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Antioxidant and Hepatoprotective Activity of a Lichen *Usnea ghattensis* in Vitro

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Abstract Antioxidative and hepatoprotective activity of a cultured lichen *Usnea ghattensis* has been studied. The methanolic extract of cultured lichen *U. ghattensis* showed good antioxidant activity by preventing lipid peroxidation by 67% and 86% in Trolox-equivalent antioxidant capacity at 20 μg/ml. It also showed superoxide, 1,1-diphenyl-2-picrylhydrazyl, nitric oxide, and hydroxyl radical-scavenging activity, 89%, 89.6%, 94.8%, and 89.6%, respectively, and found levels higher then that known for the synthetic antioxidants butylated hydroxytoluene, butylated hydroxyanisol, and quercetin at 20 μg/ml concentration. The cultured lichen extract also showed hepatoprotection against ethanol-induced toxicity in the mice liver slice culture model by a significant decrease in the antioxidant enzymes, glutathione peroxidase, catalase, and superoxide dismutase, along with a decrease in lipid peroxidation and lactate dehydrogenase release.

Keywords Lichen · *Usnea ghattensis* · Antioxidant activity · Hepatoprotective activity

Introduction

Many studies have shown that reactive oxygen species (ROS) including oxygen-free radicals are causative factors in the etiology of degenerative diseases, including some hepatopathies. According to in vitro and in vivo studies, several classical antioxidants have been shown to protect hepatocytes against lipid peroxidation or inflammation, therefore preventing the occurrence of hepatic necrosis [1].

Antioxidants have been widely used as food additives to provide protection from oxidative degradation of foods and oils. The most extensively used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone [2]. However, BHT and BHA have been suspected of being responsible for liver damage and carcinogenesis [3]. Consequently, the development of an alternative antioxidant from natural origin has attracted considerable attention, and many researchers have focused on the discovery of new natural antioxidants for quenching biologically harmful radicals [4–6].

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Lichens are symbiotic associations of fungi, green algae, or cyanobacteria. Lichens have been used by various ethnic groups from the time of early civilization. Irrespective of the advances in medical sciences, tribal people still utilize these plants. The lichens are utilized for different purposes depending on their nutritive, medicinal, decorative brewing, distilling, dyeing, cosmetic, and perfumery properties. These different uses are substantiated by the complex lichen secondary metabolism producing many polyketide-derived compounds such as depsides, depsidones, and dibenzofurans, most of which are not known from other groups of plants. Lichens have been appreciated in traditional medicine, but their importance has largely been ignored by the modern pharmaceutical industry because of the difficulties in establishing axenic cultures and conditions for rapid growth. These factors preclude their routine use in most conventional screening processes [7].

The culture of whole lichen thalli is still difficult, and many problems are unsolved. Thus, the reason that experimental studies of lichens are neglected is due to the difficulty in culturing lichens.

In recent years, workers have succeeded in developing the cell aggregates composed of fungal and algal cells from natural lichen thallus fragments. These cultured fungal and algal cells have been studied to produce high quantities of biomass and the secondary metabolites for the screening of biological activities [8].

With this background, the aim of the present study was to culture in vitro the lichen *Usnea ghattensis* and to study its antioxidative and hepatoprotective properties.

Materials and Methods

Lichen Material

The natural thalli of the lichen species namely, *U. ghattensis* (fruticose; voucher no. 03.121), producing usnic acid and norstictic acid in nature was collected from Mahabaleswar (Satara District, Maharashtra State), India. A part of the material of the species has been preserved as a dried herbarium specimen in the Ajrekar Mycological Herbarium, Pune, India.

Lichen Culture

Lichen culture was started within 7 days after the collection. The isolation and culture methods were originally reported by Yamamoto et al. [9, 10]. Lichen thalli were cut into pieces of 1 cm long and were washed with tap water for overnight and homogenized with 5 ml of distilled water under sterile conditions. The suspensions were passed through a sterilized stainless filter with a 500-μm mesh. Small segments from the second filtration were picked up with a stainless loop and was planted on slant media in Petri dishes of 9 cm diameter and cultured at 18 °C in light (400 lux) with a daily cycle (8 h light/16 h dark). The following culture media were used: malt–yeast medium, malt extract (20 g/l), and yeast extract (2 g/l) solidified with agar (20 g/l).

Identification of Lichen Substances Produced in Cultured Tissue

Lichen substances were identified by high-performance liquid chromatography (HPLC). After 3 months of culture, cell aggregates composed of the mycobiont (fungus) and phycobiont (algae) were extracted in methanol by the soxhlet extractor. HPLC analysis was

carried out on Agilent 1100 system with a 360 autosampler, C8 (ZORBAX) column (Eclipse × DB-C8, 4.5×150 mm, 5 μm) and UV spectrophotometric detector, at 28 °C with the solvent: methanol–water–phosphoric acid (80:20:0.9, *v*/*v*/*v*). The detection wavelength was 254 nm, and the injection volume was 5 μl, with a flow rate of 1 ml/min [11]. Lichen substances were identified by their peck symmetry and their retention time, by comparison with authentic substances made to the standard concentration. The retention times were 7.21 min for usnic acid and 6.00 min for nortictic acid.

Extraction

The lichen cell aggregates composed of the mycobiont and photobiont were pulled from 20 Petri plates, which amounted 24.0 g fresh weight. A fresh weight of 24.0 g of the cell aggregates of *U. ghattensis* was then extracted in 20 ml of 10% aqueous methanol by the soxhlet extractor. The methanolic extract was filtered and dried in vacuo for 8 h. Then, the dried methanol-soluble extracts were weighed. The dried methanol-soluble extract obtained was 0.235 g. From this amount of dried extract, the concentrations of 10, 50, 100, and 200 μg/ml were used in determining the antioxidant and hepatoprotective activities.

Antioxidant Activity

Trolox-equivalent Antioxidant Capacity Assay

Trolox-equivalent antioxidant capacity (TEAC) of the extract of a cultured lichen *U. ghattensis* was carried out by the following procedure recently published by Arnao et al. [12] with some modifications. The stock solutions included 7.4 mM $ABTS^+$(2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonate) solution and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml $ABTS^+$ solution with 60 ml methanol to obtain in absorbance of 1.1±0.02 U at 734 nm using the UV-Vis spectrophotometer (Shimadzu, Japan). Fresh $ABTS^+$ solution was prepared for each assay. The extract (150 μl) was allowed to react with 2.9 ml of the $ABTS^+$ solution for 2 h under a dark condition. Then, the absorbance was taken at 734 nm.

DPPH Radical-scavenging Assay

The radical-scavenging ability of lichen *U. ghattensis* extracts was measured following procedure by Kyung et al. [13], using the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). A volume of 0.1 ml of the extracts was placed in a cuvette, and 2 ml of 0.06 mM ethanolic solution of DPPH was added. Absorbance measurements commenced immediately at 515 nm using a UV-Vis spectrophotometer (Shimadzu). The decrease in absorbance was determined after 50 min when the absorbance stabilized. The absorbance of the DPPH radical without the lichen extract was measured as the control. The percent inhibition of the DPPH radical in the samples was calculated according to the formula given below:

$$\%\,\text{inhibition} = \left[\left(A_{C(0)} - A_{A(t)}\right)/A_{C(0)}\right] \times 100$$

Where $A_{C(0)}$ is the absorbance of the control at $t=0$ min and $A_{A(t)}$ is the absorbance of the antioxidant at $t=70$ min. Vitamins C and E were used as positive controls.

Superoxide Anion-scavenging Activity

The superoxide anion-scavenging ability of the lichen extract was measured by the method of Nishimiki et al. [14], with slight modification. One milliliter of nitroblue tetrazolium (NBT) solution (150 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml nicotinamide adenine dinucleotide (NADH) solution (468 μM in 100 mM phosphate buffer, pH 7.4), and 0.1 ml of extract were mixed. The reaction started by adding 100 μl of phenazine methosulfate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance was measured at 560 nm against blank samples. Decreased absorbance indicated the increased superoxide anion-scavenging activity.

Nitric Oxide-scavenging Activity

The nitric oxide-scavenging ability of the extract was measured according to the method of Marcocci et al. [15]. Aliquots of 4 ml of extract solution were added to 1 ml of sodium nitroprusside solution (25 mM) in a test tube and then incubated at 37 °C for 1 h. A volume of 0.5 ml of the incubation solution was removed and diluted with 0.3 ml of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% napthylethylene-diamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with napthylethylenediamine dihydrochloride was immediately read at 570 nm and referred to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess reagent.

Hydroxyl Radical Scavenger Activity: Deoxyribose Assay

As a major intermediate in the metabolism of ROS, hydrogen peroxide can be formed from superoxide anions by the action of superoxide dismutase (SOD), and it is metabolized by Fe (II) to hydroxyl radicals or by the enzyme catalase (CAT) to water and molecular oxygen, according to the Haber–Weiss reactions [16]:

$$Fe^{3+} + O_2^{-\cdot} \rightarrow Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

The ability of the extract to inhibit the iron-induced decomposition of deoxyribose was assessed following the procedure described by Halliwell and Gutteridge [17]. The reaction mixture contained, in a final volume of 1.2 ml, the following reagents at the final concentrations stated: deoxyribose (2.8 mM), ferric chloride $FeCl_3$ (25 μM), and nitrilotriacetic acid (NTA; 100 μM). NTA and Fe^{3+} ions are premixed at the ratio given prior to the addition of deoxyribose, phosphate buffer (K_2HPO_4–KH_2PO_4) pH 7.4 (10 mM), different concentrations of the extract (10–200 μg/ml), and hydrogen peroxide (H_2O_2; 2.8 mM). The test tubes were incubated at 37 °C for 1 h. Products of OH attack upon deoxyribose was assessed by reaction with thiobarbituric acid (TBA; 1%) in acid solution (2.8% trichloroacetic acid). After 15 min at 100 °C and cooling over ice, the absorbance of the pink chromophore was measured at 532 nm.

Inhibition of Lipid Peroxidation

The inhibition of lipid peroxidation was determined using the method of Liegeois et al. [18]. An aqueous solution of linoleic acid and 2,2′-azobis,2-amidineopropane dihydrochloride

(AAPH) solution was prepared as described [18]. A 30-μl aliquot of 16 mm linoleic acid dispersion was added to a UV cuvette containing 2.81 ml of 0.05 mM phosphate buffer, pH 7.4, prethermostated at 40 °C. The oxidation reaction was initiated at 37 °C under air by adding 150 μl of 40 mM AAPH solution. Oxidation was carried out in the presence of aliquots (20 μl) of the lichen extract. The rate of peroxidation at 37 °C was monitored by recording the increase in absorbance at 234 nm caused by conjugated diene hydroperoxides. The percentage inhibition of lipid peroxidation was calculated by the following equation:

$$\%\,\text{inhibition} = ([A_0 - A_1]/A_0) \times 100$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in presence of the extract.

Total Soluble Phenolic Content

Total soluble phenolics in the lichen extracts were determined with Folin–Ciocalteu reagent according to the method of Slinkard and Singleton [19], using pyrocatechol as a standard. An extract of 0.1 and 2 ml of 2% Na_2CO_3 was added and mixed thoroughly. After 5 min of incubation, 0.1 ml of 50% Folin–Ciocalteu reagent was added and allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm. The concentration of total phenolic compounds in the extract was determined as micrograms of pyrocatechol, equivalent by using an equation that was obtained from the standard pyrocatechol graph given below:

$$\text{Absorbance} = 0.001 \times \text{Pyrocatechol}\ (\mu\text{g}) + 0.0033$$

Thin-layer Chromatography

Thin-layer chromatography (TLC) plates, coated with silica gel G (Fluka Chemie, Switzerland) to 0.25 mm thickness, were each spotted with 20 μl of the lichen extracts at a concentration of 2.5%. The plates were then developed in a solvent system of ethyl acetate/methanol/water (10:2:1; *v*/*v*/*v*). After drying, one of the developed plates was first observed under UV light at a wavelength of 365 nm and sprayed with 0.4 mM DPPH radical in methanol [20]. Furthermore, the developed TLC plates were sprayed separately with spray 1 solution (1% solution of iron (III) chloride in water) mixed immediately before use with an equal volume of a 1% solution of potassium hexacyanoferrate (III) in water (Barton's reagent) which gave a blue color in the presence of phenolic compounds, and spray 2 solution (2% iron (III) chloride in ethanol), which, when heated to 105 °C for 5–10 min, gave either a blue color, indicating the presence of phenolics with trihydroxy groups, or a green color, indicating phenols with dihydroxy groups, or a red/brown color, indicating the presence of other phenolics.

Hepatoprotective Activity

Animals

To asses the hepatoprotective activity of lichen extract, adult albino mice (6–8 weeks old) of either sex bred in the animal house of Agharkar Research Institute, Pune, were used for

the preparation of liver slices. The approval for this work, using animals, was taken from the Institutional Animal Ethical Committee of Agharkar Research Institute, Pune.

The in vitro mice liver slice culture method of Wormser et al. [21] was used for the determination of hepatoprotective activity of the lichen extract against ethanol-induced hepatotoxicity.

Preparation of Liver Slices

Adult albino mice of either sex, weighing 18–20 g was taken and dissected after cervical dislocation. The liver lobes were removed and transferred in prewarmed Krebs–Ringers–4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (KRH) medium (2.5 mM HEPES [pH 7.4], 118 mM NaCl, 2.85 mM KCl, 2.5 mM $CaCl_2$, 1.15 mM KH_2PO_4, 1.18 mM $MgSO_4$, and 4.0 mM glucose). Liver were sliced into small pieces (4–6 mg) of about 0.5× 0.5×0.5 mm using a prep blade. Slices were incubated for 1 h in a capped Erlenmeyer flask containing approx. 30 ml of KRH medium at 37 °C in a waterbath shaker.

During this incubation, the slices were washed by carefully removing the medium and replacing it with fresh medium every 10 min. All media used for incubation and rinsing were prewarmed. Slices were divided into small portions (20–22 slices) of 100–120 mg wet weight. Slices were incubated for 1 h in 2 ml KRH medium in 20 ml capped beakers at 37 °C in a rotary waterbath shaker. The cultures were aerated in an Erlenmeyer flask with oxygen every 10 min by removing the cap.

Experiment Design

Liver slices were divided in to three sets: set 1, control, slices incubated in KRH medium; set 2, slices incubated in 112 mM ethanol; set 3, slices incubated in 112 mM ethanol+lichen extract. The 50% release of liver marker enzyme in slices treated with ethanol or in combination with lichen extract was determined by following the method of INVITOX Protocol no. 42 [22].

The Dry methanolic extract with concentrations 10, 50, 100, and 200 μg was added in the KRH medium before transferring the preincubated liver slices in the medium. Duplicate cultures were set up for each concentration to minimize the errors. Cultures were incubated for 2 h at 37 °C. Trolox is a water-soluble vitamin E analogue, and it is soluble in the KRH medium and used as a standard antioxidant to compare with the response of methanolic lichen extract.

Hepatoprotective Effect in Vitro

After completion of the incubation period, liver slices were homogenized in ice-cold 100 mM potassium phosphate buffer (pH 7.4) giving a concentration of 100 mg/ml and centrifuged at 10,000×*g* for 3 min at 4 °C. Liver marker enzyme lactate dehydrogenase (LDH) and antioxidant enzymes SOD, CAT, and glutathione peroxidase (GPx) were estimated in the medium and in the supernatant. Measurement of lipid peroxidation and glutathione (GSH) content were also measured. Effective concentration, EC_{50}, of lichen extract was estimated. It is the dose that inhibits the LDH release by 50% with respect to control.

Measurement of Lactate Dehydrogenase Activity

LDH activity was measured specrtophotometrically at 340 nm according to the method of Racher [23]. Cuvette containing 10 mM Tris–Cl buffer, pH 7.2 (2 ml), 10 mg/ml NADH

and pyruvate, and 70 μl of sample were added to this reaction mixture. Commercially available LDH (Sigma) was used as the standard.

Measurement of Superoxide Dismutase Activity

The SOD activity was assayed spectrophotometrically according to Marklund and Marklund [24] by means of inhibition of pyrogallol autoxidation. Reaction mixture containing 50 mM Tris–cacodylic acid buffer, pH 8.2, 1 mM diethylenetriaminepentaacetic acid, 0.2 mM air-equilibrated pyrogallol, iron in a trace amount, and 100 μl liver tissue homogenate was added to this mixture. Changes in absorbance at 420 nm were recorded at 30-s intervals for 5 min. SOD activity was determined from a standard curve of percentage inhibition of pyrogallol autoxidation with standard SOD. Data were expressed as SOD units per minute per milligram protein as compared with the standard.

Measurement of Catalase Activity

CAT activity was measured by the method of Aebi [25]. Liver tissue homogenate (10%, *w*/*v*) were centrifuged (10,000 rpm) at 4 °C for 10 min. One milliliter of the supernatant was added to a quartz cuvette, containing 30 mM H_2O_2 solution prepared in 50 mM potassium phosphate buffer (pH 7.0) and water. Changes in absorbance at 240 nm were recorded at 15-s intervals for 1 min. One unit was defined as the amount of the enzyme that converts 1 mol substrate to product in 1 s.

Measurement of Glutathione Peroxidase Activity

GPx activity was measured according to the method of Rotruck et al. [26]. Briefly, 2 ml of 80 mM sodium phosphate buffer (pH 7.0) contained 1 mM ethylenediamine tetraacetic acid, 1 mM sodium azide, 0.4 mM GSH, and 100 μl of the liver tissue homogenate. The reaction was started by the addition of 2.5 mM H_2O_2. The change in absorbance at 255 nm was recorded at 30-s intervals for 3 min. The values were expressed as micrograms of GSH consumed per minute per milligram protein.

Measurement of Lipid Peroxidation

Lipid peroxidation was estimated by the method of McMillan et al. [27] in terms of TBA-reactive substances (TBARS) formed in liver tissue homogenate, with some modification. Briefly, liver tissue homogenate was incubated with 1:1 of 0.67% TBA at 100 °C for 15 min. The mixture was allowed to cool at room temperature, and absorbance was read at 532 nm by a UV-Vis spectrophotometer. TBARS formation was quantified with a standard curve using known amounts of malondialdehyde (MDA).

Estimation of Glutathione

GSH content was determined after deproteinization [28]. Proteins were precipitated with metaphosphoric acid (MPA) and salt. Then filtrate was added to a cuvette containing 0.5 M phosphate buffer, pH 7.5, and the color was developed by the addition of 5, 5′-dithiobis (2-nitrobenzoic acid). The absorbance was read at 420 nm after 1 h.

Estimation of Proteins

Protein in the tissue homogenate was estimated according to the method described by Bradford [29].

In Vitro Toxicity Study

In the liver slice culture system release of LDH was used as a marker to study the hepatotoxicity of ethyl alcohol. The toxicity of cultured lichen extract and Trolox, a water-soluble vitamin E analogue, was determined. Liver slices were divided into three sets (100 mg wet weight) and treated with fixed concentrations of 10, 50, 100, and 200 mg of (1) Trolox, (2) lichen extract only, and (3) ethanol. These sets were incubated for 2 h at 37 °C. After completion of incubation period, %LDH release was calculated. No toxic effect of Trolox or lichen extract was detected even at doses of 100 and 200 mg.

Histopathology of Liver Slice

Histopathology of liver slices was carried out by fixing the liver slices in 4% formaldehyde buffer. Sections of 10 μm size were cut by a freeze microtome and stained with haematoxyline/eosin and examined under a light microscope.

Chemicals

Linoleic acid, DPPH, NBT, NADH, PMS, sodium nitroprusside, sulfanilamide, sodium acetate, 2,4,6-tripyridyl-*s*-triazine, potassium hexacyanoferrate (III), KRH, MPA, 5,5′-dithiobis (2-nitrobenzoic acid), potassium persulfate, CAT, hematoxyline, and eosin were purchased from Hi Media, India. Napthylethylenediamine dihydrochloride, deoxyribose, NTA pyrocatechol, pyrogallol, and pyruvate were purchased from SRL Chem. India. ABTS, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ascorbic acid, AAPH, LDH, SOD, GPX, GSH, Tris–cacodylic acid, and diethylenetriaminepentaacetic acid were purchased from Sigma-Aldrich Chemical, USA. Ethanol, TBA, tricarboxylic acid, folin–ciocalteu, and MDA were purchased from Merck, Germany. All other routine chemicals used were of analytical reagent grade.

Statistical Analysis Experimental results were mean±SEM of five parallel measurements. Student's *t* test was used for statistical significance between groups.

Results

Lichen *U. ghattensis* was cultured under laboratory conditions, and the produced lichen substances usnic acid and nortictic acid were identified by HPLC as they were produced in natural lichen thalli. The methanolic extract of this lichen species was screened for the antioxidant and hepatoprotective potential.

The antioxidant activity was measured in terms of lipid peroxidation inhibition and scavenging of hydroxyl radical, nitric oxide, superoxide, DPPH radical, and TEAC using various in vitro assay systems (Figs. 1 and 2). The extract has shown concentration- and time-dependent antioxidant activity with the IC_{50} value 11 to 17 μg extract/ml for 50% lipid

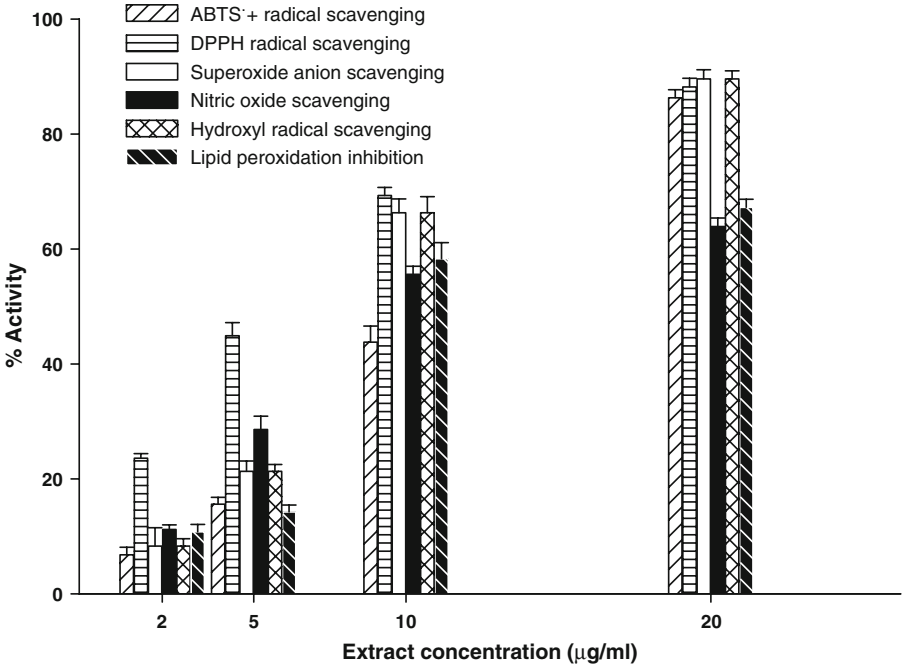

Fig. 1 Antioxidant activity of various concentrations of extract of a lichen *U. ghattensis* cultured in vitro

peroxidation inhibition and 50% scavenging of superoxide, DPPH free radicals, hydroxyl radical, and nitric oxide, in the assay system.

Effect of Lichen Extract on Lactate Dehydrogenase and Lipid Peroxidation Activity in Mice Liver Slice

The effects of various concentrations of lichen extract on the LDH activity of mice liver tissue homogenate are presented in Fig. 3. LDH activity was significantly decreased (50%) in the liver slice treated with 112 mM ethanol compared to control (untreated) or treated with Trolox 50 μg/100 mg tissue weight. LDH activity in liver tissue increased with the increase in extract concentration. It seems that the LDH activity is dose dependent.

The LDH release and lipid peroxidation of mice liver tissue under various time courses are presented in Fig. 4. Continuous LDH release (50%) was observed up to 2.0 h by the liver tissue treated with ethanol only, whereas the LDH release by the control (untreated) was constant (below 10%). However, the extract-treated liver tissue showed 20% LDH release up to 1.0 h incubation and further decreased equivalent to the control level at 2.0 h incubation.

Ethanol is known to generate oxidative stress in cells, which can be measured from the extent of lipid peroxidation in liver tissue. The lipid peroxidation of the liver tissue under various time courses was measured in terms of TBARS and is expressed as micromoles malondealdehyde (MDA) formed. The results are presented in Fig. 4.

The continuous increase of MDA (up to 0.07 μmol) was observed up to 2.0 h incubation in the liver slice treated with ethanol alone, whereas in the control (untreated), the liver tissue

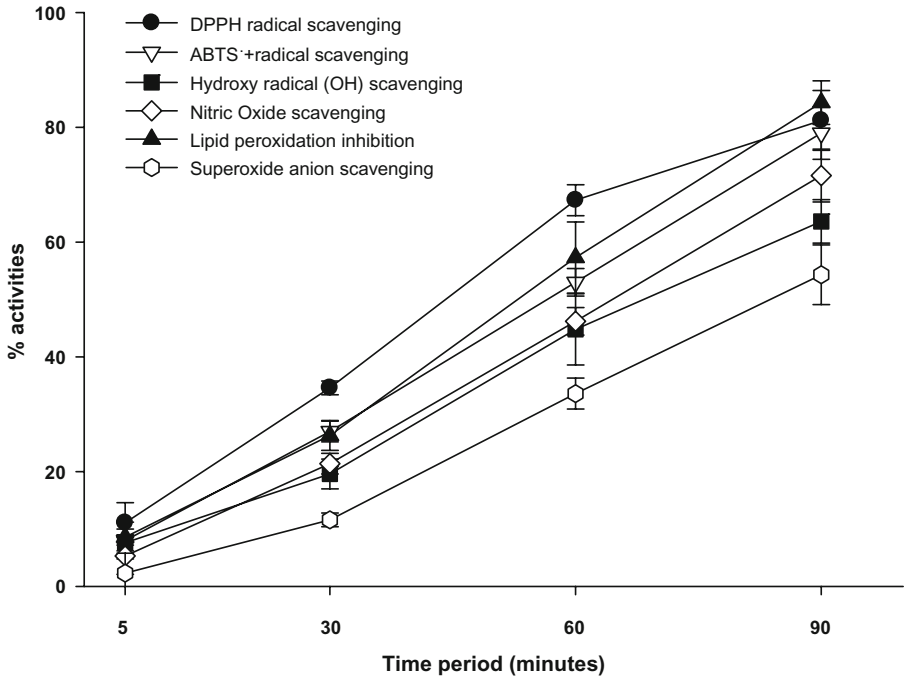

Fig. 2 Antioxidant activity of the extract of a lichen *U. ghattensis* at various time periods

showed a linear increase in MDA formation (below 0.02 μmol) up to 1.5 h. However, the formation of MDA gradually decreased, and finally a concentration below 0.005 μmol MDA was obtained at 2.0 h incubation of liver tissue treated with lichen extract along with ethanol.

The above results indicated that LDH activity and the lipid peroxidation are dose–time dependent.

Effect of Lichen Extract on the Antioxidant Status of Liver Slice Culture

Antioxidant enzymes SOD, CAT, and GPx and the nonenzymatic antioxidant GSH protect cells from oxidative stress of highly reactive free radicals. These enzymes are induced on the generation of free radicals in cells. Activities of SOD, CAT, GPx, and GSH were checked in liver slice culture treated with ethanol, ethanol+Trolox, and extract+ethanol. The activities were checked every 30 min up to 2.0 h. The results are presented in Table 1.

The activities of the antioxidant enzymes SOD, CAT, and GPx were linearly increased up to 2.0 h in the liver tissue treated with ethanol, whereas SOD and GPx linearly increased up to 1.0 h incubation except CAT, which decreases after 30 min incubation in the liver tissue treated with Trolox along with ethanol. However, the liver tissue treated with lichen extract with ethanol showed a linear increase in the activities of SOD, CAT, and GPx up to 1.0 h, and thereafter, the activities were decreased.

As far as GSH content is concerned, there was a continuous increase in the GSH content observed in the liver tissue treated with ethanol only, whereas GSH content was increased up to 1.0 h and thereafter decreased in the tissue treated with extract or Trolox with ethanol. The results indicated that the antioxidative status of liver slice is time dependent.

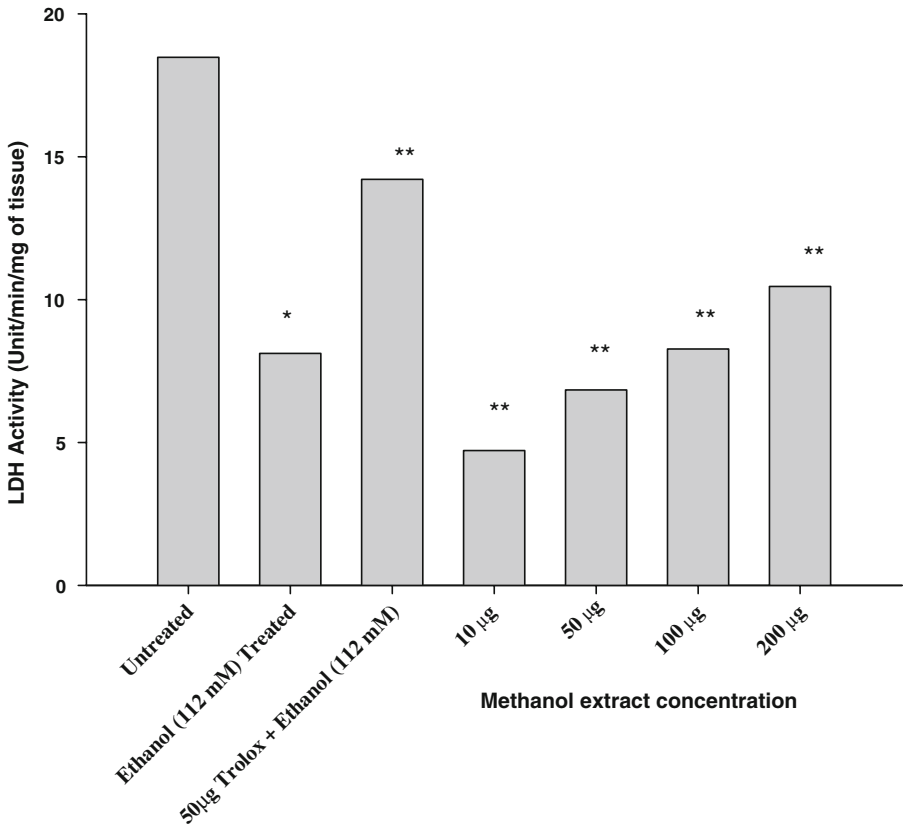

Fig. 3 Effect of lichen extract on LDH activity in mice liver slice in vitro. Results are mean of three consecutive readings. *Asterisk*, $p<0.001$, when compared to normal, *double asterisk*, $p<0.05$ when compared with that treated with ethanol

Histological Examination of Liver Slice Cultures

Histopathological studies of the liver tissue were done. Degeneration of hepatocytes in the liver tissue treated with ethanol was prominent, whereas the extract-treated liver tissue showed very less fatty degeneration up to 30 min and thereafter started regeneration of hepatocytes.

Components in the Lichen Extract

As far as the active component in the lichen extract is concerned, after qualitative analysis by HPLC and TLC, it showed the presence of lichen substances usnic acid and norstictic acid along with trihydroxy phenolic compounds in the extract.

Discussion

Antioxidative potential in terms of scavenging of DPPH, superoxide, nitric oxide, and hydroxyl radicals along with lipid peroxidative inhibitory activities of the extract of a

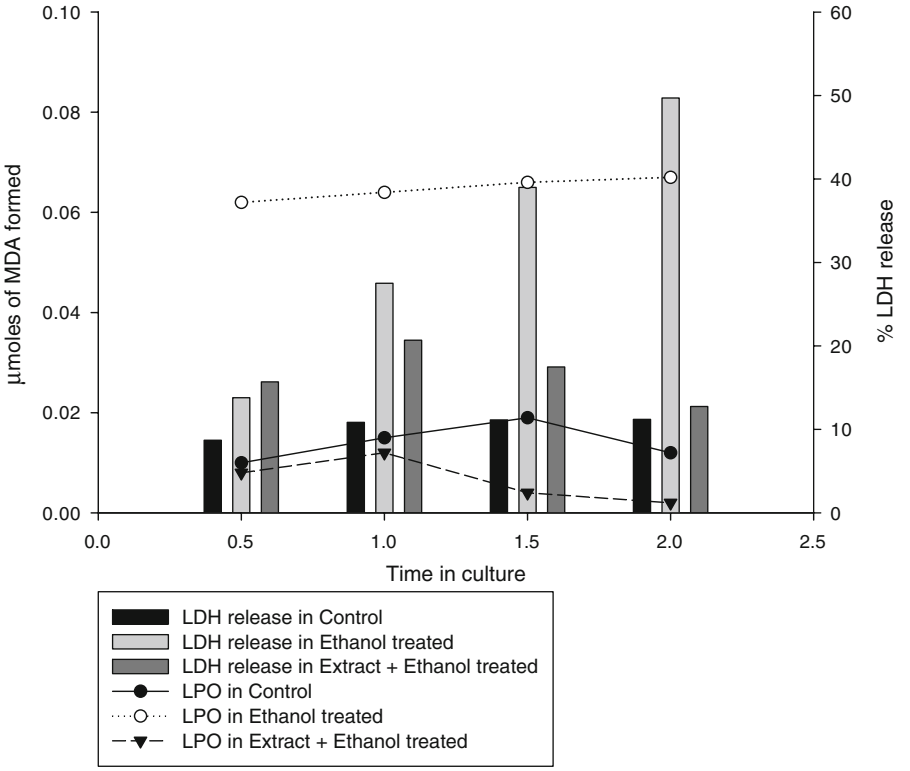

Fig. 4 Time course of lipid peroxidation of liver tissue and LDH release in the presence of ethanol and the methanolic extract of lichen *U. ghattensis*. Values are mean of five experiments

cultured lichen *U. ghattensis* was assayed in an in vitro system. The results showed that the extract has the capacity to scavenge the radicals. To prove this property, we have examined the hepatoprotective effects of the lichen extract in ethanol-induced toxicity in a liver slice culture model, and the results are discussed.

The development of hepatic disease in response to ethanol exposure associated with metabolic imbalance in the liver leads to the formation of ROS. Inadequate removal of ROS may cause cell damage by attacking membrane lipids and proteins and inactivating antioxidant enzymes thus mediating several forms of tissue damage [30–33].

The liver slice is a microcosm of the intact liver consisting of highly organized cellular communities in which the different cell types are subject to mutual contact. Liver slice culture is therefore an in vitro technique that offers the advantages of an in vivo situation and hence is a more suitable model for the experimental analysis of hepatotoxic events [34, 35].

The lipid peroxidation in terms of MDA formation was increased with the increasing of ethanol exposure up to 2.0 h in the liver tissue treated with ethanol. However, the administration of lichen extract significantly decreased the MDA formation in the liver tissue. These results would indicate that the extract could hinder their interaction with polyester fatty acids and could abolish the enhancement of the lipid peroxidation process leading to MDA formation [36, 37].

In the present study, we found that the activities of antioxidant enzymes SOD, CAT, and GPx were linearly increased up to 2.0 h in the liver tissue treated with ethanol. Our results

Table 1 Effect of cultured lichen *U. ghattensis* extract on the antioxidant status of liver slice culture in vitro against ethanol-induced hepatotoxicity.

Enzyme	Treatment	Time at which the activity was measured			
		0.5 h	1.0 h	1.5 h	2.0 h
GSH (μg/mg tissue)	Normal	7.40±0.23	8.00±0.12	8.60±0.11	6.80±0.53
	Ethanol	14.60±0.33*	15.30±0.43	17.86±0.93*	23.02±0.58*
	Trolox+ethanol	7.02±1.01	9.53±0.92	7.44±0.21	5.53±0.48
	Extract+ethanol	7.02±0.41	10.51±0.29	6.89±0.89	5.91±0.12
GPx (unit min^{-1} mg $protein^{-1}$)	Normal	0.040±0.005	0.032±0.001	0.040±0.002	0.041±0.003
	Ethanol	0.044±0.004*	0.049±0.006*	0.051±0.005*	0.069±0.01*
	Trolox+ethanol	0.028±0.002	0.034±0.003	0.034±0.001	0.020±0.006
	Extract+ethanol	0.018±0.003	0.020±0.001	0.038±0.002	0.030±0.004
CAT (unit min^{-1} mg $protein^{-1}$)	Normal	0.190±0.05	0.212±0.01	0.217±0.01	0.222±0.01
	Ethanol	0.813±0.05*	0.815±0.04	0.911±0.06*	0.971±0.06*
	Trolox+ethanol	0.836±0.003	0.574±0.003	0.434±0.001	0.262±0.02
	Extract+ethanol	0.366±0.006	0.390±0.02	0.312±0.003	0.266±0.003
SOD (unit min^{-1} mg $protein^{-1}$)	Normal	0.009±0.01	0.016±0.001	0.014±0.001	0.015±0.05
	Ethanol	0.024±0.004*	0.026±0.02*	0.028±0.02*	0.029±0.01*
	Trolox+ethanol	0.015±0.03	0.018±0.06	0.012±0.05	0.010±0.04
	Extract+ethanol	0.011±0.001	0.014±0.002	0.009±0.005	0.008±0.05

Values are mean±SEM for three sets of culture in each observation
GSH Glutathione, *GPx* glutathione peroxidase, *CAT* catalase, *SOD* superoxide dismutase
*$p<0.001$ as compared with normal group

were in agreement with the results on an increase in Mn-SOD, which was found after a short-term exposure to ethanol in a cell culture model using human hepatoma cells reported by Perera et al. [38]. This increased diminished after repeated ethanol administration. Further, the prolonged ethanol exposure may suppress the cellular adaptive response to oxidative stress [39].

The relative contribution of CAT and GPx in decomposition of endogenous hydrogen peroxide is dictated by tissue specificity. GPx has a more important contribution in the liver [40], whereas CAT predominates in the renal tissue [41]. Further, our results are in agreement with the results on an increase of CAT and GPx activities in the kidney after ethanol treatment [42]. Therefore, an increase in CAT activity would indicate an enhanced ethanol toleration of the liver.

Reduced GSH offers one of the mechanisms for the scavenging of toxic free radicals. As far as GSH content in the liver tissue after ethanol treatment is concerned, a continuous increase in the GSH content up to 2.0 h was observed in the liver tissue. In this study, our results are consistent with the reports that an increase in GSH content could be expected to prepare the cell against a potential oxidative insult [43, 44].

The results on the administration of the lichen extract or Trolox in the ethanol-induced toxicity in the liver revealed significant depletion in the lipid peroxidation, antioxidative enzymes SOD, CAT, and GPx, and the nonenzymatic antioxidant GSH in this study. This would indicate that the methanol extract of cultured lichen *U. ghattensis* has antioxidant and hepatoprotective potentials.

Usnic acid and nortictic acids are the main phenolic lichen substances in the lichen *U. ghattensis.* The key role of phenolic compounds is the ability to scavenge free radicals and ROS such as singlet oxygen, superoxide free radical, and hydroxyl radicals [45]. The

methanolic extract of cultured lichen *U. ghattensis* has antioxidative and hepatoprotective potentials. Our results are in agreement with those that reported the ability of phenolic compounds to scavenge free radicals and active oxygen species [46, 47].

In conclusion, the results of the present study suggest that the *U. ghattensis* extract could prevent oxidative liver damage. Further comprehensive pharmacological investigations will be needed to elucidate the mechanism of this hepatoprotective effect.

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