Protective efficacy of *Hippophae rhamnoides* L. extract exhibited in rat heart against hypobaric hypoxia is possibly mediated by configurations in JAK/STAT pathway

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Hypoxia is a condition deprived of oxygen at tissue level, is known to be linked to oxidative stress and inflammatory cytokines. *Hippophae rhamnoides* L., commonly called Seabuckthorn, being rich in flavonoids, is reported to reduce oxidative stress. It is hypothesized that aqueous extract of *H. rhamnoides* relieves adverse changes in rat heart induced by continuous sub-acute hypobaric hypoxia. Exposure to continuous hypobaric hypoxia for seven days resulted in elevated levels of malondialdehyde, decrease in reduced glutathione, glutathione peroxidase and superoxide dismutase activities with concomitant increase in NFκB expression in rat heart. The levels of pro-inflammatory cytokines, TNFα and IL6 and TGFβ1, AKT and ERK were found to be decreased. The expression levels of JAK1 were reduced while STAT3 and STAT6 levels were found increased following hypoxia exposure. The treatment of rats with aqueous extract of *H. rhamnoides* significantly attenuated hypobaric hypoxia induced oxidative stress, increased TNFα and IL6 and deactivated NFκB activity. *H. rhamnoides* treatment augmented expressions of JAK1, AKT and ERK proteins. Overall, results of this study indicate that the aqueous extract of *Hippophae rhamnoides* helps in inducing tolerance to rat heart at extreme altitude faster by optimizing tissue oxidative stress, preventing inflammatory response and configuring JAK1/ERK/AKT and STAT3/STAT6, at least to certain extent.

Keywords: Inflammatory response, Oxidative stress, Seaberry, Seabuckthorn

A constant supply of oxygen is essential for cardiac viability and function at high altitude. It has been reported that hypobaric hypoxia causes changes in cardiac mass, function and energy metabolism and exert an influence on heart rate variability. Further, prolonged hypoxic injury has been implicated in progression of many clinical disorders. Hypoxia-evoked reactive oxygen species (ROS) driven oxidative stress is an underlying mechanism of cell death and unchecked production of these oxidants for longer period reduces efficacy of antioxidant system resulting in disintegration of cellular architecture. It is documented that various antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are reported for their beneficial effects in protecting tissues against oxidants including hypoxia.

Hypoxia has been shown to induce a vascular inflammation and induce proinflammatory cytokine response including cytokines such as tumour necrosis factor (TNFα) and interleukin 6 (IL6). JAK/STAT pathway plays a major role in cytokine signaling. The pathway gets activated on binding of cytokines, such as TNFα, IL6, etc. to their respective receptors. Ligand binding to receptor causes phosphorylation of JAK proteins which in turn phosphorylates receptor tyrosine leading to recruitment of STATs. JAK also mediates phosphorylation of STATs that are recruited to phosphorylated receptors and dimerisation of STATs induces expression of target genes. HIF1α was also suggested to promote the expression of inflammatory cytokines which are known to be regulated by NF-κB. NFκB is a transcriptional activator of HIF1α and further HIF1α suggested promoting expression of inflammatory cytokines that are regulated by NFκB. TGFβ1 is a cytokine that exhibits cardioprotective activity by promoting cardiac development and function. Various studies have reported the beneficial effects of TGFβ1 in reperfusion injury. Hypoxia like stresses may alter the level of TGFβ1 and subsequently activate cellular signaling pathways. Further, hypoxia induces ROS which lead to cell death and may also activate signalling molecules like Akt and ERK. Moreover,
HIF1α plays a central physiological role in oxygen and energy homeostasis and is activated during hypoxia by stabilization of the subunit HIF1α and it is reported that AKT cascade and ERK kinase is also linked to activation of vascular endothelial growth factor (VEGF) via HIF1α17,18.

Further, exogenous supplementation of biologically active extracts derived from herbal plants reduces oxidant load3,19,20. One such plant, *Hippophae rhamnoides* L. commonly called Seaberry or Seabuckthorn, that grows at high altitude (2500-4000 m) in adverse climatic conditions has shown numerous medicinal benefits. The pharmacological properties of aqueous extract of seabuckthorn leaves, such as cytotoxic19, antioxidant4,5,21, adaptogenic22, wound healing23 and antidengue activity24 have been documented. Moreover, it improves physical endurance, longevity and prevents high altitude sickness25,26. Further, aqueous extract of *H. rhamnoides* has been shown to be rich in phenolics and flavonoids and possess high antioxidant activity and reported that configuration of JAK/STAT pathway is positively associated with neuronal survival under low oxygen conditions27. Therefore, the present studies were designed to study the effects of hypobaric hypoxia on rat heart, which is also equally sensitive under low oxygen conditions as of neurons, and possible association of JAK/STAT pathway in inducing tolerance in hypoxia conditions.

**Materials and Methods**

**Plant material**

*Hippophae rhamnoides* L. leaves were collected in the month of September from hilly regions of Western Himalayas, India, where the plant grows widely under natural conditions. The Field Research Laboratory, Leh, India, where the voucher specimen (DIP- HIP/2014) of the plant material is preserved in the herbarium, carried out the ethanobotanical identification of the plant. Fresh leaves of seabuckthorn were cleaned and washed thoroughly with water and re-washed with distilled water. Washed fresh leaves were dried under shade in a clean, dust free environment and crushed using laboratory blender.

**Extract preparation**

Aqueous lyophilized extract of *H. rhamnoides* dried leaves was prepared by maceration method as described earlier24. One gram of dried *Hippophae rhamnoides* leaves produced 0.2 g of lyophilized seabuckthorn aqueous extract powder.

**Animals**

Male Sprague-Dawley rats (200-250 g) bred in the animal facility of Defence Institute of Physiology and Allied Sciences (DIPAS) were used for all experiments. The animals were maintained on a bedding of rice husk in polypropylene cages under controlled environment in the institute’s animal house at 22±1°C, 55±10% humidity and 12 h light-dark cycle. Animals had access to standard rodent pellet feed and water ad libitum. The study has the approval of the Institutional Ethical Committee on Animal Experiments (IAEC) and the experiments were performed in accordance with the regulations specified by the IAEC and conformed to national guidelines on the care and use of laboratory animals, India.

**Animal exposure**

The animals were randomly subdivided into seven experimental groups of 10 animals each: Gr. I, Control; Gr. II, Hypobaric hypoxia 6 h; Gr. III & IV, Hypobaric hypoxia 6 h + *H. rhamnoides* extract 100 & 250 mg/kg, respectively; Gr. V, Hypobaric hypoxia 7 d; and Gr. VI & VII, Hypobaric hypoxia 7 d + *H. rhamnoides* extract 100 & 250 mg/kg, respectively. All the groups of animals were exposed to a simulated altitude of 25000 ft in a decompression chamber in laboratory conditions where temperature and humidity were maintained at 28°C and 55-60%, respectively for 6 h or 7 d in the absence or presence of *H. rhamnoides* extract except group one control animals. The rate of ascent was at the rate of 300 m/min. The rats were taken out of hypoxic chamber once after every 24 h exposure for 15-30 min for replenishing food and water.

**Biochemical analysis**

After hypoxic exposure, the rats were sacrificed and heart was dissected out after perfusion with sterile PBS (pH 7.4) to remove the blood and stored at −80°C for further analysis. Later, the heart was homogenized in 0.1 M phosphate buffer (pH 7.4) to obtain 10% homogenate (w/v). The homogenate was then centrifuged at 10000 rpm for 30 min at 4°C and supernatant was collected for analysis of reduced glutathione (GSH), glutathione peroxidase (GPx) and superoxide dismutase (SOD). Cellular GSH content was measured by the method of Denzoin et al.28 with slight modifications. The activities of GPx and SOD were measured by commercial diagnostic kits. Lipid peroxidation was measured by estimating malondialdehyde (MDA) formed by thiobarbituric acid
(TBA) reaction and measuring the absorbance at 532nm. 1,1,3,3-tetraethoxy propane was used as standard, and the levels of lipid peroxides were expressed as nmol MDA/g tissue.

**Protein expression studies**
Quantitative Elisa kits were used for studying expression of NF B, TNFα, IL6, TGFβ1, AKT and ERK following the manufacturer’s instructions (R&D Systems, USA, Invitrogen, USA). Total tissue lysates were prepared using (50 mM Tris-HCl, pH 7.5, with 120 mM NaCl, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 1.0 mM phenylmethylsulfonyl fluoride, 1% NP-40 and protease inhibitor cocktails added just prior to extraction) (Sigma, St. Louis, MO). In general, equal amounts of protein samples were loaded onto each specific antibody coated wells and incubated. After incubation the Elisa plates were further incubated with horseradish peroxidase-conjugated secondary antibodies following washing. The colour was generated by incubating the Elisa wells with TMB substrate and color generation was terminated by stop solution. The yellow colour generated for the samples was read at 450 nm against the specific standards provided in the kit.

**Immunoblotting**
Following indicated treatments, total tissues were washed thrice with phosphate buffered saline (PBS) and homogenized in ice-cold buffer (50 mM Tris-HCl, pH 7.5, with 120 mM NaCl, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 1.0 mM sodium orthovanadate, 1.0 mM phenylmethylsulfonyl fluoride, 1% NP-40 and protease inhibitor cocktails added just prior to extraction) (Sigma, St. Louis, MO). Homogenates were clarified by centrifugation at 12000 rpm for 30 min and protein concentrations were estimated by Bradford method. About 30 μg of protein was resolved on 10-12% SDS-polyacrylamide gel which was subsequently transferred onto PVDF membrane. The membranes were probed with respective primary antibodies for JAK1, STAT3, STAT6 and actin followed by HRP conjugated secondary antibodies. Immunoblots were detected by 3,3’-diaminobenzidine (DAB)

**Statistical analysis**
The results were expressed as mean ± SE of 10 animals in each group. The data was analyzed by one-way ANOVA followed by Dunnet's test for comparing control with various test groups, using GraphPad software. Statistical significance was estimated at the 5% level.

**Results**

**Body weights of rats exposed to hypobaric hypoxia in the absence or presence of H. rhamnoides aqueous leaf extract**
Fig. 1 shows the effects of hypobaric hypoxia on body weight of rats exposed for 6 h and 7 d in the presence or absence of *Hippophae rhamnoides* extract. No significant change was observed in the mean body weight of rats after hypobaric hypoxia exposure for 6 h (Fig. 1, bar 2). However, rats exposed to hypobaric hypoxia for 7 d showed significant reduction in body weight (Fig. 1, bar 5) and treatment with *H. rhamnoides* extract reduced this change in body weight of rats (Fig. 1, bars 3, 4, 6 & 7). The treatment of rats with 100 and 250 mg/kg dose of extract for 7 d prevented change in body weight by 19.8 g and 27.2 g, respectively in comparison to the rats exposed to hypoxia in the absence of extract.

**Effects of hypobaric hypoxia on the levels of MDA, GSH, GPx and SOD in the absence or presence of H. rhamnoides leaf extract in rat heart**
Fig. 2 depicts the protective efficacy of aqueous extract of *Hippophae rhamnoides* on hypobaric hypoxia induced oxidative stress and antioxidant status in rats. Hypobaric hypoxia exposure to rats for 6 h or 7 d resulted in an increased MDA levels (Fig. 2A, bars 2 & 5) and treatment with this extract significantly prevented rise in MDA levels in a dose dependent manner (Fig. 2A, bars 3, 4, 6 & 7). Concomitant to this increase in MDA levels, the levels of GSH were found to be decreased following 6 h or 7 d of exposure and significant reduction in
GSH levels was recorded in rat heart exposed following 7 d hypoxia exposure (Fig. 2B, bars 2 & 5). The administration of extract restored such decrease in GSH levels (Fig. 2B, bars 3, 4, 6 & 7). Further, GPx activity was found to be increased initially after 6 h of hypobaric hypoxia exposure (Fig. 2C, bar 2) but observed to be decreased after 7 d of exposure (Fig. 2C, bar 5). A reverse trend was observed in case of SOD where an initial decrease was observed after 6 h of hypobaric hypoxia exposure (Fig. 2D, bar 2) but an increase was recorded after 7 d exposure (Fig. 2D, bar 5). The treatment of rats with *H. rhamnoides* extract could restore the changes in GPx and SOD activities induced by hypobaric hypoxia exposure (Fig. 2C & D, bars 3, 4, 6 & 7).

**Effects of hypobaric hypoxia on NFκB, TNFα, IL6, TGFβ1, Akt and ERK expression in the absence or presence of *H. rhamnoides* extract in rat heart**

Fig. 3 depicts the effects hypobaric hypoxia exposure on NFκB, TNFα, IL6, TGFβ, Akt and ERK

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**Fig. 2** — Effects of hypobaric hypoxia on (A) MDA; (B) GSH; (C) GPx; and (D) SOD levels in rat heart in the presence and absence of *Hippophae rhamnoides* extract. [Values are means of 10 rats. *P* < 0.05 vs. control]

**Fig. 3** — Levels of (A) NFκB; (B) TNF-α; (C) IL-6; (D) TGFβ; (E) Akt; and (F) ERK in rat heart facing subacute hypobaric hypoxia stress for 7 d in the presence and absence of *Hippophae rhamnoides* extract. [Values are means of 10 rats. *P* < 0.05 vs. control]
expression in rat heart in the absence or presence of *H. rhamnoides* extract. Rats exposed to hypobaric hypoxia for 6 h or 7 d showed higher expression of NFκB than the respective unexposed controls (Fig. 3A, bars 2 & 5). Administration of extract to rats prevented such rise in NFκB levels in rat heart (Fig. 3A, bars 3, 4, 6 & 7). The levels of pro-cytokines, such as TNFα, IL6 and TGFβ1 were found compromised in rat heart exposed to hypobaric hypoxia for 6 h or 7 d (Fig. 3 B-D, bars 2 & 5) when compared to the unexposed control. The treatment of *Hippophae rhamnoides* extract was found to be effective in restoring levels of TNFα, IL6 and TGFβ1 altered by hypobaric hypoxia exposure (Fig. 3 B-D, bars 3, 4, 6 & 7).

The levels of AKT and ERK were found to be decreased after 6 h or 7 d hypobaric hypoxia exposure (Fig. 3 E & F, bars 2 & 5). Treatment of rat with extract prevented decrease in AKT and ERK levels after 6 h of exposure (Fig. 3 E & F, bars 3 & 4) and surprisingly augmented levels of AKT and ERK following 7 d of hypobaric hypoxia in the presence of extract (Fig. 3 E & F, bars 6 & 7).

**Effects of hypobaric hypoxia on JAK/STAT expression in the absence or presence of *H. rhamnoides* extract in rat heart**

Fig. 4 shows the immunoblot analysis of JAK/STAT proteins under hypoxia in the absence or presence of extract. Exposure to hypoxia suppressed expression of JAK1 in rat heart (Fig. 4A, lane 2) which was augmented after treatment with extract (Fig. 4A, lanes 3 & 4). Further, expressions of STAT3 and STAT6 were found elevated following hypoxia exposure (Fig. 4 B & C, lane 2) and normalized after supplementation of extract (Fig. 4 B & C, lanes 3 & 4). The phosphorylation of STAT3 and STAT6 was found to be higher following hypoxia exposure (Fig. 4 D & E, lane 2) and normalized after supplementation of extract (Fig. 4 D & E, lanes 3 & 4).

**Effect of hypobaric hypoxia on histology of rat heart in the absence or presence of *H. rhamnoides* extract**

Rat exposed to hypobaric hypoxia for 7 d resulted in lysis of myocardial muscle fibers with surrounding hemorrhage and showed presence of inflammatory cells (Fig. 5B) in comparison to control rat heart (Fig. 5A). Further, rat exposed to hypobaric hypoxia evident presence of fibrous tissue in heart tissue (Fig. 5C).
Discussion

During stressful situations supplementation of various nutrients and herbal preparation has been shown to increase stress tolerance\textsuperscript{25}. \textit{Hippophae rhamnoides} is considered as a good source of bioactive compounds and its medicinal properties are attributed to the presence of high content of flavonoids, tannins and triterpenes\textsuperscript{5}. It is well established that complete acclimatization at high altitude requires about one to three weeks. An initial few days of exposure to high altitude is crucial in deciding the course of acclimatization by the activation of adaptive machinery. In our preliminary studies, it was observed that rats exposed for 6 h, 1, 2, 3, 5 & 7 d to hypobaric hypoxia revealed changes elevated stress markers and inflammatory cytokines up to initial 3 days, restore towards normal by day 5 and re-rise after 7 days of exposure in rat heart (data not shown). It is also reported that an increase in reactive oxygen species, altered antioxidant system and changes in histoarchitecture of rat heart was observed up to 2 days of exposure and reversibility was shown after 5 days of exposure\textsuperscript{30}. Hence, for the present study, 6 h (acute) and 7 d (subacute) exposure time points were selected, where changes in oxidative markers and inflammatory markers were evident, to study protective efficacy of extract against short and long term exposures. It is reported that aqueous extract of \textit{H. rhamnoides} has presence of high phenolic and flavanoid content and had a good antioxidant activity\textsuperscript{27}. Nakanishi \textit{et al.}\textsuperscript{31} reported effects of hypobaric hypoxia on antioxidant enzymes in rat and highlighted differential pattern of these enzymes in various organs including heart. In this study, a differential expression of GPx and SOD was observed at different time points of exposure (Fig. 2 B-D). The findings indicate that various antioxidant enzymes are activated differentially to help organisms to adapt under low oxygen conditions based on exposure levels and duration of exposure.

It is reported that exposure to hypobaric hypoxia causes reduction in body weight\textsuperscript{32}. The mechanism behind hypoxia induced weight loss is still unclear\textsuperscript{33}. Several hypotheses have been formulated to explain this phenomenon, e.g. appetite suppression, loss of body water and intestinal malabsorption\textsuperscript{34}. The majority of early weight loss during hypoxia may be mainly due to loss of body water\textsuperscript{33}. The reduction in appetite may be attributed to the increase in the expression of appetite suppressing hormone leptin via hypoxia inducible factor 1α\textsuperscript{35}. In the present study, no noticeable change in the body weight of rats was observed after 6 h of hypobaric hypoxia exposure with presence or absence of extract (Fig. 1, bars 2-4). However, rats exposed to subacute hypobaric hypoxia for 7 d showed significant reduction in body weights and administration of \textit{H. rhamnoides} extract prevented this reduction in body weight of rats (Fig. 1, bars 5-7) which probably may be due to normalized energy intake.

It has been reported in our earlier studies that aqueous extract of \textit{H. rhamnoides} provides neuroprotection against hypoxia via induction of JAK/STAT pathway\textsuperscript{27}. Hence, it was hypothesized that configuration of JAK-STAT pathway may be associated with cardioprotective effects observed following extract supplementation. Many reports indicate involvement of JAK/STAT pathway in cardiopathology and further activation of JAK/STAT signaling is also evident in hypoxia\textsuperscript{36}. Schindler \textit{et al.}\textsuperscript{37} have reported that knocking out JAK1 results in impaired IL6 signaling. In the present study, decreased expressions of JAK1 and IL6 were observed in rat heart following hypoxia exposure (Fig. 3C & 4A). TNFα activates NFκB signaling and results in upregulation of NFκB target genes including IL6. Wei \textit{et al.}\textsuperscript{39} have shown that hypoxia induces IL6 expression through NFκB in cardiac myocytes. However, in the present study, expression of NFκB was observed to be higher which was concomitant with decreased levels of TNFα and IL6 in rat heart exposed to hypobaric hypoxia (Fig. 3 A-C). The findings suggest that activation of NFκB in rat heart following hypobaric hypoxia exposure was independent of TNFα levels. Further, association of TGFβ, JAK1 and IL6 signalling has been documented\textsuperscript{40,42}. TGFβ1 has role in cardio protection\textsuperscript{12} and exogenous TGFβ1 prevent heart from hypoxia reperfusion injury\textsuperscript{43}. In the present study, a decrease in TGFβ1, JAK1 and IL6 were observed in rat heart under low oxygen conditions (Fig. 3 C-D & 4A). In one of the study, it has been demonstrated that cantharidin can inhibit angiogenesis by suppressing VEGF induced JAK1/STAT3, ERK and AKT signaling pathways\textsuperscript{44} and shows interconnection...
between these pathways. Further, coactivation of JAK1-STAT3, ERK MAPK and Akt pathway has been demonstrated to be stimulated by EPO treatment in cardiac cells. AKT, a serine/threonine kinase, and ERK are known to regulate various cellular processes, such as cell survival, cell proliferation, differentiation and cell motility. In the present study, AKT and ERK levels were observed to be decreased in rat heart under low oxygen conditions which was concomitant with decrease in JAK1 levels (Fig. 3 E-F & 4A). The treatments of experimental rats with extract augmented levels of AKT, ERK and JAK1 evidencing association of these proteins in inducing tolerance under low oxygen environment (Fig. 3 E-F & 4A). It is also reported that JAK1 and STAT3 were activated by ischemia from the basal activities in in vivo studies and STAT6 is selectively activated in the rat heart subjected to ischemia/reperfusion. Further, JAK-STAT pathway has been shown to play a role in cardiac ischemic preconditioning and prevents ischemic damage to the heart. Moreover, the role of JAK-STAT activation in preventing apoptosis in cultured myocytes is also documented. However, in the present study, activation of STAT3 and STAT6 observed in rat heart exposed to hypoxia was found to be independent of JAK1 (Fig. 4 A-C). It is reported that phosphorylation of STAT3 is dependent on upregulation of Akt in response to IL10 but independent of Jak1 in cardiomyocyte. Further, association of IL6 signaling in STAT3 activation is reported. In the present, activation of STAT3 was found to be activated independent of IL6 and AKT (Fig. 3 C & E and Fig. 4B).

Conclusion
The finding from the studies reveal that coactivation JAK1, AKT and ERK is required for inducing tolerance to rat heart following hypoxia exposure and extract treatment facilitates tolerance faster by augmenting these proteins. The role of STAT3 and STAT6 seems for opposing to JAK1 probably creating a feedback loop. Collectively, results of this study suggest that the possible mechanism for protection provided by Hippophae rhamnoides against myocardial hypoxia is mediated via configuration of JAK/STAT, AKT and ERK pathway atleast to certain extent, although future studies will be needed to validate the concept.

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Conflict of Interest
The authors declare no conflict of interest.

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