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PROTECTIVE EFFECT OF ALCOHOLIC EXTRACT OF *BAUHINIA VARIEGATA* IN RAT CEREBRAL ISCHEMIA/REPERFUSION INDUCED OXIDATIVE STRESS

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ABSTRACT

Stroke is the second leading cause of death. Ischemia leads to cellular dysfunction and necrosis. Wistar albino rats were treated with alcoholic extract of *Bauhinia variegata* (200 and 400 mg/kg; p.o) for 15 days prior to Ischemia/Reperfusion. Ascorbic acid (50 mg/kg) was used as a reference standard. Ischemia/Reperfusion was induced by occluding common carotid arteries for 25 min, followed by 40 min reperfusion. Ischemia/Reperfusion caused significant depletion in superoxide dismutase, catalase, glutathione and significant increase in LPO in brain homogenate. All the alterations induced by cerebral ischemia were significantly attenuated by pretreatment with bark extract at the doses of 200 and 400 mg/kg, and the effect was comparable to that of ascorbic acid (p value < 0.05). In conclusion alcoholic bark extract containing the flavonoids and phenolic antioxidants was found to protect rat brain against Ischemia/Reperfusion induced oxidative stress, and the observed effect may be attributed to its antioxidant properties.

KEYWORDS: ***Bauhinia variegata;*** **Oxidative stress; Ischemia/reperfusion; common cerebral artery occlusion; polyphenolic compounds; flavonoids.**

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INTRODUCTION

Stroke is the second leading cause of death throughout the world and considered the most common cause of disability in adults[1]. Growing evidence supports the participation of oxidative stress in brain injury mediated by cerebral ischemia and stroke[2]. When a tissue is subjected to ischemia, a sequence of chemical events is initiated that may ultimately lead to cellular dysfunction and necrosis. If ischemia is ended by the restoration of blood flow, a second series of injurious events ensue producing additional injury. Thus, whenever there is a transient decrease or interruption of blood flow the net injury is the sum of two components-the direct injury occurring during the ischemic interval and the indirect or reperfusion injury that follows. When there is a long duration of ischemia, the "direct" damage resulting from hypoxia alone is the predominant mechanism. For shorter durations of ischemia, the indirect or reperfusion-mediated damage becomes increasingly more important[3]. Ischemia-reperfusion injury is a phenomenon whereby cellular damage in a hypoxic organ is accentuated following the restoration of oxygen delivery. Reoxygenation of the ischemic tissue may promote the generation of various reactive oxygen metabolites, which are known to have deleterious effects on various cellular functions. The roles of reactive oxygen species (ROS) and lipid peroxidation have been proposed to be important factors in reduction of cerebral blood flow and the reperfusion period[4]. The source of oxy free radicals may include stimulation of the xanthine- xanthine oxidase system in the cerebral vessels[5], electron leakage and redox state alterations of electron transport components of the ubiquinone-ubiquinol-oxidoreductase in the mitochondria, arachidonic acid metabolism or release of excitatory amino acids[6]. The oxy- free radicals formed can initiate lipid peroxidation[7] and mediate oxidative damage of cellular proteins.

Bauhinia variegata (BV) 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[8]. 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. The stem bark is used in ayurveda for its antidiabetic activity. So far, the stem bark has been investigated and reported to have antitumor & antiulcer[9], antibacterial[10], Antihyperlipidemic activity[11], Haemagglutination activity[12], Antigoitrogenic activity[13], and hepatoprotective activity[14]. Flavanone glycoside from root is reported to have anti-inflammatory activity[15]. 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. Since the flavonoids & polyphenolic compounds are present in the stem bark of *B. variegata* Linn., the present study was designed to evaluate the effect of alcoholic bark extract of *BV* on cerebral I/R induced oxidative stress 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 ^{0}C and 45–55% relative humidity with 12 h light/dark cycle. They had free access to standard pellet chow diet (Brook Bond, Lipton India) and water *ad libitum*. 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/WgI/IAEC/2010.

Chemicals

Thiobarbituric acid, DTNB reagent and 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). The other chemicals and solvents used were of analytical grade purchased from commercial suppliers.

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. Plant Specimen (voucher no: KUH 1854) was submitted in the Herbarium, Dept. of Botany, Kakatiya university. Bark was dried in shade and powdered coarsely. Extraction was done according to standard procedures using analytical grade solvent, 95% alcohol. Course powder (240 gm) was soxhlet extracted with 95% alcohol (2450 ml). 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 behaviors, 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[16]. Pilot study was carried out with various doses (50, 100, 200 and 400 mg/kg, p.o) 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* were selected in I/R induced oxidative stress model.

Drug Treatment Protocol

Animal are divided into six groups. Normal control group (Group I) received only vehicle (5% acacia solution) without ischemia reperfusion, whereas animals from model control group (Group II) received only ischemia reperfusion without any treatment. Animals from Group III to Group V received test drugs such as Ascorbic acid (50 mg/kg p.o.), alcoholic extract of *BV*, (200mg/kg; 400 mg/kg; p.o.), Group VI (*BV* 400 mg/kg only) received only *BV* 400 mg/kg without stress, once daily for 15 days prior to ischemia reperfusion.

Induction of Cerebral Ischemia/Reperfusion (I/R) Injury in Rats.[17]

At the end of 14th day food was withdrawn and animals were fasted overnight. On the next day, the last dose of drug was given and after 2 h the animals were anesthetized by Thiopentone sodium (45 mg/kg). Common carotid arteries were exposed over a midline incision, and a dissection was made between the sternocleidomastoid and the sternohyoid muscles parallel to the trachea. The induction of ischemia was performed by clamping the right common carotid artery, using bulldog clamp for 25 min followed by reperfusion for 40 min by unclamping the artery. After the completion of reperfusion period the animals were sacrificed.

Preparation of Brain Tissue for Estimation of Oxidative Stress Markers

Brain was dissected out and immediately stored at -20^0 C. One gram of tissue was homogenized with 10 ml Tris- Hcl (20 mM, pH 5.8-7.2). The prepared homogenates were centrifuged at 10000 rpm for 10 min and supernatant was divided into two portions, one of which was used for measurement of lipid peroxidation. The remaining supernatant was again centrifuged at 12,000 rpm at 4^0C for 15 min and used for the measurement of superoxide dismutase, catalase, and reduced glutathione.

Measurement of lipid peroxidation (LPO)

The amount of lipid peroxidation products present in the brain homogenates was estimated by the thiobarbituric acid reactive substances (TBARS) method of Ohkawa et al. (1979)[18],which measures the malondialdehyde (MDA) reactive products by using UV-Visible spectroscopy at 532 nm. The concentration of

MDA formed is expressed as n mole/mg of protein.

Estimation of superoxide dismutase (SOD)
The enzyme superoxide dismutase was determined in brain homogenate using photo-oxidation method of Arutla et al. (1998)[19] at 460 nm. The change in the optical density was determined. The SOD content was determined from the standard graph prepared using pure bovine SOD. The results are expressed as SOD nmol/ mg of protein.

Estimation of catalase (CAT)
Catalase measurement was done based on the ability of catalase to oxidize hydrogen peroxide by Beer and Seizer method[20] at 240 nm. The average change in absorbance per minute for each assay was calculated and the results were expressed as CAT IU/ mg of protein.

Estimation of Reduced glutathione (GSH)
Glutathione forms a coloured complex with DTNB, which is measured spectrophotometrically by George Ellman method[21] at 412 nm.The glutathione content was determined from standard graph by using pure glutathione. The concentration of reduced glutathione was expressed as µmol/mg of protein.

Estimation of total protein
Protein was measured by the method of Lowry et al., (1951)[22].

Statistical analysis
Results were expressed as mean ± SEM. 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
We found that there was no mortality up to 2000 mg/kg dose. 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, secretary signs as well as their body weight and water intake.

Effect of BV and Ascorbic acid in MDA and anti oxidant enzyme levels
The effect of *BV* and Ascorbic acid in LPO levels was represented in Fig. 1.

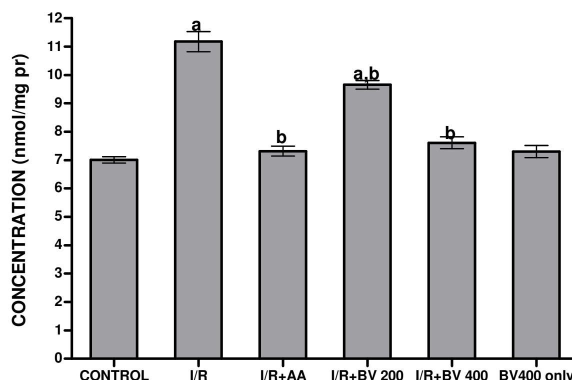

Figure 1
Protective effect of ethanolic extract of Bauhinia variegata & AA on MDA (lipid peroxidation) levels in rats subjected to cerebral ischemia and reperfusion injury. All the values are expressed as mean ± SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001 vs I/R. where I/R= cerebral ischemia reperfusion, AA= Ascorbic acid, BV= Bauhinia variegata

Ischemia for 25 min followed by 40 min reperfusion resulted in elevation of LPO marker malondialdehyde levels in brain. The amount of MDA formed in I/R brain (11.8±0.36 nmol/ mg protein) was high as compared to control group (7.01±0.11 nmol/ mg protein). Pre-treatment with *BV* (at both doses) and Ascorbic acid significantly ($P < 0.001$) lowered the MDA levels of the I/R rats brain as compared to I/R group. The significant effect of the *BV* (400 mg) in decreasing the MDA levels in brain was comparably equal to Ascorbic acid. We found that there is no significant difference in the MDA levels in animals treated with highest dose of *BV* (400 mg/kg). The effect of *BV* and Ascorbic acid on SOD levels in brain was shown in Fig. 2. In the I/R group, depletion of SOD levels was observed in brain (22.41±1.84 nmol/ mg protein) compared to that of control treated animal group (177.9 ± 27.73 nmol/ mg protein). Prior Administration of *BV* (200 and 400 mg/kg) significantly ($p < 0.05$ & 0.001 respectively) elevated the SOD levels in brain regions of the I/R rat groups compared to the I/R group. Interestingly *BV* (400 mg/kg) increased the levels of SOD more than Ascorbic acid in I/R rats. We found that there is no significant difference in the SOD levels in animals treated with highest dose of *BV* (400 mg/kg) without any stress.

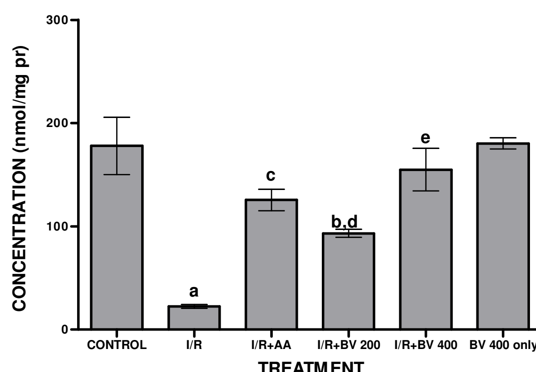

Figure 2

Protective effect of ethanolic extract of Bauhinia variegata and AA on SOD levels in rats subjected to cerebral ischemia and reperfusion injury. All the values are expressed as mean ± SEM, n = 6. a = p< 0.001 & b = 0.01 vs control; c = p< 0.01, d = 0.05 & e = 0.001 vs I/R. I/R. where I/R= cerebral ischemia reperfusion, AA= Ascorbic acid, BV= Bauhinia variegata

The CAT activity in brain of I/R rat brain was shown in Fig. 3. In I/R group decrease in catalase activity was noted in the brains (4.13±0.33 IU/ mg of protein) after 40 min of reperfusion. Administration of *BV* (400 mg/kg) and Ascorbic acid significantly ($p < 0.001$) reversed the decreased catalase activity in brain regions compared to I/R group alone. *BV* at a dose of 200 mg/kg was less effective compared to *BV* 400 mg/kg and Ascorbic acid to increase the CAT activity in the brain. Our findings showed that there is no significant difference in the CAT levels in animals treated with *BV* (400 mg/kg) without any stress.

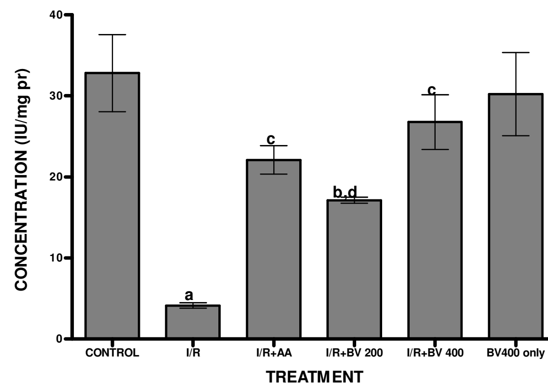

Figure 3

Protective effect of ethanolic extract of Bauhinia variegata and AA on catalase levels in rats subjected to cerebral ischemia and reperfusion injury. All the values are expressed as mean ± SEM, n = 6. a = p< 0.001& b = 0.01 vs control; c = p< 0.001& d = 0.05 vs I/R. I/R. where I/R= cerebral ischemia reperfusion, AA= Ascorbic acid, BV= Bauhinia variegata

Fig. 4 represents the effect of *BV* and Ascorbic acid on I/R-induced changes in GSH level in brain. Depletion of GSH level was noted in brain of I/R rats induced by ischemia for 25 min followed by 40 min reperfusion as compared to the control group. There were no much significant changes in GSH in the *BV* low dose (200 mg/kg) treated group. *BV* at 400 mg/kg significantly elevated the GSH level in the I/R rats ($p < 0.001$). The effect of *BV* 400 mg/kg in restoring the GSH level in the brain was comparable to that of Ascorbic acid ($p < 0.001$). We found that there is no significant difference in the GSH levels in animals treated with highest dose of *BV* (400 mg/kg) without any I/R.

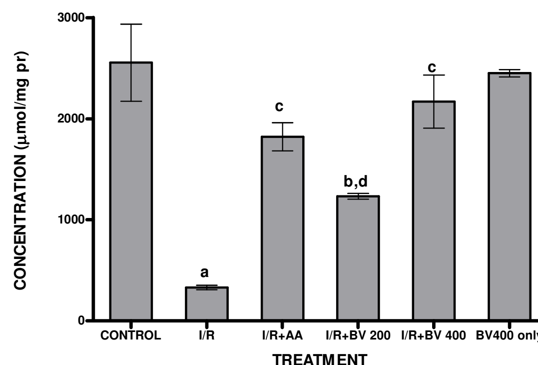

Figure 4

Protective effect of ethanolic extract of Bauhinia variegata and AA on glutathione (GSH) levels in rats subjected to cerebral ischemia and reperfusion injury. All the values are expressed as mean ± SEM, n = 6. a = p< 0.001& b = 0.01 vs control; c = p< 0.001& d = 0.05 vs I/R. where I/R. I/R= cerebral ischemia reperfusion, AA= Ascorbic acid, BV= Bauhinia variegate

DISCUSSION

Generation of reactive oxygen species is a central event in the reperfusion period, among which, $O_2^{\bullet-}$ and $OH^{\bullet}$ are potent inducers of lipid peroxidation[23]. Excessive production of ROS can cause cellular damage and subsequent cell death, because ROS may oxidize vital cellular components such as lipids, proteins and DNA[24], and alter several signalling pathways that ultimately promote cellular damage and death during cerebral

I/R[25]. During reperfusion, Hypoxanthine gets accumulated, which is converted to xanthine. In this process, superoxide radicals (O_2) are generated and converted to hydrogen peroxide (H_2O_2) or hydroxyl radical ($OH^·$). There is a considerable evidence supports that the role of ROS in the pathogenesis of I/R induced oxidative stress in brain[26]. Physiologically, SOD reacts with $O_2^{•-}$ to form H_2O_2; CAT and GSH are involved in the detoxification of H_2O_2. During cerebral ischemia, a number of events predispose the brain to the formation of ROS such as rapid decrease in adenosine triphosphate levels, calcium release from intracellular stores, loss of Ca^{2+} homeostasis, excitotoxicity, arachidonic acid release and metabolism, mitochondrial dysfunction, acidosis and edema[27]. This spectrum can modulate the antioxidants enzymes and its gene expression in I/R insult[28]. Calcium plays a unique role on the ischemic pathophysiology since it causes several damaging events by activation of a variety of Ca^{2+} dependent enzymes, including protein kinase C, phospholipase A2, phospholipase C, cyclooxygenase, calcium-dependent nitric oxide synthase, calpain and various proteases and endonucleases. As a result of formation of cytotoxic products such as free radicals and leukotrienes, irreversible mitochondrial damage, and inflammation, both necrotic and programmed cell death are triggered by excess of intracellular Ca^{2+}.[29]

In case of excessive free-radical generation the endogenous protective system may not prove sufficient, resulting in damage. Exogenous antioxidants such as vitamin C, vitamin E, and ß-carotene can also scavenge potent radicals and prove useful in preventing damage[30]. In the present study, we estimated the levels of antioxidants like SOD, GSH and CAT which served as oxidative indices in brain of the common carotid artery occluded rats. Decrease in the levels of SOD and CAT were noted in brain of the ischemic rats indicating participation of superoxide radical which is known to produce highly toxic hydroxyl radical through its reaction with H_2O_2 (Haber–Weiss reaction)[31]. These in turn decreases the SOD through a modification in histidine residue located in the active site of the enzyme[32]. On the other hand this over production of H_2O_2 can be inactivated by catalase enzyme and there by reduction in CAT. *BV* (200 and 400 mg/kg) was found to elevate the activity of two major oxygen radical species metabolizing enzymes in brain of ischemic brain. Reduced glutathione is one of the primary endogenous antioxidant defense systems in the brain, which removes hydrogen peroxide and lipid peroxides[33]. Decline in GSH levels could either increase or reflect oxidative status[34]. In our experiment, depletion in GSH was observed in brain of ischemic rats. This could be explained by the consumption of GSH due to scavenging of the rapidly generated hydrogen peroxide and lipid peroxides. It has been shown that depletion of GSH in IS/RP injury can be attributed to several factors such as cleavage of GSH to cysteine, decrease in synthesis of GSH and the formation of mixed disulfides with GSSG, causing their cellular stores depleted[35.] Interestingly, those rats fed with *BV* at the higher dose (400 mg/kg) increased the GSH levels in brain.

The thiobarbituric acid reacting substance assay is used as an indicator of lipid peroxidation and levels of free radicals. The assay is based on the reactions of thiobarbituric acid with malondialdehyde produced during lipid peroxidation[36]. As observed in our study, the increase in malondialdehyde in brain affected by ischemic-reperfusion injury suggested enhanced lipid peroxidation. In our study, alcoholic *BV* stem bark extract pre-treated animals showed significantly less lipid peroxides due to ischemic-reperfusion injury than the untreated animals. Ascorbic acid treated animals also showed lesser degree of lipid peroxidation than control group. Hence, it is possible that the mechanism of protection of brain by alcoholic *BV* stem bark extract might be due to its antioxidant effect. This is supported by previous findings that numerous flavonoids, e.g. those found in green tea extract and other antioxidant preparations containing biflavones and polyphenolics protect the neurons against ischemia-reperfusion induced alteration in activities of intra cellular antioxidant complexes[37]. The antioxidant effect of *BV* observed in brain of I/R rats explains the potential permeation of *BV* across the blood–brain barrier (BBB) and utilization of the *BV* on brain. *BV* stem bark contains flavonoids such as quercetin,

flavonone glycosides such as 5,7 dihydroxy[38] and 5,7 dimethoxy flavanone-4-O- α -L rhamnopyrosyl- ß -D-glycopyranosides[39], flavonol glycosides characterised as kaempferol-3-glucoside[40], stigmasterol[41] and phenolic compounds, and these pharmacophores have been shown to possess potent antioxidant and free radical scavenging activity.

The present investigation showed the neuroprotective potential of alcoholic extract of *BV* against I/R induced oxidative stress. It was observed that *BV* attenuated the impaired neurological deficit of the ischemic rats. The activity of *BV* appears to restore the altered antioxidants enzymes as well as decrease the production of LPO in various brain regions induced by I/R. This study suggest that *BV* administration to the normal rat did not showed any effect on the levels of endogenous antioxidant enzymes and oxidative stress marker in brain regions of normal rat. Interestingly *BV* exerts its antioxidant effect only in the presence of ischemia and reperfusion induced oxidative stress. This may be due to direct scavenging effect of biflavone on free radical released during ischemia reperfusion and also modulate the endogenous antioxidants enzymes mediated neuroprotection.

CONCLUSION

The present investigation demonstrates the protective effect of *BV* tested in cerebral ischemia and reperfusion induced oxidative stress. The results suggest that the protective effect may be by mechanisms involving inhibition of free radical generation, reactive oxygen species scavenging, modulation of intracellular antioxidants against I/R induced decrease. It may possess potential as a therapy for the oxidative stress—related disorders. *BV* extract is cocktail of antioxidants which may act coperatively at various stages of free radical generation and protect the neurons from cerebral ischemia reperfusion injury. However, further work on infarct size measurement in rats is required for clinical use of *BV*.

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