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Prophylactic Efficacy of *Rhodiola rosea* Extract against Hypobaric Hypoxia-Induced Pulmonary Injuries in Rats and its Toxicity Evaluation.

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ABSTRACT

People travel to high altitude for leisure and work purposes and if not acclimatized, develop high altitude illnesses. In traditional medicine system, *Rhodiola rosea* is being used for the prevention of high altitude sickness and lung injuries. The present study was designed to evaluate the effects of prophylactic administration of *Rhodiola rosea* hydro-ethanolic extract (RRE; 100 and 250 mg/kg body weight) on pulmonary injuries of rats exposed to hypobaric hypoxia (7620 m, 6 h). The protective efficacy was assessed using pulmonary damage markers, oxidative stress markers, and lung histology. The results demonstrated that RRE significantly ($p < 0.05$) attenuated the hypoxia-induced increase in lung water content; protein, albumin, and lactate dehydrogenase levels in bronchoalveolar lavage fluid; lipid peroxidation and antioxidants (GSH, SOD, and CAT) in lung homogenate, and also restored changes in lung histology. The toxicity profile of RRE indicated that LD_{50} was > 5000 mg/kg b.w. and its long-term administration (28 days) didn't cause any toxic effects; RRE was free from heavy metals and its HPLC analysis revealed the presence of active constituents, salidroside and rosavins. These results suggested that RRE is non-toxic and its pulmoprotective effects may be attributed to the anti-oxidant properties of RRE.

Keywords: edema, hypoxia, lung, oxidative stress, *Rhodiola rosea*

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INTRODUCTION

Exposure to high altitude induces physiological changes that lead to acute mountain sickness (AMS) and its life-threatening complications, high altitude pulmonary edema (HAPE) or high altitude cerebral edema (HACE). Due to low partial pressure of oxygen at high altitude, people travel to these areas often experience altitude sickness (AMS) characterized by headache, dizziness, light headedness, fatigue, weakness, gastrointestinal disturbances, and insomnia [1]. Rapid ascent to high altitude above 2500 m causes brain and lung problems in unacclimatized persons which is sometimes preceded by AMS. As lungs are the first interface between environment and metabolism, it is the first organ that is affected on exposure to hypoxia and cold**.** HAPE is a form of non-cardiogenic pulmonary edema that develops in otherwise healthy people within 2-5 days of altitude exposure**.** It occurs as a result of fluid accumulation in the lungs (pulmonary edema) which may be caused due to excessive hypoxic pulmonary vasoconstriction that result in increased pulmonary vascular permeability and pulmonary hypertension [1].

It was reported that contact to oxygen deficient environment leads to oxidative stress in lungs, and the generation of reactive oxygen species (ROS)/reactive nitrogen species is one of the main causative factors for the etiology of AMS, HAPE, and HACE [2]. Increased ROS formation under hypoxic environment may not only increase pulmonary arterial pressure but also resulted in increased pulmonary capillary permeability [2, 3]. Acetazolamide is used as a prophylactic drug against AMS [4] and HAPE [5] but associated with adverse effects such as paresthesia, urinary frequency, and dysgeusia [4]. There are reports which suggested that antioxidants supplementation viz. plants may provide protection against hypoxia-induced oxidative stress [2, 6] and may curtail high altitude induced pulmonary dysfunctions [7, 8].

Rhodiola rosea L. ("Golden root" or "Arctic root") is an arctic-alpine species from the family Crassulaceae, distributed in high altitudes of Asia, Europe, and North America. It is widely used in the traditional medicine system of Europe and Asia for decreasing depression, enhancing work performance and physical endurance, stimulating nervous system, eliminating fatigue, and preventing high altitude sickness [9]. *R. rosea* has been shown to possess pharmacological properties, including adaptogenic, hepatoprotective, cardioprotective antidiabetic, anticancer, antibacterial, anti-inflammatory, anti-stress, anti-aging, immunostimulating, and central nervous system stimulating [9, 10]. *R. rosea* contain more than 140 compounds but most of the pharmacological activities of *R. rosea* are attributed mainly to the presence of salidroside and rosavins (combination of rosavin, rosarin, and rosin), which are considered to be the active constituents of *R. rosea* (Figure 1) [9, 10, 11]. Salidroside and rosavins are known to possess antioxidant properties [11]. Many species of Rhodiola is available worldwide but *R. rosea* is the most studied and commercially used. There are many variants of standardized Rhodiola extracts available in the market. Thus, it becomes very crucial to evaluate the efficacy and safety of the commercial version to avoid any kind of toxicity related issue [12]. Though traditional medicine system recommended *R. rosea* for prevention of high altitude sickness but very little scientific evidence is available. Recent studies have shown only the computational approach to determine therapeutic use of *R. rosea* in the treatment of AMS [13]. Researchers also utilized philosophical model to explore benefits of *R. rosea* as a therapeutic strategy for the treatment of pulmonary hypertension [14]. Other species of Rhodiola (*R. crenulata*) has shown beneficial effects in alleviating hypoxia-induced pulmonary injuries in rats [8] but *R. rosea* extract (RRE) has not been studied for its prophylactic efficacy against hypobaric hypoxia induced pulmonary injuries in experimental rats.

The present study was undertaken to evaluate the prophylactic efficacy of RRE against hypobaric hypoxia (7620 m) induced pulmonary injuries in rats in terms of evaluating pulmonary damage markers, oxidative stress markers, and histopathological alterations. The presence of active constituents and heavy metals were determined in RRE using high performance liquid chromatography (HPLC) and atomic absorption spectrophotometer (AAS), respectively. RRE was also studied for its toxicity (acute and sub-acute), if any, in rats.

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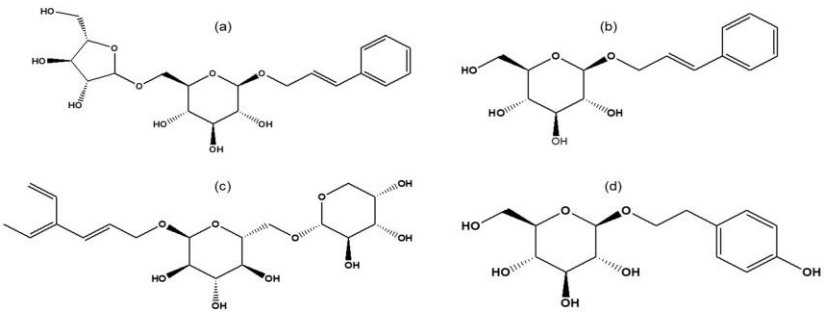

Figure 1: Bioactive compounds of *Rhodiola rosea,* (a) Rosarin, (b) Rosin, (c) Rosavin, and (d) Salidroside

MATERIALS AND METHODS

Test Material

Commercial hydro-ethanolic extract of the roots of *R. rosea* (RRE) was purchased from M/s Panacea Phytoextracts, Ahmedabad. The certificate of analysis provided by the manufacturer stated it to contain 3% salidroside and 1% rosavin. *R. rosea* extract was prepared by dissolving in 1:1 ratio of ethanol and water. Acetazolamide (powder) was purchased from Sigma-Aldrich.

Chemicals & reagents

Salidroside, rosavin, rosarin, and rosin standards were purchased from Sigma-Aldrich (Saint Louis, MO 63103, USA). Water, methanol, and acetonitrile were HPLC grade (Merck, Germany). All the other chemicals used in the study were of analytical grade.

HPLC analysis

RRE (1.0 mg) was dissolved in ethanol and water (1:1) to make a total volume of 1 mL. The standards (1.0 mg) were accurately weighed and dissolved in 1 mL of distilled water. All the standards and extracts were freshly prepared.

HPLC was performed using SPD-10A system consisting of binary HPLC pump, ultraviolet detector, and a rheodyne manual injector equipped with 20 µL loop (Shimadzu, Japan). Separation was achieved on reverse phase C_{18} column (5.0 µm, 150 × 4.6 mm) (Thermo Fischer) using the isocratic mobile phase mixture of phosphate buffer (pH 6.7) and acetonitrile (30:70 v/v). 20 µL of the sample was injected at a flow rate of 1.0 mL min^{-1} and wavelength 205-254 nm was used for detection. The chromatographic separation of compounds was achieved within 20 min. The system was controlled using class VP software (Shimadzu, Japan). Standard stock solutions of rosarin, rosin, rosavin, and salidroside were prepared and dissolved in appropriate amounts of diluent stock (water and methanol; 9:1 v/v). RRE and standards were dissolved in appropriate amounts of diluent stock (water and methanol; 8:2 v/v) to achieve concentration of 50 µg/mL. Calibration standards were plotted at concentrations of 5, 10, 20, 30, 40, 50, 100, 250, and 500 µg/mL. The accuracy and precision was assessed by using replicates of standard samples. The inter-day and intra-day precision was assessed by interpretation of three different batches at three different days. The compounds peaks were assigned on the basis of retention time and spectral matching. The peaks of observed standards were compared with RRE.

Heavy metals analysis in RRE

Wet digestion method was followed to determine heavy metals in RRE. Briefly, 0.2 g of RRE was taken in 100 mL volumetric flask and 4 mL HNO_3 was added. The solution was heated and then 4 mL perchloric acid was added. Filtered solution was made up to 100 mL with distilled water. The digested samples were used for metal analysis using AAS, Model 932 AA. All the samples were analyzed three times to estimate the concentration of arsenic (As), lead (Pb), mercury (Hg), and cadmium (Cd). Pb (II) and Cd (II) was determined by flame AAS at wavelength 217 nm and 228.8 nm, respectively while As (III) and Hg (II) was determined by using hydride generation technique at wavelength 193.7 nm and 253.7 nm, respectively. The measurements were

made by using spectral lines and minimum detection limits (MDL) of the instrument. Heavy metal values analyzed in the RRE (mg/kg) were compared with the available World Health Organization (WHO) maximum allowable limits for assessing quality of herbal medicines with reference to contaminants and residues [15].

Total flavonoid and total phenolic contents in RRE: in vitro assay

Aluminium chloride colorimetric method was used for the determination of total flavonoid content [16]. The color developed was read spectrophotometrically at 510 nm and expressed in mg quercetin equivalent (mg QE/g RRE) using standard curve of quercetin (1 mg/mL). Total phenolic content was estimated in RRE using Folin-Ciocalteu reagent based assay [17]. The absorbance was recorded at 765 nm. The phenolic content was calculated as gallic acid equivalents (mg GAE/g RRE) using standard curve of gallic acid (1mg/mL).

In vivo experimental studies

Experimental animals

Inbred male and female Sprague-Dawley rats, weighing 180 ± 20 g were taken for the study. Animals were maintained under controlled environment at 25 ± 1 °C and 12 h light–dark cycle in the Institute's Animal Experimental Facility. The rats were housed in polyvinyl cages, fed standard animal food pellets (Golden feed, Delhi), and were offered tap water *ad libitum*. All experiments were conducted after approval of Institutional Animal Experimentation Ethics Committee in accordance with the regulations specified by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Experimental design

The whole study was divided into two experiments. Experiment 1 was conducted to evaluate the efficacy of RRE in countering hypobaric hypoxia-induced pulmonary injuries in rats. Experiment 2 was carried out to evaluate the toxicity of RRE.

Efficacy studies

Experiment 1: Total 50 rats were divided into 5 groups with 10 rats in each group. Group 1: control animals, without hypoxia exposure; Group 2: Hypoxia alone (H), animals were exposed to hypoxia (H) for 6 h; Group 3: Hypoxia treated with 100 mg/kg b.w. RRE (RRE100 + H), Group 4: Hypoxia treated with 250 mg/kg b.w. RRE (RRE250 + H), and Group 5: Hypoxia treated with 25 mg/kg b.w. acetazolamide, used as positive control (PC + H).

For hypoxia exposure, the rats were kept in cages and exposed to a simulated altitude of 7620 m or 25000 ft. (280 mmHg) in a hypobaric chamber (Decibel Instruments, India) for 6 h. The temperature of the hypobaric chamber was maintained at 25 °C with an air flow rate of 4 L h^{-1} and humidity 55%. Group 1 animals were administered 0.5 mL distilled water only and were not exposed to hypoxia. Group 2 animals were administered orally 0.5 mL distilled water through gastric cannula, 30 min before hypoxia exposure. The animals of groups 3 and 4 were administered RRE doses of 100 and 250 mg/kg b.w. 30 min before hypoxia exposure. The extract doses of 100 and 250 mg/kg b.w. were selected based on previous study [6] and also to see the dose dependent effect. In group 5 animals, acetazolamide was administered to rats as single oral dose of 25.0 mg/kg b.w., 30 min before hypoxia exposure.

Immediately after exposure, animals of all the groups were first anesthetized and then sacrificed humanely by cervical dislocation. The lungs were dissected out and immediately stored at −80 °C for further assays. Out of 10 animals in each group, 5 animals were used to collect bronchoalveolar lavage fluid (BALF) to determine pulmonary edema markers viz. levels of total protein, albumin, and lactate dehydrogenase (LDH). Another five animals were used to prepare homogenate of lung tissue for estimation of lipid peroxidation (malondialdehyde, MDA); enzymatic antioxidants [(superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST)], and non-enzymatic antioxidant [reduced glutathione (GSH)]. Part of the lung tissue was kept for histology. The other part of the lung tissue was used to estimate lung water content (marker of pulmonary edema). All the enzyme activities and colorimetric detection were measured at 25 ± 1 °C using ELISA reader (PowerWave XS2, BIOTEK, VT).

Lung water content

The lung lobes were cut, blot dried, and placed on pre-weighed glass plates. The wet weight of the tissue was noted immediately. The lung tissues were kept in hot air oven at 80 °C for 72 h to obtain a dry weight. The wet-to-dry weight ratio was considered as edema index.

BALF collection and determination of total protein, albumin and LDH levels in BALF

For BALF collection, an incision was made through midline thorax to neck, the ribs removed, and a tracheal cannula (18-gauze) was placed. 5 mL of sterile phosphate buffer saline (PBS; pH 7.4) was lavaged in the lungs and returned lavage fluid (BALF) was collected Zhang et al**.** [18]. The fluid was centrifuged at 3000 g for 10 min at 4 °C. Total protein was measured using Bradford method [19] and albumin contents were measured using fully automated biochemistry analyzer (Erba; Model No: EM-360). LDH (E.C.1.1.1.27) activity in cell free BALF was measured spectrophotometrically using the method of Kornberg et al. [20]. LDH activity was calculated based on oxidation of NADH for 3 min, using a molar extinction coefficient of 6.22 x 10^{-3}/M/cm and expressed as nmol NADH oxidized/min/mg protein.

Determination of Lipid peroxidation & antioxidants

Lung samples were homogenized with PBS (pH 7.4) and centrifuged at 3000 g for 15 min at 4 °C to prepare lung homogenate. To estimate lipid peroxidation, MDA formation per unit protein was measured by the method of Dousset et al. [21]. Absorbance was recorded at 531 nm and MDA values were expressed as nmol/mg protein. Beutler et al. [22] method was used to determine the levels of GSH. Absorbance of colored complex was measured at 412 nm. SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) activities were determined using the methods of Marklund and Marklund [23] and Aebi [24], respectively. Enzyme kinetics of SOD was recorded at 540 nm for 3 min (every 15 s) and change in absorbance per min was used to calculate the percentage of auto-oxidation inhibition to derive SOD units (U). CAT activity was assayed by recording change in absorbance at 240 nm for 150 s (every 15 s) and was calculated using molar extinction coefficient of 0.041 $cm^2/\mu mol$.

Toxicity evaluation

Experiment 2: Acute oral toxicity and sub-acute oral toxicity (28 days repeated oral toxicity) studies of RRE were conducted as per Organization for Economic Cooperation and Development (OECD) guidelines 420 [25] and 407 [26], respectively.

Acute oral toxicity

Total 20 overnight fasted female rats were used to study acute toxicity. Animals were divided into two groups, control and experimental, with 10 animals in each group. Control animals were administered 1 mL of distilled water orally. Experimental animals were administered RRE dose of 5000 mg/kg b.w. (RRE5000) orally one time in a day for single day to see the acute effect of RRE. After treatment, animals were provided with food and water 4 h after this procedure. Body weight of the animals were measured and recorded daily. All the rats were observed for changes in any mortality and sign of toxic effects such as eye, hairs and skin color, tremors, convulsions, salivation, diarrhea, lethargy, motor activity, general behavioral, for 24 h and thereafter daily for 14 days. After 14 days, number of deaths was recorded to calculate LD50. Any died animal was subjected to macroscopic and microscopic necropsy.

Sub-acute oral toxicity (28 days repeated oral toxicity)

For repeated dose oral toxicity, female rats were randomly divided into four groups with 5 rats in each group. Control group was administered distilled water orally and three experimental groups were administered RRE doses of 100, 250, and 500 mg/kg b.w. respectively, by gavage once a day for 28 days. Body weight of the animals was recorded daily. After 28 days, the rats were anesthetized and then sacrificed humanely by cervical dislocation for analyzing biochemical markers (liver function test, kidney function test and lipid profile) and hematology parameters. Blood was collected by direct puncture of the abdominal vena cava and collected in tubes without EDTA; centrifuged at 3000 g at 4 °C for 10 min for collection of serum. Liver

markers viz. serum aspartate transaminase (SAST), serum alanine aminotransferase (SALT), alkaline phosphatase (ALP) and bilirubin; kidney markers viz. creatinine, urea, and uric acid, and lipid markers viz. cholesterol and triglycerides (TG), were determined using diagnostic kits obtained from Agappe Diagnostics Ltd., Kerala, India. Hematological changes were determined using hematology analyzer (Celltac α, Nihon Kohden, Japan). Lung, kidney, liver, and spleen tissues were excised for histology.

Histology

All the organs (lungs, liver, kidney and spleen) from toxicity study and lung tissues from efficacy study were fixed in 10% neutral buffered formalin, dehydrated in graded alcohol, and embedded in paraffin wax. Sections (5 µm) were stained with hematoxylin and eosin (H&E). Microscopic observations were recorded by using light microscope (Axio Scope Observer D1, Carl Zeiss, Germany) at 200× magnification.

Statistical analysis

Data is expressed as mean ± standard error (SE). Student's t test was used for analyzing the results. For analyzing variations between the different treatment groups, ordinary two way analysis of variance (ANOVA) was used followed by post hoc Bonferroni's multiple comparison tests. GraphPad Prism 6.0 software was used for finding significant values of data. Value of $p < 0.05$ was considered as statistically significant.

RESULTS

HPLC analysis

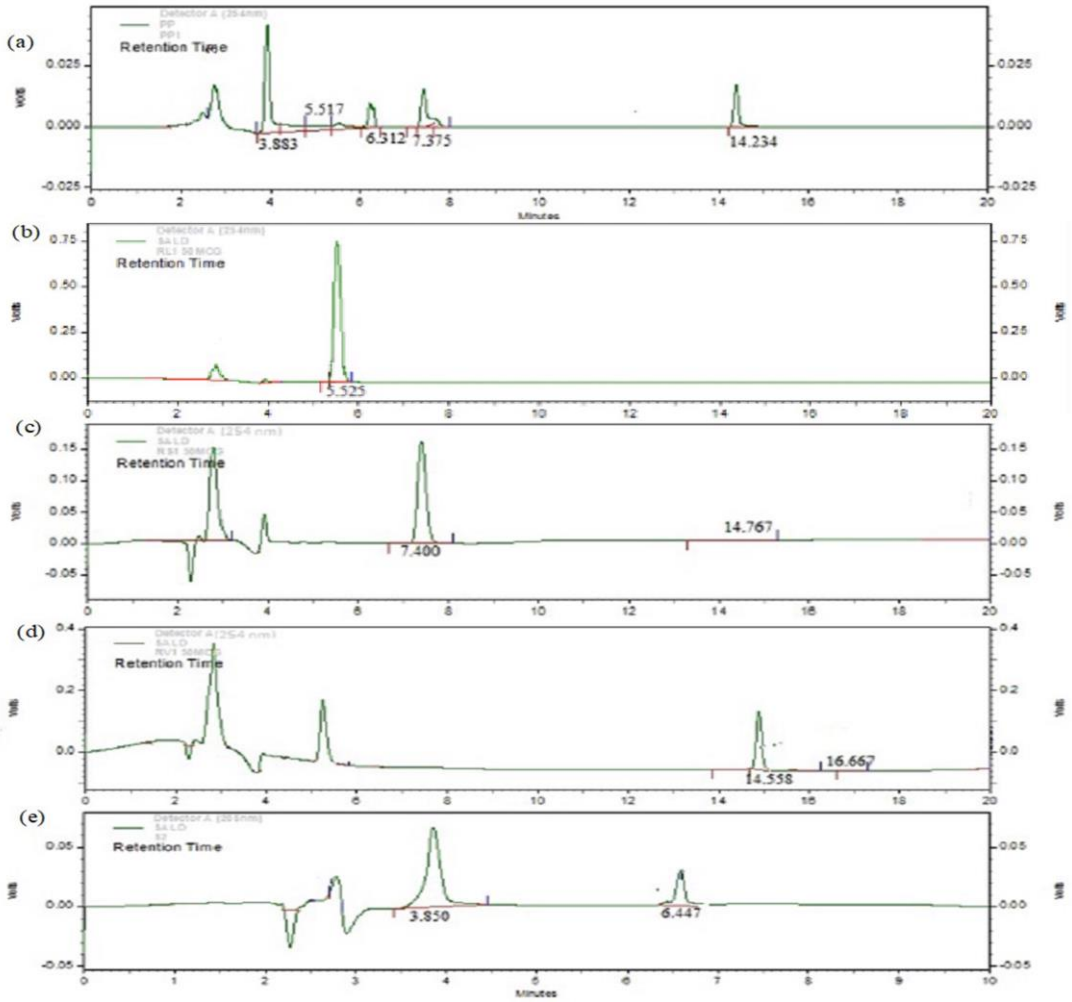

Figure 2. HPLC chromatogram of (a) *Rhodiola rosea* extract (RRE), (b) Rosarin, (c) Rosin, (d) Rosavin, and (e) Salidroside

The retention time of salidroside, rosarin, rosin and rosavin was found to be 3.85, 5.525, 7.4, and 14.558 min, respectively (Figure 2). In RRE, salidroside, rosarin, rosin, and rosavin was observed at their particular retention time when compared to standards. The linearity of the analytical method was in the calibrating range of 5-500 µg/mL. Accuracy (± 20), precision (< 20%), limit of quantification was within 0.7-0.9 ng/mL for all standards. Limit of detection for rosarin, rosavin, and rosin was within 0.19-0.25 µg/mL and for salidroside 0.68 µg/mL. The correlation coefficient of standards was between $r^2 > 0.996$-0.999. The accuracy of analytical method was determined using recovery test. The recoveries of standards in comparison to RRE were similar from 95.6 to 100.1% with RSD less than 3.0%. The precision of the standards was determined by its reproducibility. The RSD of peak area were ranged from 0.90 to 2.15%.

Heavy metal analysis

The concentration of As, Cd, and Hg in RRE was found to be below the limits specified by WHO (2007) whereas the concentration of Pb was found to be below the MDL (Table 1).

Table 1: Heavy metal analysis of *Rhodiola rosea* extract (RRE)

Elements	Concentration (mg/kg)	Upper Limit (mg/kg) specified by WHO (2007)
As	1.85 ± 0.051	5.0
Cd	0.158 ± 0.081	0.30
Pb	< MDL	10.0
Hg	0.204 ± 0.031	0.50

Data presented is mean ± SD of 3 independent experiments. As- arsenic, Cd- cadmium, Pb- lead, Hg- mercury; MDL- minimum detection limit

Total flavonoid and total phenolic contents in RRE

Total flavonoid and total phenolic contents calculated from calibration curve were 92.8 mg of QE/g and 63.7 mg of GAE/g of RRE, respectively.

Effect of RRE on pulmonary damage markers

Exposure to hypobaric hypoxia (group 2) caused a significant increase (2.1-folds, $p < 0.001$) in lung Wet/Dry (W/D) weight ratio when compared to control (group 1) animals (Figure 3a). This hypoxia-induced increase in W/D weight ratio was significantly decreased ($p < 0.05$) by RRE treatment (1.5-folds and 1.6-folds for 100 and 250 mg/kg b.w. resp.). Lung W/D weight ratio remained low in RRE 250 mg/kg b.w. and acetazolamide treated groups, when compared with RRE 100 mg/kg b.w dose which showed normalized W/D weight ratio (Figure 3a).

Figure 3b indicated that hypoxia exposure significantly ($p < 0.05$) decreased the LDH (32%) levels in lavage fluid, when compared to control animals. Pre-treatment with RRE caused significant ($p < 0.05$) increase in levels of LDH by 35% and 18% for 100 and 250 mg/kg b.w. doses, in comparison to hypoxia exposed animals. LDH levels of RRE 100 mg/kg b.w. and acetazolamide treated animals were found to be comparable with control animals but levels remained low in comparison to hypoxia group.

Figure 3c and 3d showed levels of albumin and proteins in BALF, respectively. Both albumin and protein contents in hypoxia exposed animals were significantly ($p < 0.05$) increased by 31.5%, when compared with control animals. Treatment with RRE doses (100 mg/kg b.w.) significantly reduced the albumin (18%) and

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protein (25%) levels in lavage fluid, in comparison to hypoxia group. However, 250 mg/kg b.w. of RRE dose was able to bring down the protein (30%) contents only. RRE dose of 100 mg/kg b.w. showed better efficacy in attenuating the hypoxia-induced increase in albumin and protein levels in BALF. Acetazolamide treatment before hypoxia exposure significantly ($p < 0.05$) decreased the lung W/D weight ratio (1.5-folds), protein (24%) and LDH (29%) levels in BALF, when compared with hypoxia exposed animals.

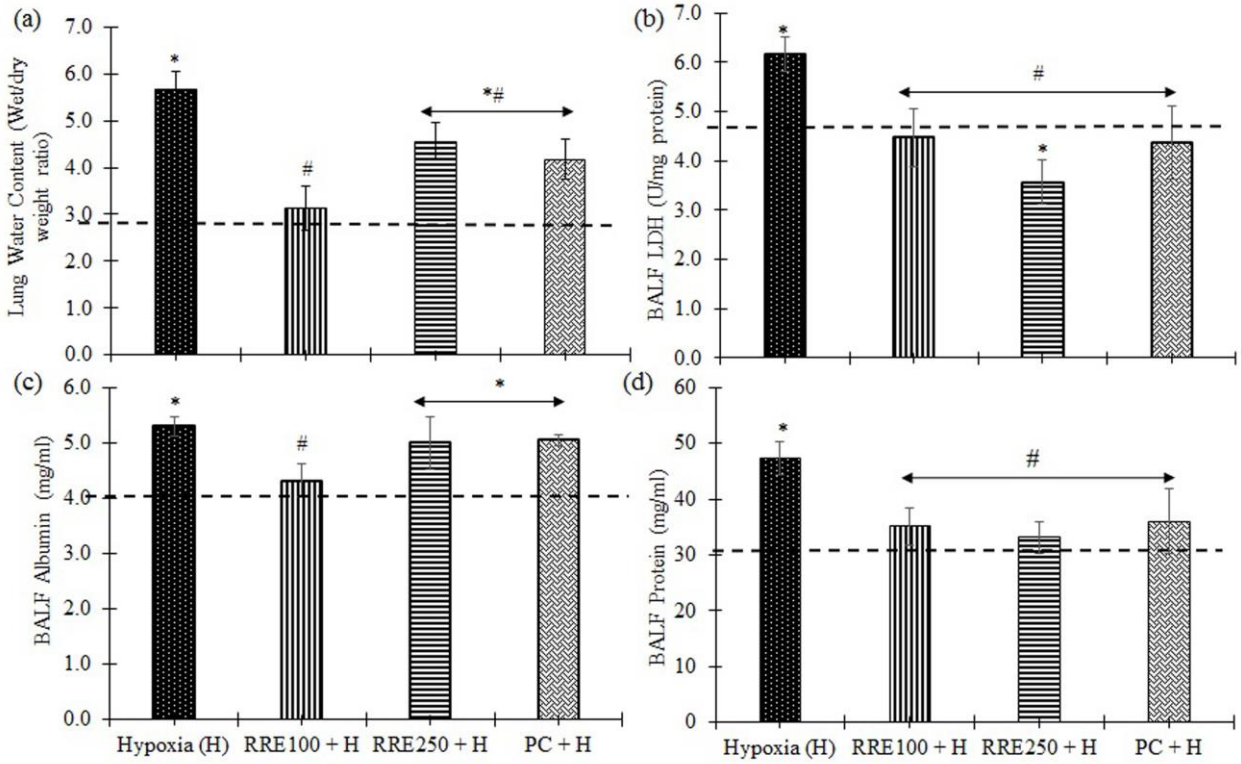

Figure 3: Modifying effect of oral *Rhodiola rosea* extract (RRE) on hypobaric hypoxia-induced changes in (a) lung water content (wet-to-dry weight ratio); levels of (b) LDH, (c) albumin, and (d) protein in bronchoalveolar lavage fluid of rats. Control (unexposed, presented by black dashed line ---) and Hypoxia (H, 6 h) exposed animals were administered distilled water; RRE (100 and 250 mg/kg b.w). and acetazolamide [25 mg/kg b.w.; positive control (PC)] were administered 30 min before hypoxia (H) exposure. Data presented is mean ± SE of six rats in each group. $^{*}p < 0.05$ in comparison to control group and $^{\#}p < 0.05$ in comparison to H exposed group

Effect of RRE on lipid peroxidation and antioxidants

Table 5 shows the protective effect of RRE on hypoxia-induced oxidative stress. Results demonstrated a significant ($p < 0.05$) increase in MDA (121%) levels and decrease in GSH (23.5%), SOD (18.2%) and CAT (50%) levels in the lung tissue upon hypoxia exposure. Pre-treatment with RRE 100 and 250 mg/kg b.w. doses significantly ($p < 0.05$) curtailed the MDA levels (50% and 42.8%) and increased the GSH (38.5% and 53.8%), SOD (45.3% and 83%) and CAT (40.1% and 50%) levels resp., in comparison with hypoxia exposed animals. Acetazolamide pre-treatment significantly decreased the MDA (52.1%) and increased the GSH (7.7%) levels, when compared with hypoxia exposed group.

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Table 5: Effect of *Rhodiola rosea* extract (RRE) on hypoxia-induced changes in the levels of malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) in lung tissue of rats

	MDA (nmol/mg protein)	GSH (μmol/mg protein)	SOD (U/mg protein)	CAT (μmol H_2O_2/min/ mg protein)
Control	1.92 ± 0.3	1.72 ± 0.03	3.42 ± 0.13	0.12 ± 0.001
Hypoxia (H)	4.21 ± 0.71*	1.32 ± 0.02*	2.78 ± 0.43*	0.06 ± 0.003*
RRE100 + H	2.14 ± 0.03$^{\#}$	1.81 ± 0.03$^{\#}$	4.04 ± 0.31$^{\#}$	0.11 ± 0.001$^{\#}$
RRE250 + H	2.41 ± 0.13$^{\#}$	2.01 ± 0.03$^{*\#}$	3.91 ± 0.14$^{\#}$	0.09 ± 0.008$^{*\#}$
PC + H	2.01 ± 0.55$^{\#}$	1.43 ± 0.02*	2.56 ± 0.62	0.05 ± 0.001*

Data presented is mean ± SE of six rats in each group. $^{*}p < 0.05$ in comparison to control (group 1) animals and $^{\#}p < 0.05$ in comparison to hypoxia exposed (H, group 2). RRE100 + H (group 3), RRE250 + H (group 4), PC + H (group 5) animals were administered different doses of RRE (100 and 250 mg/kg b.w.) and acetazolamide [25 mg/kg b.w.; positive control (PC)] 30 min before hypoxia (6 h) exposure

Effect of RRE on lung histology

Lung histology of animals of different treatment groups are depicted in Figure 4. Normal lung parenchyma with well-structured bronchiole, alveoli (marked as thick black arrows), thin lined interalveolar septa (marked as black dashed arrow), and well-organized alveolar spaces were observed in control animals. Hypoxia exposure caused thickened alveolar spaces which resulted in reduction of alveolar space area. Alveoli were also seen filled with small inflammatory infiltrate (solid black arrows) and inter-alveolar septae were severely thickened (marked as black dashed arrow). Treatment with RRE (100 and 250 mg/kg b.w.) before hypoxia exposure maintained the integrity of the alveolar-capillary barrier as seen by lessened collapsed alveolar spaces with less widened and thickened alveolar septum in lung tissue, when compared with hypoxia exposed animals. However, animals treated with 250 mg/kg b.w RRE dose showed mild edema, inflammatory infiltration, and hemorrhage. Pre-treatment with acetazolamide brings the hypoxia-induced structural changes in lungs to near normal but fewer inflammatory infiltrations were observed in alveoli.

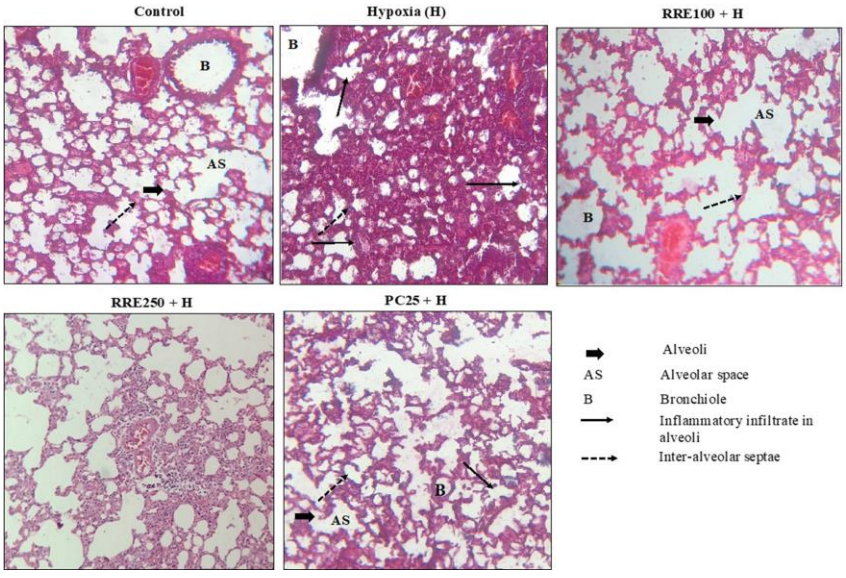

Figure 4: Effect of *Rhodiola rosea* extract (RRE) on the lung histology of rats exposed to hypobaric hypoxia (6 h). Microscopic observations of H&E stained tissue sections are presented at magnification 200×. Control (unexposed, group 1) and hypoxia exposed (H, group 2) animals were administered distilled water; RRE doses of 100 and 250 mg/kg b.w. and acetazolamide dose of 25 mg/kg b.w. [positive control (PC)] were administered 30 min before hypoxia (H) exposure.

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Toxicity profile of RRE

The acute oral administration of RRE at a dose of 5000 mg/kg b.w. showed no mortality within 24 h of treatment and even up to 14 days of drug treatment. In both control and RRE treated groups, no significant change was observed in body weight of animals up to 14 days (Table 2). No toxic signs appear in animals during 14 days observation period. LD_{50} of RRE was found to be > 5000 mg/kg b.w.

Table 2: Effect of acute oral administration of *Rhodiola rosea* extract (RRE) on body weight gain of animals administered orally 5000 mg/kg body weight, single dose once in a day for one day

	Body weight (g)		
Groups	**Day 1**	**Day 7**	**Day 14**
Control	190.4 ± 4.78	196.3 ± 4.99	205.2 ± 5.23
RRE 5000 mg/kg	192.5 ± 3.57	197.2 ± 6.29	208.9 ± 5.67

Data is presented as mean ± SE of 5 female rats in each group

Repeated dose oral administration of RRE doses (100, 250, and 500 mg/kg b.w.) for 28 days showed no significant changes in organ weight/b.w. ratio (Table 3). Administration of different RRE doses to rats did not cause any significant changes in liver, kidney, and lipid markers as well as in hematology parameters, when compared with control group (Table 4). No changes in histology of lung, kidney, liver, and spleen were observed (data not shown).

Table 3: Effects of sub-acute oral administration of *Rhodiola rosea* extract (RRE) at doses of 100, 250, and 500 mg/kg b.w. (single dose per day for 28 days) on the organ weight by body weight ratio of rats

		Sub-acute RRE doses (mg/kg body weight)		
	Control	**100**	**250**	**500**
Liver × 10^{-3}	40.17 ± 3.2	38.78 ± 3.6	40.32 ± 3.8	41.21 ± 2.21
Kidney × 10^{-3}	4.29 ± 0.3	4.19 ± 0.79	4.29 ± 0.7	4.55 ± 0.87
Spleen × 10^{-3}	3.67 ± 1.8	4.06 ± 1.41	3.67 ± 0.8	3.43 ± 0.93
Adrenal × 10^{-4}	3.60 ± 1.1	3.88 ± 0.82	3.60 ± 0.8	3.89 ± 0.76
Lung × 10^{-3}	3.58 ± 0.92	3.85 ± 0.67	3.99 ± 0.59	4.21 ± 0.78

Data is presented as mean ± SE of 5 female rats in each group. No significant difference was recorded at $p < 0.05$

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Table 4: Effect of sub-acute oral administration of *Rhodiola rosea* extract (RRE) at doses of 100, 250, and 500 mg/kg body weight, single dose/day consecutively for 28 days on biochemical (liver, kidney, and lipid) and hematological parameters.

		Sub-acute RRE doses (mg/kg body weight)		
Parameters	**Control**	**100**	**250**	**500**
SAST (IU)	18.6 ± 3.01	18.0 ± 2.21	20.7 ± 2.91	19.9 ± 3.92
SALT (IU)	10.9 ± 4.22	7.91 ± 1.89	8.76 ± 1.56	9.97 ± 1.98
ALP (IU)	314.7 ± 14.3	311.0 ± 4.32	299.2 ± 19.81	301.9 ± 14.92
Bilirubin (mg/dL)	0.82 ± 0.08	0.67 ± 0.05	0.82 ± 0.08	0.66 ± 0.06
Creatinine (mg/dL)	14.0 ± 3.89	12.7 ± 0.87	9.9 ± 3.39	10.9 ± 2.32
Urea (mg/dL)	30.3 ± 2.61	29.9 ± 1.92	33.0 ± 3.45	32.9 ± 4.89
Uric acid (mg/dL)	2.8 ± 0.23	2.78 ± 0.84	2.99 ± 0.78	3.01 ± 0.54
TG (mg/dL)	87.9 ± 3.71	90.2 ± 2.26	91.7 ± 7.21	83.7 ± 1.89
Cholesterol (mg/dL)	125.4 ± 8.21	120.9 ± 3.87	113.8 ± 6.23	119.9 ± 3.12
WBC (x10^3 µ/L)	6.9 ± 1.76	7.1 ± 1.81	7.3 ± 2.01	6.6 ± 1.21
RBC (x10^6 µ/L)	8.8 ± 0.76	9.1 ± 0.88	8.7 ± 0.38	7.9 ± 0.95
Hemoglobin (g%)	15.3 ± 2.01	14.1 ± 2.71	14.3 ± 1.67	13.9 ± 0.98
Hematocrit (g%)	40.8 ± 3.94	39.8 ± 3.01	41.2 ± 2.04	40.3 ± 1.29
MCV (fL)	52.3 ± 1.24	50.7 ± 1.83	51.9 ± 1.28	54.5 ± 2.19
Platelets (x10^3 µ/L)	702.3 ± 49.9	687.3 ± 37.8	698.3 ± 12.5	711.0 ± 53.2

Data is presented as mean ± SE of 5 female rats in each group. No significant difference was recorded at $p < 0.05$, SAST, Serum aspartate transaminase; SALT, Serum alanine aminotransferase; ALP, Alkaline phosphatase; TG, triglycerides; WBC, white blood cells; RBC, red blood cells; MCV, mean corpuscular volume.

DISCUSSION

In the current study, we attempted to evaluate the effect of *R. rosea* extract (coded as RRE) in prevention of pulmonary injuries induced by hypobaric hypoxia (7620 m) exposure. Acetazolamide, a diuretic approved by Food and Drug Administration, was used as a positive control in this study. It was found to be useful in prevention of HAPE in rats exposed to hypobaric hypoxia [5]. However, acetazolamide has undesirable adverse effects that seriously limit its applications [4]. We have observed that RRE provided effects equally comparable to acetazolamide against hypoxia-induced lung injuries in terms of countering pulmonary edema, oxidative stress and by restoring histology of lung tissue.

HAPE is caused by the leakage of highly proteinaceous fluid specifically albumin into the lung from the pulmonary vasculature [27] and the main cause for the occurrence of pulmonary edema may be the oxidant injury to alveolar and endothelial cells [3]. We have observed that after exposure to hypoxia for 6 h, pulmonary edema occurred in animals as shown by significant increase in lung water content, total protein, albumin, and LDH levels in lavage fluid. Further, increased LDH levels in lavage fluid indicated damage to pulmonary membrane (Figure 3). LDH is a cytotoxicity marker for assaying membrane damage and it occurs extracellularly

in BALF only in the presence of damaged cells [28]. This study also showed significantly increased lipid peroxidation (MDA) and decreased antioxidants (GSH, SOD, and CAT) (Table 5) on hypobaric-hypoxia exposure, indicated oxidative stress in lung tissue. The findings of our study corroborated with other studies where exposure of animals to hypoxia caused oxidative damage in liver, muscle, blood, and lung tissues of rats [6, 29]. We have also seen alterations in the lung histology of rats exposed to hypoxia which revealed the deterioration of alveolar membrane as seen by increased infiltration of alveolar spaces especially protein-rich fluid (Figure 4). Such changes in pulmonary vasculature after hypoxia exposure have been reported by other researchers also [30]. This study, therefore, indicated that exposure to hypobaric hypoxia for 6 h caused oxidative stress which caused damage to membrane lipids resulting in extravasation of protein and albumin in lavage fluid.

Oral administration of 100 mg/kg b.w. RRE dose curtailed the hypoxia-induced increase in lung water content, protein and albumin leakage, as well as LDH levels in BALF (Figure 3), suggesting that besides reducing fluid leakage it also inhibited leakage of proteins into the alveoli by maintaining cell membrane permeability. This could be further supported by normalization of hypoxia-induced changes in the lung histology. However, 250 mg/kg b.w. of RRE dose was not able to prevent leakage of plasma albumin in lavage fluid, which was reflected in lung histology also (Figure 3), suggesting better efficacy provided by 100 mg/kg b.w. of RRE dose.
Oxidative stress occurs when there is an imbalance between pro-oxidants and antioxidant defense. Hypoxia generates oxidative stress in lungs may be due to lower ventilation and greater hypoxemia inducted by hypobaric hypoxia. SOD has an important role in catalyzing the conversion of superoxide radicals to H_2O_2 and O_2 which are decomposed to O_2 and water by enzyme CAT. GSH is found abundant in lung epithelial cells and is an important pulmonary antioxidant that plays a key role in the control of pro-inflammatory processes in the lungs [2]. Single oral doses of RRE (100 mg/kg b.w.) before hypoxia exposure normalized the lipid peroxidation (MDA) and also restored the levels of most antioxidants. Normalization of oxidative stress by RRE could probably ameliorate the transvascular leakage in the lungs of rats by maintaining cell membrane permeability and hence prevented pulmonary edema. Earlier studies reported that antioxidant agents attenuated not only oxidative stress but also high altitude induced pulmonary dysfunction [7, 8]. Our study also suggested that RRE attenuated the hypoxia-induced oxidative stress which might have curtailed the manifestations of HAPE.

R. rosea has been reported to be a valuable medicinal plant world-wide in traditional medicine system to treat lung injuries and high-altitude maladies. The presence of bioactive constituent's viz. salidroside and rosavins makes *R. rosea* unique from other Rhodiola species. Now-a-days, adulteration of Rhodiola is a major concern [31] and therefore, it became important to evaluate the authenticity of extract. In this regard, we have attempted to evaluate that RRE is safe to consume and possesses anti-oxidant properties. RRE was found to be rich in phenols (63.7 mg of GAE) and flavonoids (92.8 mg of QE). Furthermore, HPLC analysis revealed the presence of salidroside and rosavins (Figure 2), which are known to exhibit antioxidant activities [11]. It was reported that salidroside alone can provide protection against pulmonary hypertension in animals exposed to hypobaric hypoxia [32] and also protected lung injury in mice exposed to LPS, by reducing inflammatory cells in the BALF [33]. *R. rosea* and rosavins were reported to protect pulmonary fibrosis induced by bleomycin in lung tissue of mice [34, 35]. All these reports and the results of our study suggested that the presence of active constituents in RRE may have contributed in the prevention of lung damage in rats probably by rendering its antioxidant properties. However, further studies are warranted to confirm the beneficial effect of RRE in countering pulmonary injuries with elaborate mechanistic studies.

Toxicity evaluation of RRE demonstrated LD_{50} > 5000 mg/kg b.w. which as per GHS considered safe for human consumption. This study also showed that long-term administration of RRE doses (100, 250 and 500 mg/kg b.w.) for 28 days didn't cause any toxic effect in animals. Moreover, all the parameters related to liver, kidney, and lipids, were not effected by treatment with RRE doses for 28 days, indicated its safe use for longer period. RRE was also found to be free from heavy metals contamination, further confirmed that it is safe.

CONCLUSION

In conclusion, we proposed that pretreatment with RRE (100 mg/kg b.w.) exhibits potential to counter hypobaric hypoxia induced pulmonary changes by rendering its anti-oxidative properties which helped in alleviating pulmonary edema. Thus, our findings showed the beneficial effect of RRE for its probable use in countering pulmonary injuries and also proved the traditional use of *R. rosea* in treatment of lung injuries.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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