ISSN 0976 – 3333

Available Online at www.ijpba.info

International Journal of Pharmaceutical & Biological Archives 2013; 4(5): 956 - 964

ORIGINAL RESEARCH ARTICLE

Antistress/Adaptogenic Activity of*Bauhinia variegata* Against Different Stress Paradigms

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Received 17 Jun 2013; Revised 09 Oct 2013; Accepted 19 Oct 2013

ABSTRACT

Aim: The present study was designed to investigate the effect of ethanolic bark extract of Bauhinia variegata on oxidative stress induced by cold restraint stress (CRS) and iron overload (IO) oxidative stress.

Materials and Methods: Wistar albino rats of either sex (200-250 g) were treated with alcoholic extract of *Bauhinia variegata*(200 and 400 mg/kg; p.o) for 7 days. Ascorbic acid (AA) (50 mg/kg) was used as a reference standard. The animals were subjected to cold restraint stress and iron overload oxidative stress to assess the anti-stress activity of *B.variegata* in stress condition, fresh Wistar rats were subjected to cold restraint stress (4^{o}C for 2 h) and iron overload (ferrous sulphate 30 mg/kg i.p) for 7 days. After sacrifice, blood and liver samples were prepared and levels of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and lipid peroxidation (LPO) were estimated in brain homogenate.

Results: There was alteration in the antioxidant enzymes like GSH, CAT, SOD and LPO. Treatmentwith the extract significantly ameliorated the stress-induced variations in these biochemical levels and antioxidant enzymes in both CRS and IO stress models. The extract treated animals showed increase in antioxidant enzymes and decrease in lipid peroxidation, hepatic marker enzymes inboth stress models,respectively. Treatment groups also reverted the increase in liver, adrenal gland weights and atrophy of spleen caused by IO stress and CRS models.

Conclusion: The results indicate thatethanolic extract of *B.variegata*has significant adaptogenic activity against a variety of biochemical and antioxidant perturbations in different stress models.

Key words*:* cold restraint stress, iron overload, antioxidants, adaptogenic activity.

INTRODUCTION

Biological stress is a response to physical, chemical, biological and emotional changes, consisting of a pattern of metabolic and behavioural reactions that helps to strengthen the organism. Stress has been postulated to be involved in the etiopathogenesis of a diverse variety of diseases, ranging from psychiatric disorders such as depression and anxiety, immunosuppression, endocrine disorders including diabetes mellitus, male sexual dysfunction, cognitive dysfunctions, peptic ulcer, hypertension and ulcerative colitis[1].

During stressful situations, the energy requirement of the organism is increased, resulting in enhanced generation of free radicals[2]. Stress induced sympathetic stimulation results in increased respiration rate to make available more oxygen supply to active tissues and maximize cellular energy yield. But enhanced metabolic rate also generates excess free radicals, causing an imbalance between reactive oxygen species (ROS) production and anti-oxidant defence mechanism.

Although essential for the growth of cells, in excessive amounts iron is toxic. The liver is especially subject to this toxic effect because it is the major site of iron storage. Oxidative stress seems to be a common cytotoxic response; however, the pathways triggering this redox unbalance are different. In fact, iron is a potent catalyst of reactions leading to free radical generation, such as those of Fenton and Haber-Weiss[3].

Free radicals cause oxidation of nucleic acids and proteins. Free radicals also damage bio

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membranes, reflected by increased lipid peroxidation, thereby compromising cell integrity and function. It is possible to support the body's adaptation to stress by using food supplements, dietary elements, herbs and minerals for increasing physical and mental performance. Such substances have been described as'adaptogens' [4].

An adaptogen (i) produces a non-specific responsein an organism; i.e., an increase in power of resistanceagainst multiple stressors including physical,chemical or biological agents; (ii) has a normalizinginfluence on physiology, irrespective of the directionof change from physiological norms caused by thestressor, and (iii) is incapable of influencing normalbody functions more than required to gainnon-specific resistance[5]. Anti-stress and adaptogenic agents have opened a new vista in themodern therapeutics in the management of the stress related clinical disorders.

The polyphenolics including flavonoids, which are found in many herbal extracts, have been shown to be strong ROS scavengers, antioxidants. *Bauhinia variegata* Linn. (Ceasalpiniaceae) is a medium-sized deciduous tree found throughout India. It is traditionally used in bronchitis, leprosy, and tumors. The stem bark is used as astringent, tonic, and antihelmintic[6].Infusion of the leaves is used as a laxative and for piles. Dried buds are used in the treatment of worm infestations, tumors, diarrhea, and piles [7]. The stem bark is used in ayurveda for its antidiabetic activity.

So far, the stem bark has been investigated and reported to have antitumor [8],antibacterial, antifungal, antiulcer, and hepatoprotective activity [9]. Flavanone glycoside from root is reported to have anti-inflammatory activity[10].The stem bark is reported to contain 5,7 dihydroxy and 5,7 dimethoxy flavanone-4-O-α-L rhamnopyrosyl- ß -D-glycopyranosides, Kaempferol-3-glucoside, lupeol, and betasitosterol. Seeds contain protein, fatty oil-containing oleic acid, linoleic acid, palmitic acid, and stearic acid. Flowers contain cyanidin, malvidin, peonidin, and kaempferol. Root contains flavanol glycosides[11].

Since polyphenolic compounds are present in the ethanolic extracts of stem bark of *B. variegata* Linn.The aim of our present study was to evaluate the alcoholic bark extract of *BV*on oxidative stress induced by cold restraint stress and iron overload in rats.

MATERIALS AND METHODS

Animals

Wistar strain albino rats of either sex weighing 200–250 g were used for this study. Animals were housed in cages at an ambient temperature of 25±2 ˚C and 45–55% relative humidity with 12 h light/dark cycle. They had free access to standard pellet chow (Brook Bond, Lipton India) and water *ad libitum*. Animals were divided in to 5 groups of six animals each. The experimentations on animals were approved by the Institutional Animal Ethical Committee (IAEC) under the regulation of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi. Approval no: **1018/SPIPS/Wgl/IAEC/2010**.

Chemicals

Thiobarbituric acid,DTNB reagent, Glutathione (HiMedia Laboratories Ltd., Mumbai), Trichloroacetic acid (Qualigens Fine Chemicals, Mumbai), 1,1,3,3,-Tetraethoxy propane, O-Dianisidine (Sigma, St. Louis, USA), Riboflavin(Astra IDL, Bangalore), Ascorbic acid (Medrich company, Bangalore), Sodium dihydrogen phosphate, Disodium hydrogen phosphate(S.D. Fine Chemicals, Mumbai), SGOT, SGPT, ALP, GGT, Albumin, Bilurubin, Triglycerides, Glucose, Cholesterol, And Total Protein (Auto analyser kits from Coral clinical bio systems, Hyderabad).The other chemicals and solvents used were of analytical grade purchased from commercial suppliers.

Plant Material

Shade dried stem bark of *Bauhinia variegata(BV)* was used for the preparation of plant extract.

Extraction of the Plant Material

The stem bark of *BV* Linn was collected from the Botanical Garden, and authenticated from the Dept. of Botany, Kakatiya University (KU). Specimen voucher no: **KUH 1854** was deposited in Herbarium of Department of Botany, KU. Bark was dried in shade and powdered coarsely. Extraction was done according to standard procedures using analytical grade solvent, 95% alcohol. Course powder (240gm) was soxhlet extracted with 95% alcohol (2450ml). The resultant alcoholic extract was concentrated by rotary vacuum evaporator. The extracts were then freeze-dried and stored in a vacuum desiccator (yield 25%, w/w). The extract was stored in an airtight container in a cool place and used throughout the project.

Acute Toxicity Study and Gross Behaviour in Rats

Acute toxicity study – up and down procedure – was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD). The maximum upper limit dose 2000 mg/kg of *BV* was administered orally to mice. Animals were observed individually after dosing. Observation included mortality and clinical signs, such as changes in skin fur, eyes and mucous membranes. The gross behaviours, e.g. body positions, locomotion, rearing, tremors, gait was observed. The effect of BV on passivity, grip strength, pain response, stereotypy, vocalization, righting reflex, body weight and water intake was assessed[12].Pilot study was carried out with various doses (50, 100, 200 and 400 mg/kg, per oral route to rats) of *BV*. At doses of 200 and 400 mg/kg, it was active and at 50 mg/kg it was inactive. Based on this observations two different doses (200 and 400 mg/kg) of*BV* was selected in oxidative stress models.

Drug Treatment Protocol

Animal are divided into various groups. Normal control group (Group I) received only vehicle (5% acacia solution) without stress, whereas animals from model control group (Group II) received only stress without any treatment. Animals from Group III to Group V received test drugs such as standard drug vitamin C (50 mg/kg) p.o., alcoholic extract of *BV*, (200mg/kg) p.o., alcoholic extract of *BV* (400 mg/kg) p.o. once daily for 7 days 45min prior to stress.

STRESS PROCEDURES

Cold restraint stress[13]

Cold stress is produced by restraining the animal inside an adjustable cylindrical plastic tube, rats are confined individually and exposed continuously to cold stress at 4^0C for 50min once a day for 7days. Drugs are administered orally 45 min prior to stress regimen up to 7 consecutive days, except that the rats are kept fasted overnight on the 6^{th} day after drug feeding and stress exposure. On 7^{th} day rats are sacrificed immediately after stress by decapitation and blood is collected in tubes containing 4%EDTA and kept on ice. Blood is centrifuged; plasma is separated out and stored at -20^0C for biochemical assay of lipid peroxidation (LPO), glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), triglycerides (TGL), cholesterol (CHO), total protein (TOP), and glucose (GLU).

Iron overload hepatotoxicity[14]

Iron overload is induced by the administration of ferrous sulphate (30 mg/kg i.p). Standard drug (vita-C) and test drug are given orally. The rats were sacrificed after 1 h of drug administration on 7^{th} day; the liver was removed and washed with saline, weighed and processed for estimation of lipid peroxidation, hepatic marker enzymes (SGOT, SGPT, ALP, and GGT), albumin(ALB), bilirubin (BIL), TOP, and TGL.

Biochemical estimations

Biochemical tests to estimate plasma/ liver lipid peroxidation by Ohkawa et al., method, glutathione by George Ellman method, catalase by Beers and Sizer method, superoxide dismutase by Arutla et al method, SGOT&SGPT by Mod. IFCC method, ALP by Mod. Kind & King's method, GGT by Carboxy substrate method, albumin by BCG method, bilurubin by Mod. Jendrassik and Grof's method, triglycerides by GPO/PAP method, glucose by GOD/POD method, cholesterol by CHOD/PAP method, and total protein by Biuret methodwere performed using standard procedures reported in the literature.

Statistical analysis

Results are reported as mean±S.E.M. Statistical analysis was performed using one-way analysis of variance (ANOVA). If the overall *P*-value was found statistically significant ($p< 0.05$), further comparisons among groups were made according to post hoc Tukey's test. All statistical analyses and the diagrammatic representation of the data were performed by using Graph pad PRISM, Version 5 software.

RESULTS

Effect of BV in acute toxicity and gross behaviours in rats

The rats treated with *BV* at the dose of 2000 mg/kg were well tolerated and exhibited normal behaviour. Rats were alert with normal grooming, touch response, pain response and there was no sign of passivity, stereotypy, and vocalization. There was no abnormal change in motor activity, secretory signs as well as their body weight and water intake.

Effect of BV in cold restraint stress

We observed that there was decease in levels of GSH**(Fig 1)**, CAT**(Fig 2)**, SOD and increased levels of LPO**(Fig 3)**, TGL, GLU, CHO**(Fig 4**), TOP**(Fig 5)** in stress control group compared to vehicle control group.

Pretreatment of animals with *BV* at both doses significantly inhibited the stress induced alterations in plasma lipid peroxidation,

glutathione, catalase, superoxide dismutase, triglycerides, cholesterol, total protein, and glucose levels in cold restraint stress model. The results shown by *BV* at 400 mg/kg dose were similar to Ascorbic acid.

Effect of BV in Iron overloads stress

Iron overload induced stress resulted in significant increase in liver and adrenal glands weight and concomitant decrease in spleen weight in stress control group, which was significantly reverted by *BV* treatment at 200 mg/kg and 400 mg/kg **(Table 1)**.

Treatment of animals with *BV* at both doses also significantly restored back iron overload induced alterations in the liver enzymes SGOT, SGPT, GGT **(Fig 6)**, ALP **(Fig 7)**, BIL**(Fig 8)**, ALB, TOP **(Fig 9)**, TGL **(Fig 10)**, and LPO **(Fig 11)** levels.We observed that there was increase in all the enzyme levels in iron overload group compared to normal control.

Glutathione

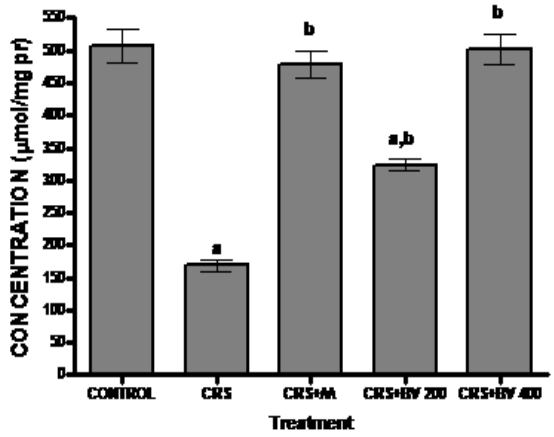

Figure 1: Protective effect of ethanolic extract of *Bauhinia variegata* (*BV*) on Glutathione levels in rats subjected to cold restraint stress. All the data were expressed as mean ± SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001 vs CRS.

Catalase

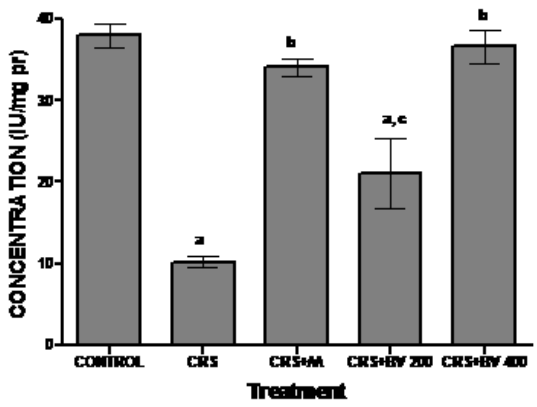

Figure2: Protective effect of ethanolic extract of *Bauhinia variegata* (*BV*) on Catalase levels in rats subjected to cold restraint stress. All the data were expressed as mean ± SEM, n = 6. a = p< 0.001vs control; b = p< 0.001& c = 0.05 vs CRS.

SOD and LPO

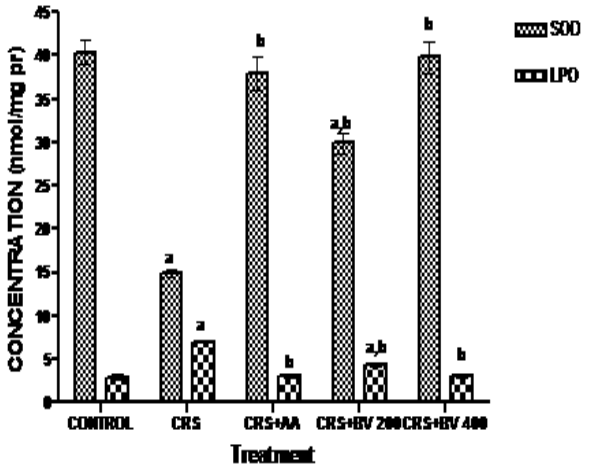

Figure 3: Protective effect of ethanolic extract of *Bauhinia variegata* (*BV*) on SOD and LPO levels in rats subjected to cold restraint stress. All the data were expressed as mean ± SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001 vs CRS.

TGL, Glucose and Cholesterol

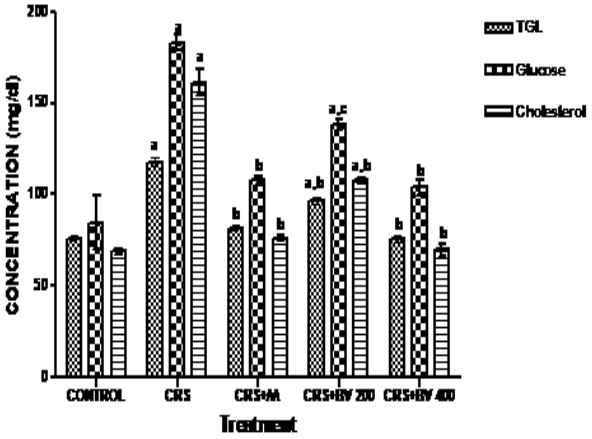

Figure 4: Protective effect of ethanolic extract of *Bauhinia variegata* (*BV*) on Triglycerides, Glucose, and Cholesterol levels in rats subjected to cold restraint stress. All the data were expressed as mean ± SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001 & c = p<0.01vs CRS.

Total Protein

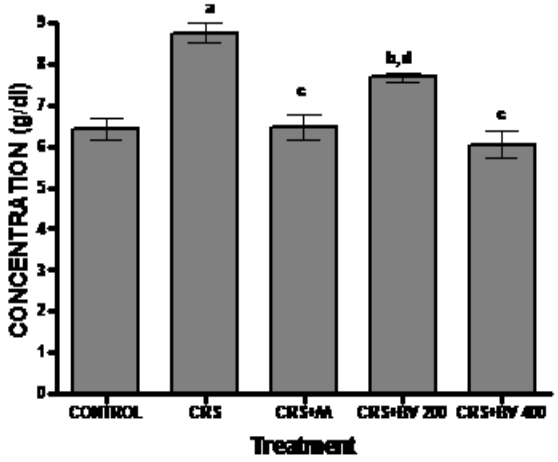

Figure 5: Protective effect of ethanolic extract of *Bauhinia variegata* (*BV*) on Total protein levels in rats subjected to cold restraint stress. All the data were expressed as mean ± SEM, n = 6. a = p< 0.001 & b = 0.05 vs control; c = p< 0.001 & d = 0.05 vs CRS.

SGOT, SGPT and GGT

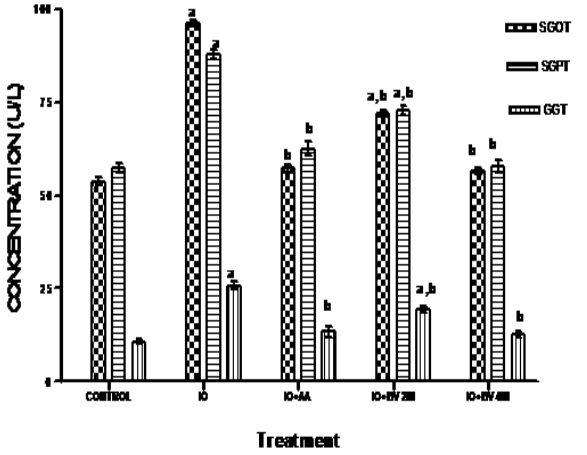

Figure 6: Protective effect of ethanolic extract of *Bauhinia variegata* (*BV*) on SGOT, SGPT, and GGT levels in rats subjected to Iron overload stress. All the data were expressed as mean ± SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001 vs. Iron overload.

Alkaline Phosphatase

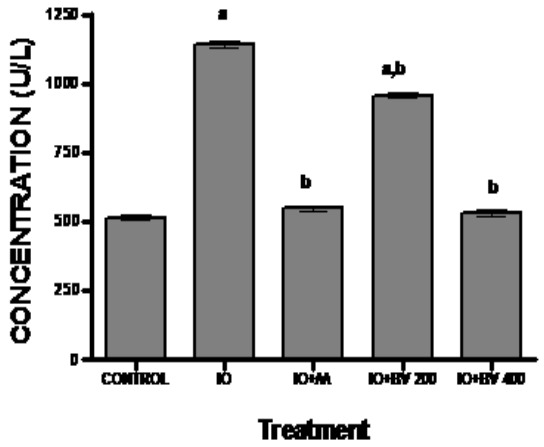

Figure 7: Protective effect of ethanolic extract of *Bauhinia variegata* (*BV*) on Alkaline phosphatase levels in rats subjected to Iron overload stress. All the data were expressed as mean ± SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001 vs Iron overload.

Bilurubin

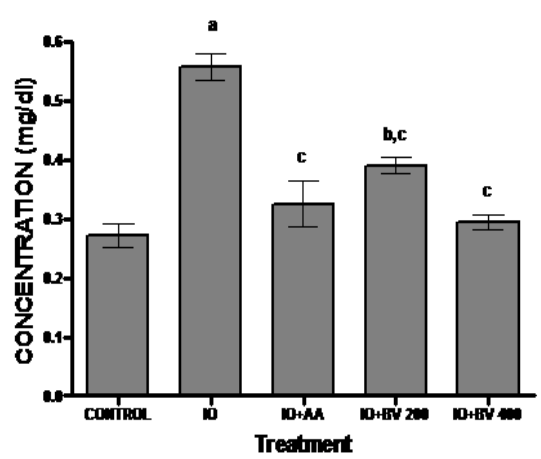

Figure 8: Protective effect of ethanolic extract of *Bauhinia variegata* (*BV*) on Bilurubin levels in rats subjected to Iron overload stress. All the data were expressed as mean ± SEM, n = 6. a = p< 0.001 & b = p< 0.05 vs control; c = p< 0.001 vs. Iron overload.

Albumin and Total Protein

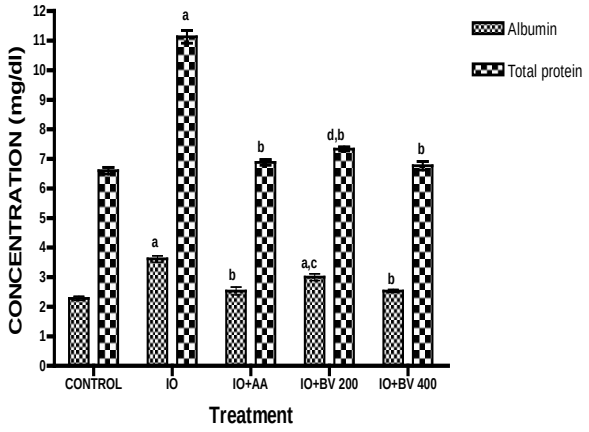

Figure 9: Protective effect of ethanolic extract of *Bauhinia variegata* (*BV*) on Albumin and Total protein levels in rats subjected to Iron overload stress. All the data were expressed as mean ± SEM, n = 6. a = p< 0.001 & d = 0.01 vs control; b = p< 0.001, c = 0.01vs Iron overload.

Triglycerides

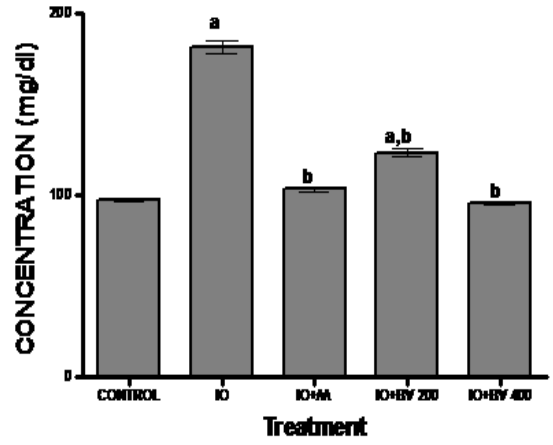

Figure 10: Protective effect of ethanolic extract of *Bauhinia variegata* (*BV*) on Triglyceride levels in rats subjected to Iron overload stress. All the data were expressed as mean ± SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001 vs. Iron overload.

Lipid Peroxidation

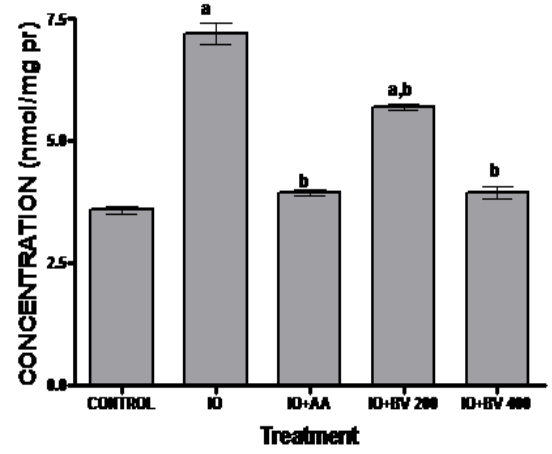

Figure 11: Protective effect of ethanolic extract of *Bauhinia variegata* (*BV*) on Lipid peroxidation levels in rats subjected to Iron overload stress. All the data were expressed as mean ± SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001 vs. Iron overload.

Mechanisms of iron toxicity

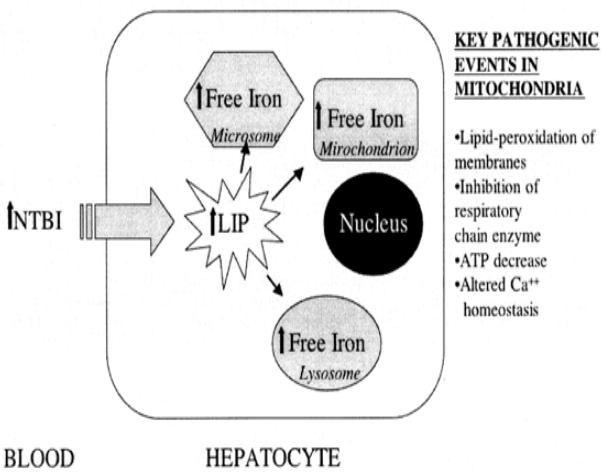

Figure 12: Mechanisms of iron toxicity. NTBI = Non-transferrin-bound iron; LIP = labile iron pool; Free iron = redox-active iron.

Table 1: Protective effect of ethanolic extract of *Bauhinia variegata* (*BV*) on Liver weight, adrenal glands weight and spleen weight in rats subjected to Iron overload stress.

Group	Liver weight (g/100 g)	Adrenal glands (mg/100 g)	Spleen weight (mg/100 g)
Control (vehicle)	2.36±0.33	19.18±0.41	251.3±3.20
Iron overload(IO)	3.65±0.06[a]	39.45±0.30[a]	145.7±2.40[a]
IO +Vit C (50 mg/kg)	2.45±0.04[b]	20.10±0.49[b]	248.1±3.55[b]
IO +*BV*200 mg/kg	3.08±0.06[a,b]	29.25±0.22[a,b]	206.1±3.84[a,b]
IO +*BV*400 mg/kg	2.35±0.04[b]	20.02±0.28[b]	245±1.92[b]

All the data were expressed as mean ± SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001 vs Iron overloa

DISCUSSION AND CONCLUSION

The present investigation showed the antistress potential of alcoholic extract of *BV* against oxidative stress. It was observed that *BV* attenuated the alterations brought by the cold restraint stress and iron overload stress in rats. The activity of BV appears to work by restoring the altered antioxidants enzymes as well as decrease the production of LPO.

We examined the antioxidants like SOD, GSH, and CAT, which served as oxidative indices in stress rats. Decrease in the levels of SOD and CAT were noted in cold restraint stress rats that indicates participation of superoxide radical which known to produce highly toxic hydroxyl radical through its reaction with H_2O_2 (Haber–Weiss reaction).[15] These in turn decrease the SOD through a modification in histidine residue located in the active site of the enzyme [16]. On the other hand this over production of H_2O_2 can be inactivated by catalase enzyme and there by reduction in CAT. Depletion in GSH was observed in cold restraint stress rats. This could be explained by the consumption of GSH due to scavenging of the rapidly generated hydrogen peroxide and lipid peroxides. *BV* (200 and 400 mg/kg) was found to elevate the activity of three major oxygen radical species metabolizing enzymes in cold restraint stress rats.

The increase in weight of adrenals in stressed animals was due to the stress-induced adrenomedullary response leading to increased production of corticotropic hormone that leads to increase in weight of adrenals[17].*BV* has significantly reduced the liver, adrenal gland weight; this may be due to the reversal of the stress-induced adrenomedullary response and hence decreased production of corticotropic hormone. The decrease in weight of spleen may be due to recruitment of lymphocytes to blood from spleen which results in squeezing of the spleen[18].The treatment with the *BV* and reference standard ascorbic acid significantly increased the spleen weight. This may be due to inhibition of recruitment of lymphocytes to blood from spleen.

The hypothalamus is a major integrating center for receiving messages from divergent centers and converting them to hormonal signals, via the control of the pituitary gland and by neural pathways[19].The activation of this HPA system results in secretion of corticotrophin hormone, adrenocorticotropin hormone (ACT), β-endorphin and glucocorticoids into the circulation. Release of ACT in stress stimulates adrenals to increase production of hormones- epinephrine, norepinephrine and corticosteroids[20].These hormones have profound effect on metabolic functions. Increased plasma cortisol influences the mobilisation of stored fat and carbohydrate reserves[21], which in turn increase blood glucose, total protein cholesterol and triglyceride levels.

The marked increase in serum cholesterol, triglycerides and glucose, total protein levels in stress-induced animals was due to stimulation of hypothalamo–pituitary axis (HPA) and sympathetic system, resulting in,liberation of catecholamines and glucocorticosteroids, whichinhibits the immune system at multiple sites like liver, kidney[22].*BV* reduced the elevatedserum cholesterol, triglycerides and glucoselevels, which may be dueto inhibition of stimulation of sympathetic nervous system.

ROS can cause oxidative damage to polyunsaturatedfatty acids of membrane phospholipids (LPO)releasing cytotoxic and reactive aldehyde metabolitessuch as MDA. These cytotoxic products may impair cellularfunctions, including nucleotide and protein synthesis[23].Iron is involvedin the initiation of this free radical

chain reaction bycausing decomposition of hydrogen peroxide to formthe highly reactive hydroxyl radical in the Fenton reaction.

In addition, oxygen is activated by ferrous andferric compounds [23]. Increased LPO is an importantexpression of both acute and chronic iron toxicityand is a proposed driving force for hepatocarcinogenesisin hereditary haemochromatosis. Cells are normallyprotected against ROS by non-enzymatic antioxidants [24].However, an imbalance betweenthe production and detoxification of ROS in hepatocytesresults in oxidative stress and favours the induction ofDNA mutations[25].This in turn elicits lipid peroxidation of membrane lipids in the presence of oxygen generated by metabolic leakage from mitochondria. All these events culminate in loss of integrity of cell membranes and damage of hepatic tissue.

Assessment of liver function can be made by estimating the activities of SGPT, SGOT, GGT, and ALP which are enzymes originally present in higher concentration in cytoplasm. When there is hepatopathy, these enzymes leak into the blood stream in conformity with the extent of liver damage. The elevated levels of these marker enzymes in iron overload rats in the present study corresponded to the extensive liver damage induced by the toxin. Treatment with the test drug BV (in both doses) as well as the standard drug ascorbic acid significantly reduced the elevation in liver enzymes, thereby showing that *BV* has hepatoprotective action in iron overload induced stress. Due to oxidative stress induced by iron overload there was increased levels of albumin, bilurubin, total protein, which is significantly attenuated by *BV* extract similar to that of standard drug ascorbic acid.

The form of intracellular iron responsible for the inductionof oxidant stress in subcellular organelles is still unknown.A likely possibility is that an increase in theintracellular transit pool of iron may account for the inductionof lipid peroxidation **(Fig 12)**.

It is reasonable to assumethat the destabilization of one (or more than one) componentof the electron transport chain, occurring after the rise inthe level of chelatable iron, enhances the potential for autooxidationand increases the production of $O_2^-$and otherreactive oxygen species, so exacerbating the effects of irontoxicity. The treatment with *BV* extract containing flavonoidswith reactive oxygen species–scavenger activity and iron chelatingproperties counteracts hepatic mitochondrial oxidativedamage and ATP decrease,the derangement in the mitochondria energy-transducing capability,and the reduction in the respiratory chain enzymes due to iron [27]. Treatment with BV significantly reducedboth mitochondria functional anomalies and the associatedtissue fibrosis by inhibiting the expansion of themitochondrial “free iron” pool. Thereported reduction in mitochondria enzyme activity maybe due to several mechanisms: reduction in the synthesis ofrespiratory chain enzymes; iron-induced peroxidativedamage of inner membrane phospholipids, especially cardiolipin, leading to a reduced cytochrome *c* oxidase activity; andinactivation of protein on adducts formation with reactivealdehydic end-products of lipid peroxidation.

Thus, an antioxidant that protects from iron toxicity is a substance that can: (a) chelate ferrous iron and prevent the reaction with oxygen or peroxides; (b) chelate iron and maintain it in a redox state that makes iron unable to reduce molecular oxygen; (c) trap already formed radicals, which is a putative action of any substance that can scavenge free radicals in biological systems, regardless if they are originated from iron-dependent reactions or not.
Interestingly, *BV* extract containing flavonoids can play a double role in reducing the rate of oxidation, asthey can participate in: (a) iron chelation; and (b) trapping radicals [28]. Several reports indicate that certain flavonoids can protect lipids in liposomes from iron-mediated oxidation [29]. In vivo, dietary flavonoids have been reported to inhibit carbonyl iron-induced lipid oxidation in rat liver [30].

The present investigation demonstrates the antioxidant effects of *BV* tested in cold restraint stress and iron overload induced oxidative stress. The results suggest that the *BV*is protective against oxidative stress by mechanisms involving inhibition of free radical generation, reactive oxygen species scavenging, modulation of intracellular antioxidants against oxidative stress induced decreases and that this may have potential as a therapy for the oxidative stress related disorders. *BV* extract is cocktail of antioxidants and these may act cooperatively at various stages of free radical generation and protect the body from various forms of oxidative stress.

ACKNOWLEDGEMENTS

Author is grateful to the AICTE, New Delhi, India for providing financial support. We sincerely acknowledge Dr. V. Prabhakar Reddy for his support during this study.

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