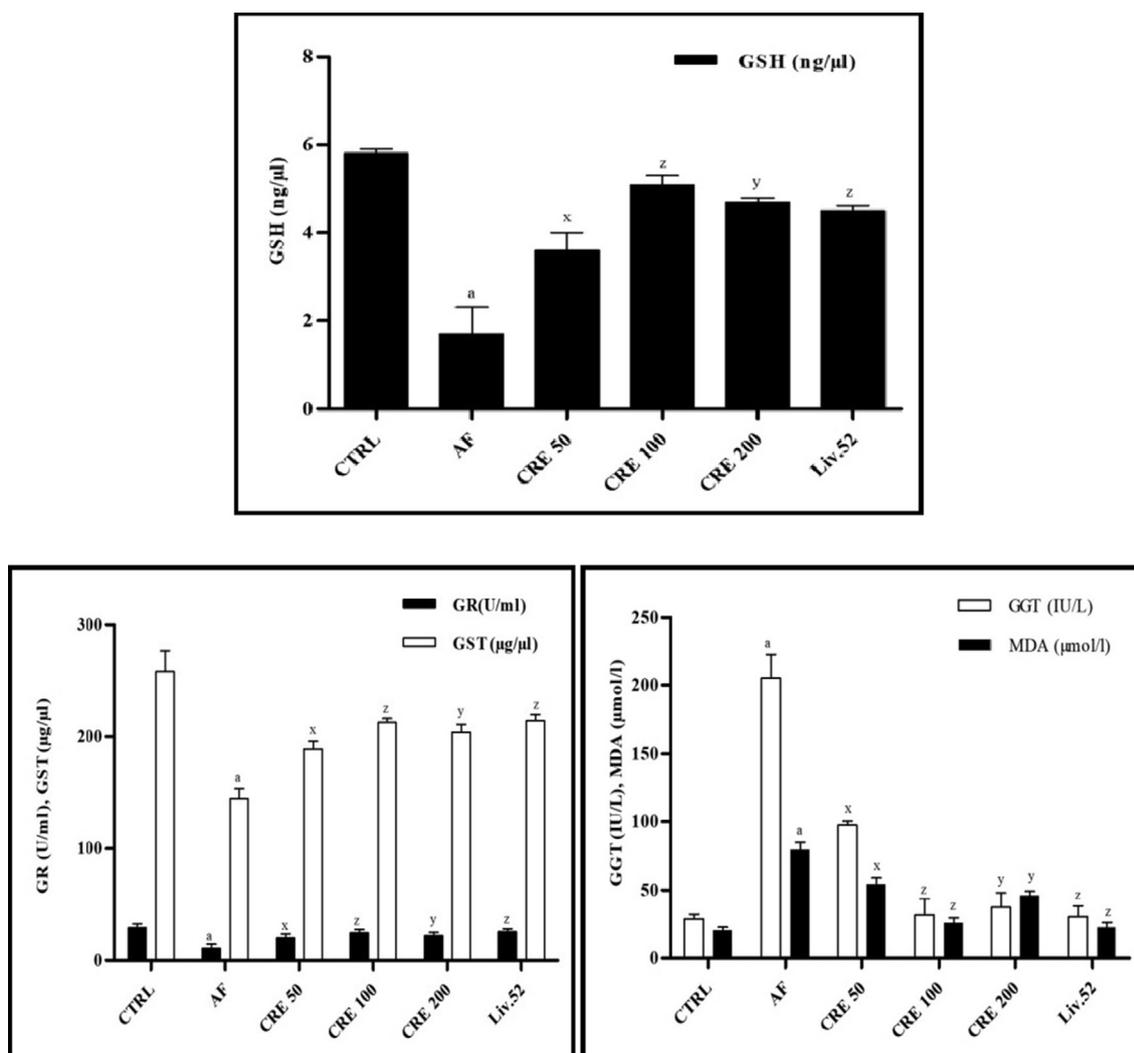


Fig. 1. Effect of CRE and standard drug (Liv. 52) on GSH, GR, GST, GGT, and MDA. Statistical analysis was carried out by 1-way ANOVA and after that Dunnett's multiple comparison assay was done. ^aDenotes significance when compared to control group (P less than 0.05); ^xDenotes significance when compared to AF (P less than 0.05); ^yDenotes significance when compared to AF (P less than 0.01); ^zDenotes significance when compared to AF (P less than 0.001) ($n = 5$).

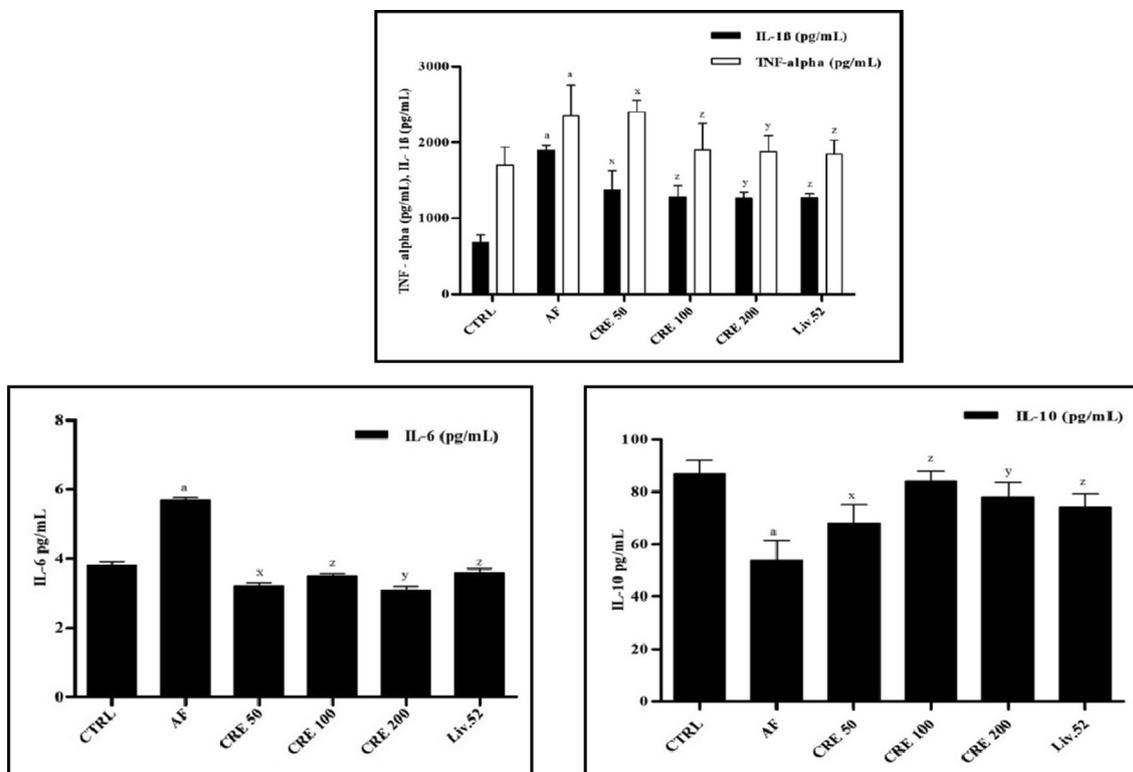


Fig. 2. Effect of CRE and standard drug (Liv. 52) on inflammatory mediators (IL-1 β , IL-6, IL-10, and TNF- α level). Statistical analysis was carried out by 1-way ANOVA and after that Dunnett's multiple comparison assay was done. ^aDenotes significance when compared to control group (P less than 0.05); ^xDenotes significance when compared to AF (P less than 0.05); ^yDenotes significance when compared to AF (P less than 0.01); ^zDenotes significance when compared to AF (P less than 0.001) ($n = 5$).

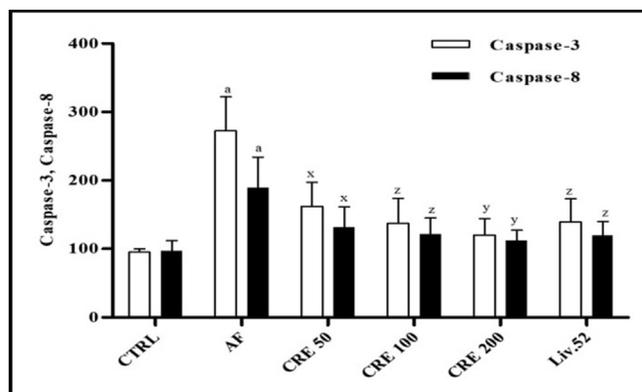


Fig. 3. Effect of CRE and standard drug (Liv. 52) on caspase activity. Statistical analysis was carried out by 1-way ANOVA and after that Dunnett's multiple comparison assay was done. ^aDenotes significance when compared to control group (P less than 0.05); ^xDenotes significance when compared to AF (P less than 0.05); ^yDenotes significance when compared to AF (P less than 0.01); ^zDenotes significance when compared to AF (P less than 0.001) ($n = 5$).

few TUNEL-positive cells were seen in the hepatic tissue acquired from CRE (100 mg/kg) treated rats and Liv. 52 treated rats. Also, the effect of CRE on DNA fragmentation was examined (Fig. 4).

The typical DNA ladder (Fig. 5) was seen in the alcohol-fed group; though, the DNA laddering was appreciably reduced in CRE (100 mg/kg) group, indicating that CRE may reduce hepatocyte apoptosis occurring due to alcohol administration in rats.

3.5. Western blot analysis

This analysis was carried out for recording the hepatoprotective activity of CRE (100 mg/kg) concerning its effect over the

expressions of Fas/FasL and NF- κ B p65. Fas is a member of the death receptor family. Stimulation of Fas leads to the induction of apoptotic signals, such as caspase 8 activation, as well as "non-apoptotic" cellular responses, notably NF- κ B activation. Convincing experimental data have identified NF- κ B as a critical promoter of cancer development, creating a solid rationale for the development of antitumor therapy that suppresses NF- κ B activity. On the other hand, compelling data have also shown that NF- κ B activity enhances tumor cell sensitivity to apoptosis and senescence. Furthermore, although the stimulation of Fas activates NF- κ B, the function of NF- κ B in the Fas-mediated apoptosis pathway remains largely undefined. Engagement of Fas with FasL triggered NF- κ B activation. (Liu et al., 2012)

As shown in Fig. 6(1) and (2), protein expressions of Fas and FasL were augmented by almost two times and five times in the liver of rats of AF group as compared to the rats of a control group, whereas their expressions were appreciably reduced in CRE and Liv. 52 rats. NF- κ B p65 expression was amplified by almost six-folds in ethanol-treated rats while CRE and Liv. 52 treated rats partially prevented this effect (Fig. 6(3)). Majorly two pathways are involved in cell apoptosis (death receptor pathway and mitochondrial pathways). Reported findings say that interaction of death receptor and its ligand e.g., the interaction of Fas/FasL, are important for initiating apoptosis (extrinsic pathway). Prior studies have shown that by suppressing Fas and FasL proteins leads to a reduction of hepatic cell death due to liver injury. Fas/FasL interaction causes activation of cascades involving caspases, which is a vital factor in the occurrence of apoptosis in hepatic damage. In this study, CRE considerably reduced alcohol-dependent up-regulation of Fas and FasL. This fact demonstrates that by suppressing the expression of proteins Fas and FasL, inhibiting the caspase-3 and caspase-8 enzymes, CRE showed a significant effect by shielding against alcohol-induced hepatic injury (Qu et al., 2012).

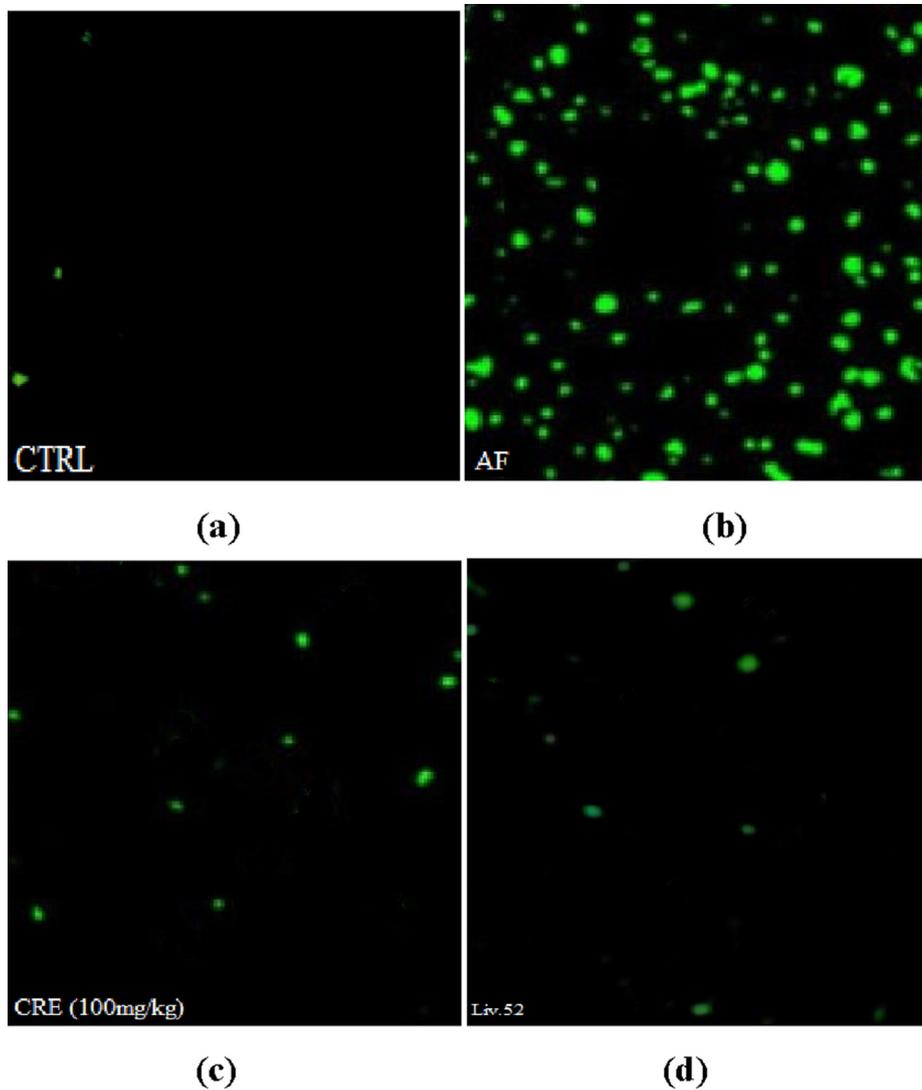


Fig. 4. Hepatic apoptosis was assessed by the TUNEL method. Histological analysis was carried by employing the fluorescence microscopy on Hepatic tissues (original magnification: $\times 100$). AF: is having a huge number of TUNEL-positive cells; CRE (100 mg/kg) having only a few TUNEL-positive cells; Liv. 52: similar results as CRE (100 mg/kg).

3.6. RT-PCR analysis

Expressions of Bcl-2, Bak, and Bax mRNA are demonstrated in Fig. 6(4)–(6). Bax (Bcl-2 associated X, apoptosis regulator) is a protein coding gene. The protein encoded by the Bax gene belongs to the Bcl-2 protein family. Bcl-2 family members form hetero- or homodimers and act as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities. This protein forms a heterodimer with Bcl-2 and functions as an apoptotic activator. This protein is reported to interact with, and increase the opening of, the mitochondrial voltage-dependent anion channel (VDAC), which leads to the loss in membrane potential and the release of cytochrome C. The expression of this gene is regulated by the tumor suppressor P53 and has been shown to be involved in p53-mediated apoptosis. During apoptosis, Bax and Bak puncture the mitochondrial outer membrane.

For excluding the variations due amount and nature of RNA, the results recorded were adjusted according to the expression of GAPDH. Liver injury in AF group was indicated by considerably amplified the levels Bak & Bax and significantly declined the levels of Bcl-2. Their levels were appreciably inverted in CRE (100 mg/kg) and Liv. 52 group. CRE 100 group showed the best results which

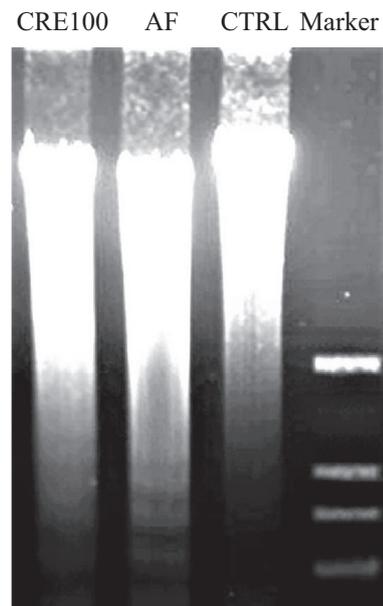


Fig. 5. Agarose gel (1% agarose gels) electrophoresis was used for DNA laddering.

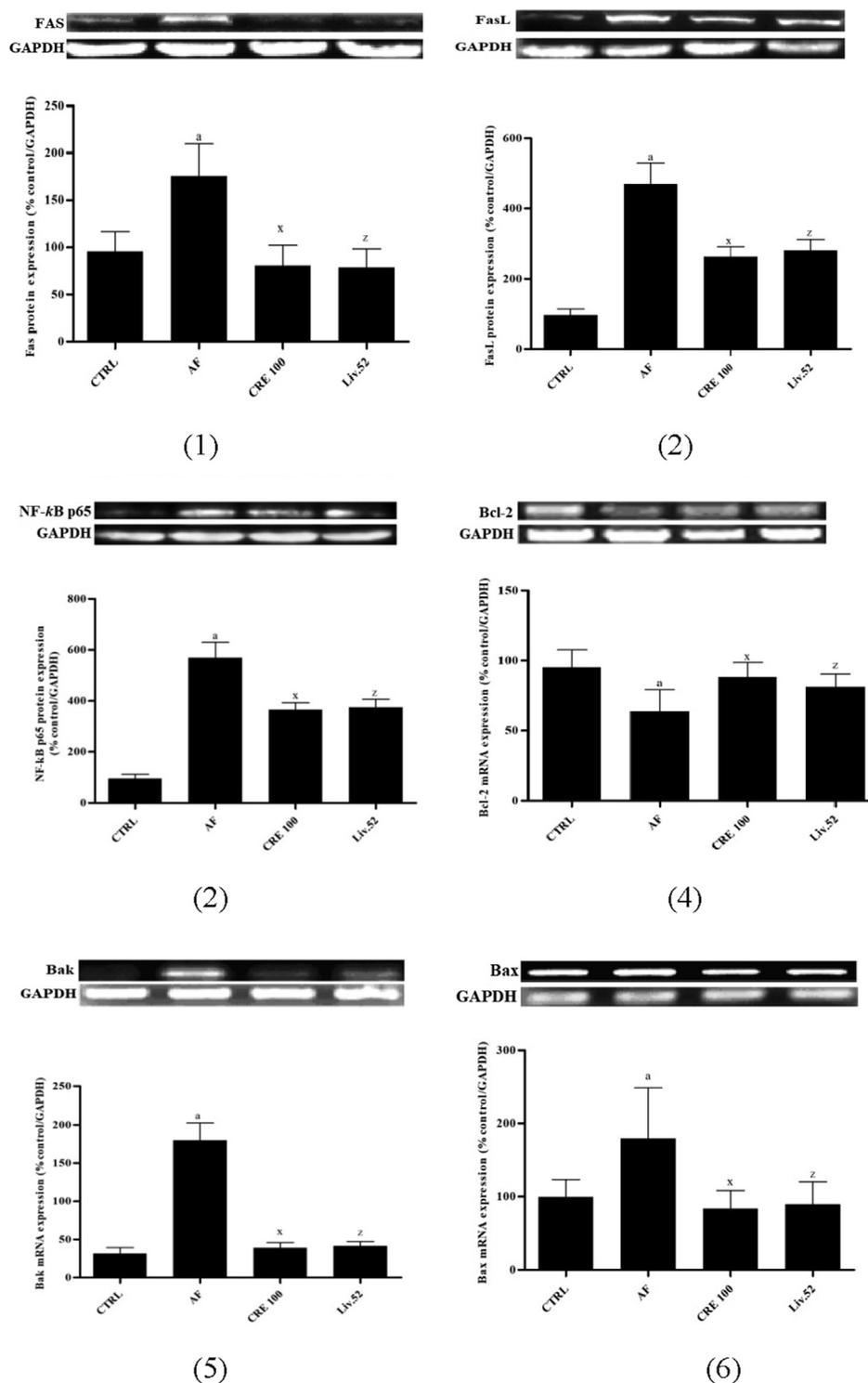


Fig. 6. (1–3) Showing protein expressions of Fas, FasL, and NF-κB p65 in livers of the experimental rats in Western blot analysis (4–6) Showing expressions of Bcl-2, Bak, and Bax mRNA in livers of the experimental rats in RT-PCR analysis. Statistical analysis was carried out by 1-way ANOVA and after that Dunnett's multiple comparison assay was done. ^aDenotes significance when compared to the control group (P less than 0.05); ^xDenotes significance when compared to AF (P less than 0.05); ^zDenotes significance when compared to AF (P less than 0.001) ($n = 5$).

were almost comparable to the Liv. 52 group. In comparison to Liv. 52, CRE (100 mg/kg) demonstrated a more significant effect in the up-regulation of Bcl-2 protein and down-regulation of p53 protein. In mitochondrial pathway cell apoptosis chiefly involves the Bcl-2 g. Bcl-2 and Bax (both belong to the Bcl family) control the secretion of proapoptotic factors from mitochondria. In the present study, Bax and Bak mRNA (proapoptotic) were down-regulated,

while Bcl-2 mRNA and protein which are anti-apoptotic were up-regulated in CRE (100 mg/kg) group opposite to AF group. Also, p53 regulates the Bcl-2 family proteins its expression in the given experiment was appreciably reduced in CRE (100 mg/kg) group opposite effect was recorded in the AF group. These findings indicated that CRE could exert its hepatoprotection by interacting with these proteins.

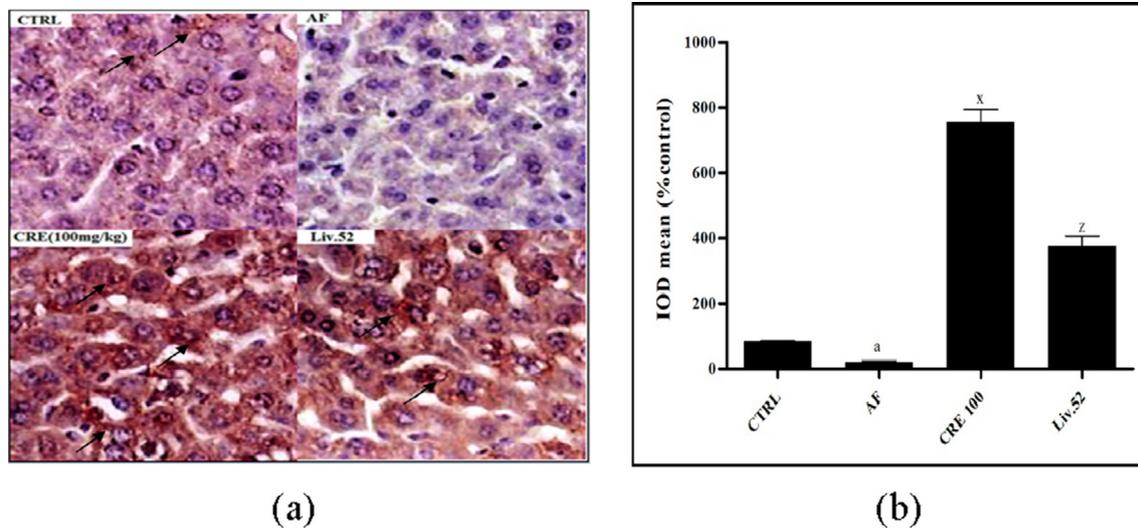


Fig. 7. (a) Immunohistochemical analysis of Bcl-2 in the livers of experimental rats. Black colored solid arrows are pointing towards the damaged areas, which is stained brown. (b) A graph is showing the results obtained from the immunohistochemical analysis of Bcl-2 in the livers of experimental rats. Statistical analysis was carried out by 1-way ANOVA and after that Dunnett's multiple comparison assay was done. ^aDenotes significance when compared to the control group (P less than 0.05); ^xDenotes significance when compared to AF (P less than 0.05); ^zDenotes significance when compared to AF (P less than 0.001) ($n = 5$).

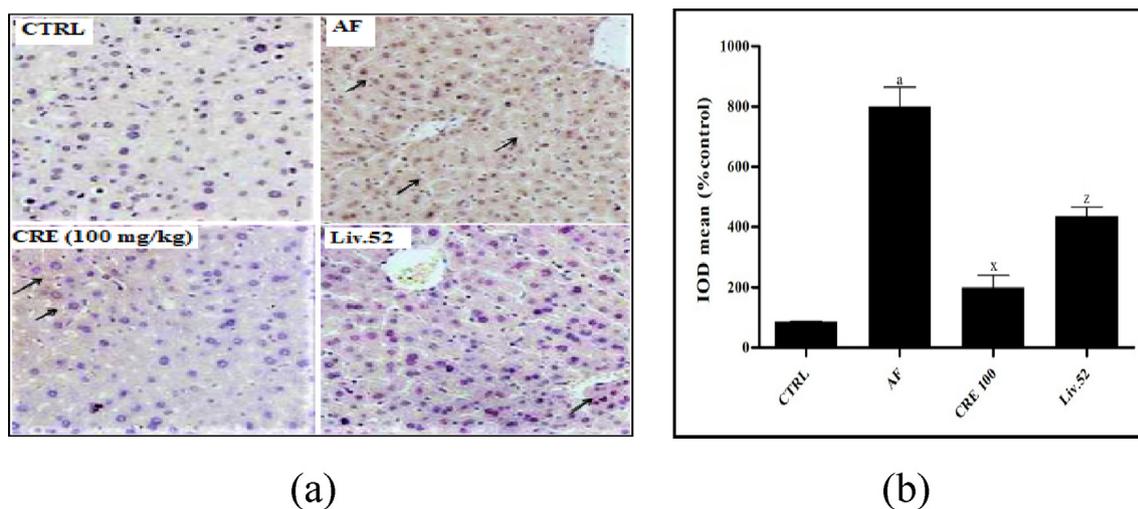


Fig. 8. (a) Immunohistochemical analysis of p53 in the livers of experimental rats. Black colored soil arrows are pointing towards the damaged areas, which is stained brown. (b) The graph is showing the results obtained from the immunohistochemical analysis of p53 in the livers of experimental rats. Statistical analysis was carried out by 1-way ANOVA and after that Dunnett's multiple comparison assay was done. ^aDenotes significance when compared to the control group (P less than 0.05); ^xDenotes significance when compared to AF (P less than 0.05); ^zDenotes significance when compared to AF (P less than 0.001) ($n = 5$).

3.7. Immunohistochemical analysis

Immunohistochemistry (IHC) is the most common application of immunostaining. It involves the process of selectively identifying antigens (proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. The AF group was stained brown due to the presence of apoptotic proteins.

The immunohistochemical study was performed for observing the expression of proteins, Bcl-2 and p53. As depicted in Fig. 7, the Bcl-2 expression in the AF group was five times lower in comparison to the control group. CRE (100 mg/kg) reversed the effect, and the expression of Bcl-2 expression of CRE at 100 mg/kg was higher as compared to the control and Liv. 52 groups. The p53 expression in the AF group was eight times more than the levels in the control group and the levels were appreciably reduced in CRE (100 mg/kg) and Liv. 52 groups (Fig. 8).

4. Conclusions

Inflammatory cytokines, such as TNF- α , induce liver injury in the rat model of alcoholic liver disease (ALD). Hepatoprotective cytokines, such as IL-6, and anti-inflammatory cytokines, such as IL-10, are also associated with ALD. IL-6 improves ALD via activation of the signal transducer and activator of transcription 3 (STAT3) and the subsequent induction of a variety of hepatoprotective genes in hepatocytes. IL-10 inhibits alcoholic liver inflammation via activation of STAT3 in Kupffer cells and the subsequent inhibition of liver inflammation. Interactions between pro- and anti-inflammatory cytokines and other cytokines and chemokines are likely to play important roles in the development of ALD (Kawaratani et al., 2013). But continued alcohol consumption overrides this protective mechanism of body and liver damage progresses from fibrosis to cirrhosis. In the current study, the serum TNF- α , IL-6 and IL-1 β levels increased while IL-10 decreased in

alcohol treated group (Aldred et al., 1999, Hill et al., 1992). TNF- α seems to be responsible for regulating products that stimulate inflammation and fibrosis in alcohol-induced hepatotoxicity (Aldred et al., 1999). CRE (100 mg/kg) treatment inhibited the increase of TNF- α and IL-6, suggesting CRE (100 mg/kg) attenuated an alcohol-induced inflammatory cascade in the liver. Considerable evidence suggested that TNF- α and IL-6 contribute to the pathogenesis of liver inflammatory diseases by activating the NF- κ B signaling pathway (Nanji et al., 1999). CRE (100 mg/kg) treatment corrected the disturbed levels of inflammatory mediators and brought back to near normal.

The TUNEL assay detects DNA breaks associated with necrotic cell death (Ansari et al., 1993; Nishiyama et al., 1996). Also, it detects active DNA repair (Kanohe et al., 1999). Therefore, TUNEL staining is a general method for the detecting of DNA breaks, one of several in situ DNA end-labeling techniques. DNA laddering is a distinctive feature of DNA degraded by caspase-activated DNase (CAD), which is a key event during apoptosis. CAD cleaves genomic DNA at internucleosomal linker regions, resulting in DNA fragments that are multiples of 180–185 base-pairs in length. Separation of the fragments by agarose gel electrophoresis and subsequent visualization, for example by ethidium bromide staining, results in a characteristic “ladder” pattern. The results here showed CRE (100 mg/kg) treatment lowered the number of tunnel positive cells and effectively reduced the formation of the DNA ladder formed due to alcohol. CRE (100 mg/kg) could hold down the activities of these two caspases and hence restrain hepatocyte apoptosis. Western blot and RT-PCR allowed to study the detailed mechanism involve in hepato-protection by lichen extract.

The results of this study reveal that *Cladonia rangiferina* (CR), may prove to be helpful in the treatment of alcohol-induced hepatotoxicity and oxidative stress. Results of different markers have shown that CRE (100 mg/kg) has demonstrated the best hepato-protective activity among CRE treated groups (CRE 50 mg/kg and CRE 200 mg/kg). These observations say about the importance of the components of the extract i.e., depsides and depsidones as found in the analysis of lichen extract. The ameliorative action of CR in alcoholic liver damage may exist due to antioxidant, anti-inflammatory, and anti-apoptotic activities. Exhaustive clinical studies have to be carried out for confirmation of the safety and benefits of CR before it can be used in human beings.

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Declaration of Competing Interest

No.

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