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Effect of *Bauhinia variegata* on stress induced changes in plasma corticosterone and brain monoamines in rats

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ABSTRACT

In the present study, effect of Bauhinia variegata (BV) was evaluated on chronic unpredictable stress (CUS) induced changes in plasma corticosterone and monoamines-noradrenaline (NA), dopamine (DA) and serotonin (5-HT) in cortex and hippocampus regions of brain in rats. Wistar albino rats of either sex (200-250 g) were subjected to CUS and treated with alcoholic extract of Bauhinia variegata (200 and 400 mg/kg; p.o) and diazepam as standard (4 mg/kg i.p.) for 7 days. Levels of plasma corticosterone, triglycerides, glucose, cholesterol and biological amines levels in cortex and hippocampus were estimated. CUS resulted in significant elevation in plasma corticosterone levels and biochemical parameters, whereas levels of NA, DA and 5-HT were significantly depleted in cortex and hippocampus regions of brain, which was significantly countered by treatment with BV similar to the effects of diazepam at 4 mg/kg i.p. Hence, our study indicates that the adaptogenic activity of BV might be due to the normalization of stress induced alteration in plasma corticosterone and levels of monoamines like NA, 5-HT and DA in cortex and hippocampus regions of the brain, which are more vulnerable to stressful conditions analogous to the effects of diazepam.

Key words: *Bauhinia variegate*, Diazepam, Chronic unpredictable stress, Monoamines, Corticosterone.

INTRODUCTION

Stressful stimuli can disrupt the physiological homeostasis of the body and inability to cope with such aversive stimuli has widespread deleterious effects on the biological system [1]. Emotional and environmental stressors reportedly influence brain function and is known to be a key factor in the genesis of neuropsychiatric disorders [1, 2]. Exposure to such stressors are known to evoke responses such as reduced locomotor activity marked anorexia, decrease growth rate and hypertension [3]. The central nervous system (CNS) is a crucial mediator during such stress related responses. Complex interactions between central nervous system viz. limbic system, hypothalamus-pituitary- adrenal (HPA) axis and several components of visceral system occur in response to a variety of stressful inputs [4] and some limbic structures, particularly amygdaloid complex and its interactions with lower brain stem areas, have been implicated in stress [5].

The HPA axis is activated to prepare the body for "adaptation" during stressful conditions. Chronic stressful conditions lead to consistent hyperactivity of HPA axis and are known to influence several physiological responses that adversely affect the normal psychosomatic homeostasis [6]. HPA axis activation releases glucocorticoids (corticosterone in rodents and cortisol in humans) whose actions are mediated through glucocorticoid receptors that are abundantly expressed in brain regions such as the cortex, hypothalamus, hippocampus, amygdala, various brain

stem nuclei and pituitary involved in the stress response [7]. Central neurotransmitters are important mediators in physiological and behavioural responses to stress [8, 9]. Among these central neurotransmitters noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT) are important monoamines, which are widely distributed in brain and are studied extensively and their role was well established in various stress-mediated disorders [10-12]. Changes in monoaminergic activity results in behavioural changes as well as a cascade of hormonal release from the HPA axis. Dysfunction of these monoamines due to prolonged stressful conditions has been associated with a wide range of central and peripheral disorders like depression, anxiety, drug abuse, obsessive compulsive disorder, eating and sleeping disorders, hyperglycaemia, and decreased immune response [13-17].

The drugs of plant origin are gaining importance and are being investigated for remedies of a number of disorders including stress. *Bauhinia variegata (BV)* Linn (Caesalpiniaceae) is a medium-sized deciduous tree found throughout India. It is traditionally used in bronchitis, leprosy, and tumours. The stem bark is used as astringent, tonic, and antihelmintic [18].

Since the flavonoids & polyphenolic compounds are present in the stem bark of *B. variegate*, the present study was designed to evaluate the effect of alcoholic bark extract of *BV* under chronic unpredictable stress (CUS) in relation to the changes in the level of plasma corticosterone and noradrenaline, dopamine and serotonin in brain and compared with Diazepam.

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

Pure HPLC grade corticosterone, Adrenaline, Noradrenaline, Dopamine, Serotonin (Sigma, St. Louis, USA), Diazepam (Gift sample from Dr. Reddys laboratories, Hyderabad), HPLC grade Dichloromethane, Methanol, Acetonitrile (Qualigene Fine chemicals, Mumbai).

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). Plant Specimen (voucher no: KUH 1854) was submitted in the Herbarium, Dept. 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 (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). If animal dies at particular dose, lower dose is given to the next animal and if animal survives at a particular dose, next higher dose was given for remaining animals. 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 [19]. 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 chronic unpredictable stress (CUS) model.

Drug Treatment Protocol
Animal are divided into six groups. Normal control group (Group I) received only vehicle (5% acacia solution) without CUS, whereas animals from model control group (Group II) received only CUS without any treatment. Animals from Group III to Group V received test drugs such as standard drug diazepam (4 mg/kg, i.p.), alcoholic extract of *BV*, (200mg/kg, p.o.), alcoholic extract of *BV* (400 mg/kg, p.o.) respectively. Animals from group VI received only highest dose (400 mg/kg, p.o.) of test without CUS, once daily for 7 days 45 minutes prior to CUS.

Stress procedure
Chronic unpredictable stress model was used to produce the stress. Animals are divided into 6 groups. Each group consists of 6 rats. 45 min after feeding the drug or vehicle, rats were subjected to stressors except the control group. In CUS, the drugs were fed daily 45 min prior to exposure to stress regimen up to 7 consecutive days except that the rats were fasted overnight on the sixth day. A parallel group of control group was also taken as described above and sacrificed on seventh day along with the CUS group of rats.

In our experiments, CUS regimen involves subjecting animals to two different stressors of variable intensity on every day in an unpredictable manner for 7 days as described earlier [31]. Various stressors include immobilization (150 min), forced swimming (20 min), overnight soiled cage bedding, foot shock (2mA for 20 min), day–night reversal and fasting (12 h).

Estimation of corticosterone
Immediately after the last stress regimen, animals were sacrificed by decapitation and blood was collected in EDTA coated tubes kept in ice and centrifuged at 1000×*g* for 20 min at 4°C. Plasma was separated and aliquots were stored at −70 °C for corticosterone estimation.

The plasma concentrations of Corticosterone were determined by a HPLC assay according to Woodward and Emory (1987) with minor modifications using Exemestane as the internal standard. Briefly, 15µl of Exemestane (25µg/ml), as the internal standard, and 5ml of Dichloromethane were added to 0.5 ml of the plasma sample. It was then mixed for 5min using a cyclomixer (REMI co, Italy) and centrifuged at 5000rpm for 15min. Then the Organic layer was collected and evaporated to dryness. Then the samples were reconstituted with 100µl of methanol. 20µl of this reconstituted solution was taken into the Hamilton syringe and injected directly into HPLC column.

The chromatographic system was composed of LC-20AT Prominence liquid chromatograph pump, Rheodyne injector, SPD-20A/SPD-20AV Prominence UV–Vis detector (Shimadzu, Kyoto, Japan). The detector wavelength was set to 250 nm, with the sensitivity of 0.005AUFS and the column, Luna C18 (2) 100A (250×4.6mm, 5µm) was used at room temperature. Mixtures of methanol: Water (70:30 v/v) was used as the mobile phase at a flow rate of 1.0 ml/min. The retention times are as follows: internal standard, 8.7 min; Corticosterone, 7.0 min. The calibration curve of Corticosterone was linear within range 0.1-6.4µg/ml (r^2 = 0.9934). Detection limit was defined below 25 ng/ml.

Estimation of monoamines
Estimation of Dopamine, Noradrenaline, and 5-HT levels in the Brain (both in cortex and hippocampus) was done by LC-MS/MS using Adrenaline as the internal standard.

Sample Extraction
The brain (cortex and hippocampus separately) was homogenized with Ringer's solution under the cold conditions (approximately, -40^{0}C), sample (20µL) was acidified with 50 μL of formic acid (98-100%, J.T Baker, USA), Samples were extracted from the supernatant by solid-phase extraction using mixed-mode strong cation-exchange and reversed-phase cartridges (Oasis MCX 150 mg, 6 cc, Waters, MA). The cartridge was conditioned with 3 mL of methanol and 3 mL of 0.1% aqueous formic acid. After addition of 5 mL of supernatant, the cartridge was washed with 4 mL of 2% aqueous formic acid and 4 mL of methanol, and the compounds were eluted with 10 mL of methanol containing 2% ammonia. To the collection tubes, 2 mL of methanol and 750 μL of formic acid were added to neutralize the ammonia used in the elution.

Methanol was evaporated with a rotary evaporator, and monoamines was fractionated from the residue with an Agilent HP 1200 liquid chromatography (Hewlett-Packard GmbH, Waldbronn, Germany) equipped with a quaternary pump, an auto sampler, a column compartment, UV diode array detector, and fraction collector. The

separation of monoamines and Adrenaline was performed on a Discovery HS F5 column (150 mm × 4 mm, 3 μm, Sigma-Aldrich, Bellefonte, PA) using Acetonitrile (ACN) and aqueous 0.1% formic acid as eluents.

Flow gradient
Buffer: 0.1% TFA (Trifloro acetic acid) containing HPLC grade water.

A linear gradient of 5-25% ACN for 0-1 min, 25-80% ACN for 1-1.5 min, 80% ACN for 1.5-2.5 min, 80-5% ACN for 2.5-3.0 min, and 5% ACN for 3.0-3.5 min was used.

The flow rate was 0.6 mL/min, and injection volume was 20 μL.

Estimation of biochemical parameters
Biochemical parameters like blood glucose by GOD/POD method (Trinder, 1969), triglycerides by GPO/PAP method [20], and cholesterol by CHOD/PAP method [20], were performed using standard procedures reported in the literature using the auto analyser (Biological systems international Arezzo, Italy).

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
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 on Plasma Corticosterone Levels
The results were graphically represented in Fig. 1. Exposure to CUS (311.3, $p < 0.001$) significantly increased the plasma corticosterone level compared to untreated control group. Pretreatment with Diazepam ($p < 0.001$) and *BV* at 400 mg/kg ($p < 0.001$) and 200 mg/kg ($p < 0.01$) significantly countered CUS induced elevation in levels of plasma corticosterone. Dose dependent increase in activity was found. *BV* at 400 mg/kg showed similar results as that of standard drug diazepam. We found no significant change in corticosterone levels in animals treated with highest dose (400 mg/kg) of test drug without any stress.

Effect of BV on monoamine changes in brain with CUS
CUS exposure significantly decreased the levels of NA (92.07 and 103.8 in cortex and hippocampus respectively, $p < 0.001$) (fig.2).

Continuous stress exposure for 7 days resulted in significant depletion in levels of DA (53.05 and 90.38, $p < 0.001$) (fig.3) and 5-HT (39.22 and 56.12, $p < 0.001$) (fig.4) in both cortex and hippocampus respectively. However, pretreatment with *BV* and Diazepam significantly countered the deleterious effect of CUS resulting in significant elevation in levels of three monoamines in cortex and hippocampus. Decrease in monoamine levels were attenuated by pretreatment with *BV* in a dose dependant manner. 400 mg/kg dose showed the results similar to diazepam.

Fig.1. Bar diagram representing the changes in levels of plasma corticosterone in CUS
All the data were expressed as mean ±SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001, c = p<0.01 vs CUS. Where BV= Bauhinia variegata, CUS = chronic unpredictable stress.

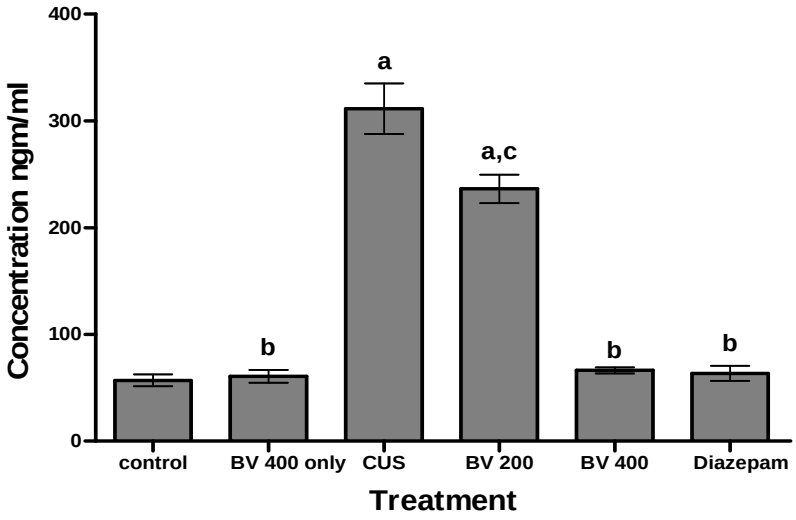


Fig.2. Bar diagram representing the changes in levels noradrenaline in cortex and hippocampus regions in CUS
All the data were expressed as mean ±SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001vs CUS. Where BV= Bauhinia variegata, CUS = chronic unpredictable stress.

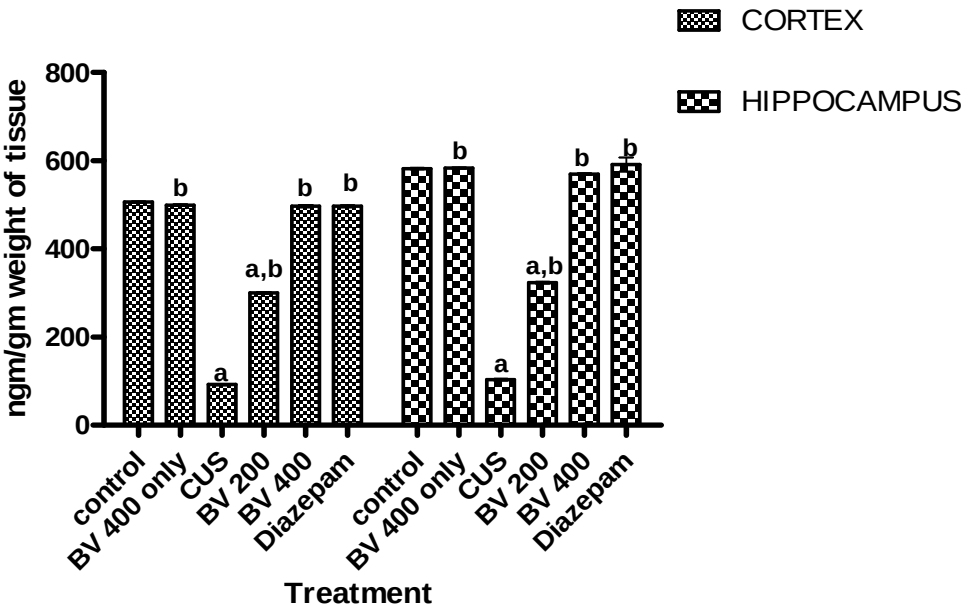


Fig.3. Bar diagram representing the changes in levels of dopamine in cortex and hippocampus regions in CUS
All the data were expressed as mean ±SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001vs CUS. Where BV= Bauhinia variegata, CUS = chronic unpredictable stress.

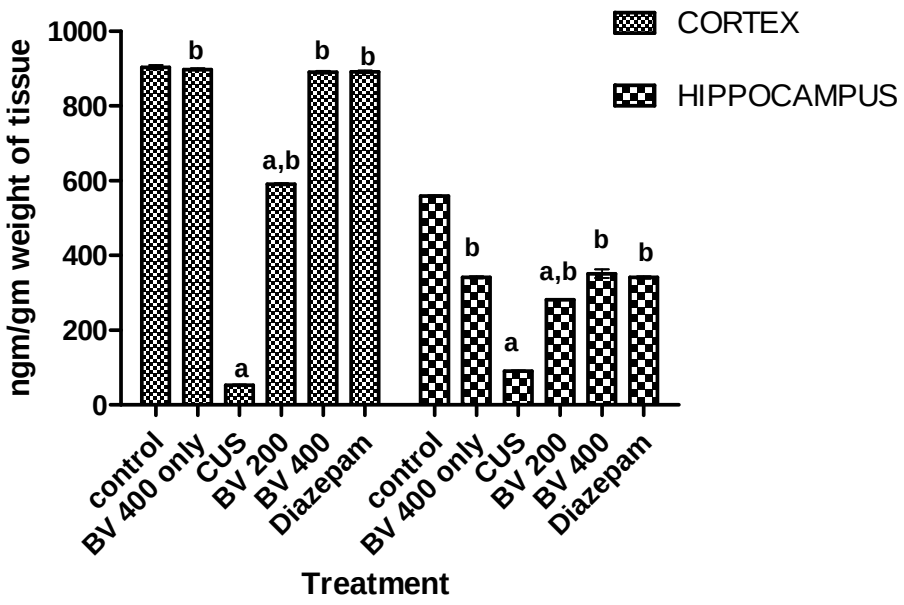
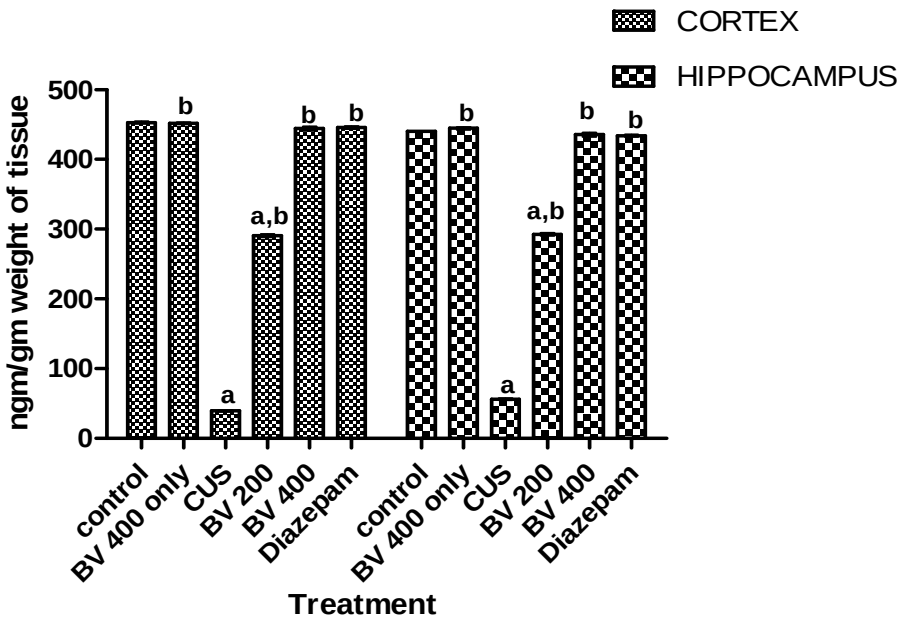


Fig.4. Bar diagram representing the changes in levels of serotonin in cortex and hippocampus regions in CUS
All the data were expressed as mean ±SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001vs CUS. Where BV= Bauhinia variegata, CUS = chronic unpredictable stress.



Effect of BV on Biochemical Parameters

We observed that there are increased levels of Triglycerides, Glucose, and Cholesterol in stress control group compared to vehicle control group. Pretreatment with *BV* significantly ($p < 0.001$) (fig.5) reverted the elevated levels, compared to stress control group. Test drug at 400 mg/kg dose was found to be showed good inhibitory action on elevated levels of triglycerides, glucose, and cholesterol than diazepam.

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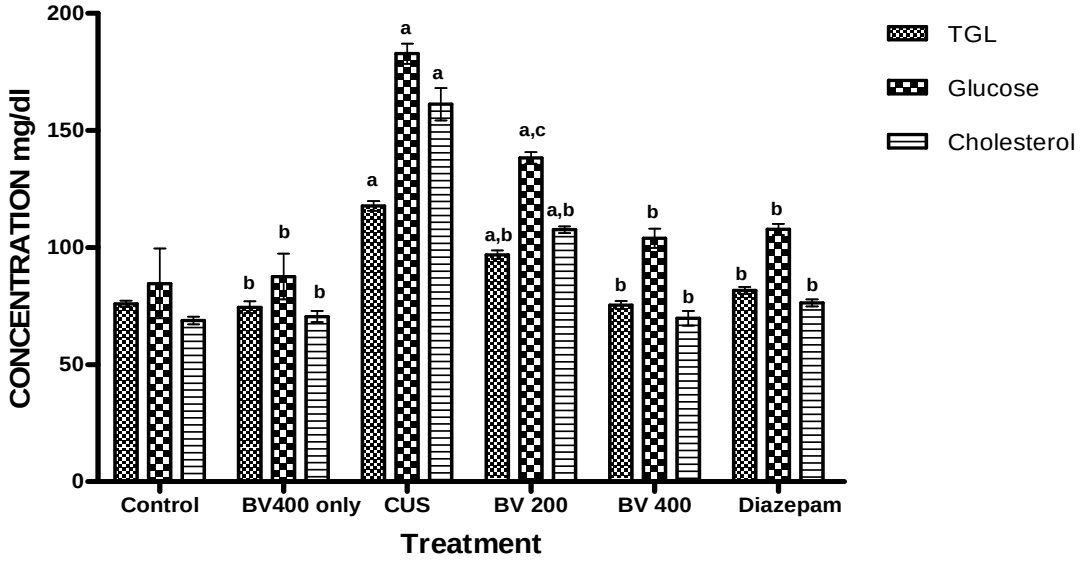
Fig.5. Bar diagram representing the changes in levels of plasma Triglycerides, Glucose and Cholesterol in CUS
All the data were expressed as mean ± SEM, n = 6. a = p< 0.001 vs control; b = p< 0.001, c = 0.01 vs CUS. Where BV= Bauhinia variegata, CUS = chronic unpredictable stress.



DISCUSSION

In our previous studies we found that there is increase in the total protein, lipid peroxidation and decreased antioxidant enzymes in CUS. 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 [21]. The activation of this HPA system results in secretion of corticotrophin hormone, adrenocorticotropin hormone (ACTH), β-endorphin and glucocorticoids into the circulation. Release of ACTH in stress stimulates adrenals to increase production of hormones- epinephrine, norepinephrine and corticosteroids [22]. These hormones have profound effect on metabolic functions. Increased plasma cortisol influences the mobilisation of stored fat and carbohydrate reserves [23], which in turn increase blood glucose, cholesterol and triglyceride levels.

In present study, in chronic stress model, the significant increase in blood glucose level was observed because; under stressful conditions adrenal cortex secretes cortisol in man and corticosterone in rats. Hyper secretion of cortisol helps in maintenance of internal homeostasis through the process of gluconeogenesis and lipogenesis [24]. During CUS release of various adrenal hormones such as catecholamines and glucocorticoids results in elevated plasma glucose levels because excess of cortisol causes insulin resistance leading to increased gluconeogenesis and eventually hyperglycaemia. During CUS, the increased level of glucose may be important for maintaining the ATP availability to muscles, CNS, and the organ of demand [25, 26]. Pretreatment with the *B. variegata* as well as reference standard drug diazepam significantly ($P < 0.001$) reduced the elevated glucose levels indicating their suppressant effect on hyper activity of adrenal cortex and maintained the homeostatic mechanism. Hyperglycaemic effect of corticosterone is reportedly due to increased glycogenolysis of glycogen in liver during stress [25, 27].

The marked increase in serum cholesterol, triglycerides and levels in stress-induced animals is due to stimulation of hypothalamo– pituitary axis (HPA) and sympathetic system, resulting in, liberation of catecholamines and glucocorticosteroids, which inhibits the immune system at multiple sites like liver, kidney [28]. Release of corticosteroids may also induce hyperinsulinemia resulting in an increased synthesis of cholesterol. Stress has profound effect on metabolic functions of body [29]. *B. variegata* as well as reference standard drug diazepam significantly ($P < 0.001$) reduced the elevated serum cholesterol, and triglycerides, which may be due to inhibition of stimulation of sympathetic nervous system.

The increase in weight of adrenals in stressed animals is due to the stress-induced adrenomedullary response leading to increased production of corticotropic hormone that leads to increase in weight of adrenals [24]. *B. variegata* and diazepam has significantly ($P < 0.001$) 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 [30, 31]. The pretreatment with the *B. variegata* and reference standard diazepam significantly ($P < 0.001$) increased the spleen weight. This may be due to inhibition of recruitment of lymphocytes to blood from spleen.

Chronic unpredictable stress resulted in significant increase in adrenal gland weight with concomitant decrease in spleen weight in stress control group, which was significantly reverted by *BV* pretreatment at 200 mg/kg and 400 mg/kg (data not mentioned here). Pretreatment of animals with *BV* at both doses also significantly restored back CUS induced alterations in plasma corticosterone, glucose, triglyceride, and cholesterol levels. Further, CUS significantly decreased NE, DA and 5-HT levels in brain.

Diazepam is reported to possess a non-specific anti- stress activity involving the mesocortical dopamine system and the norepinephrine and 5HT levels of whole brain and hypothalamus [32-34]. The mesocortical dopamine system is thought to play an important role in the etiology of the stress response. Dopamine (DA) has been shown to accumulate in the rat frontal cortex in response to a wide variety of stressors. Diazepam, an anxiolytic benzodiazepine, can reverse the effects of stress on cortical DA levels. It is proposed that this effect is produced through an enhancement of GABAergic neurotransmission by diazepam [32]. Exposure of animals to immobilization stress markedly and rapidly decreases the concentration of NE in brain and hippocampus [8, 9]. But pretreatment of rats with diazepam significantly attenuated stress-induced depletion of NE of cortex and hippocampus. As NE has been implicated in the activation of H-H-A axis (hypothalamo-hypophyseal-adrenocortical axis) [9, 35] during stress. Simultaneous attenuation of stress- induced elevation of plasma corticosterone justifies the anti-stress action, though diazepam does not affect the cortex and hippocampal 5-HT and plasma corticosterone in control rats; it attenuates stress-induced depletion of cortex and hippocampus 5-HT and also simultaneously diminishes the stress induced enhancement of plasma corticosterone levels.

Pretreatment with both the doses of *BV* effectively reduced the CUS induced elevation in the levels of plasma corticosterone. This normalizing effect on plasma corticosterone is one of the possible reasons for its “adaptogenic” properties. The adaptogenic effects were further supported by measuring biochemical markers like plasma glucose, triglycerides and cholesterol which are sensitive to the levels of plasma corticosterone. However, the major constituents responsible for this effects needs to be resolved in future studies.

Stress effects depend on the type and duration of stressor. In CUS, overload by stressors lead to disrupt the homeostatic conditions, which is generally achieved by means of unpredictable schedule by which the effects of stressors are more prominent. CUS is considered to be more clinically relevant model to observe stress manifestations. Severe stressful conditions decrease monoamine levels which are mainly due to increased stress sensitization and their preferential and higher utilization during severe stressful conditions [36, 37]. In our study, CUS significantly decreased monoamines in all the both brain regions i.e cortex and hippocampus. *BV* at a dose of 200 and 400 mg/kg was effective in normalizing NA, DA and 5-HT in cortex, and in hippocampus of brain.

From our study it is clearly evident that *BV* normalizes stress mediated transient deregulation of plasma corticosterone and monoamine changes in brain which may be one of the reasons for its adaptogenic activity [30]. Deregulated function of monoamines is one of the principle reasons for memory dysfunction during stressful conditions. Further this study provides an insight into the central mechanisms responsible for adaptogenic effect of *BV* similar to the effects of diazepam.

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