Protective effects of *Butea frondosa* leaves against stress induced immune impairment in sprague dawley rats

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Abstract: Stress is thought to impair immune function through emotional or behavioral manifestations thus the present study was done to assessed the effect of ethanolic extract of *Butea frondosa* (BF) leaves on behaviour, immunomodulatory activity and brain acetyl cholinesterase activity in normal and stress induced male rats. Neuroprotective effects of BF, doses (100,200,400mg/kg p.o) were measured by assessing the changes in the behaviour and the immunity of the rats. In stress control, the results indicated that the retention transfer latency, time spent in a closed arm, agglutination , total leukocytes counts (TLC), total paw edema ,size of spleen , decreased significantly ($p<0.01$) while glucose level, size of the kidney and the liver, AChE activity increased significantly ($p<0.01$) in comparison with normal control. In BF (200mg/kg) treated rats, the results indicated that the time spent in a closed arm ($p<0.01$), agglutination ($p<0.01$), TLC ($p<0.01$), total paw edema ($p<0.05$), size of spleen($p<0.01$) , increased significantly while glucose level ($p<0.01$), size of the kidney and the liver ($p<0.01$), AChE activity ($p<0.01$) decreased significantly in comparison with stress control. This study therefore concluded that the ethanolic extract of BF (200mg/kg) showed a protective effect against the stress induced impaired immune system and the psychological disorders.

Keywords: Acetylcholinestrase, agglutination, immunity, neuroprotective.

INTRODUCTION

Psycho Immunology (PI) is the study of the interaction between the psychological processes and the immune systems of the human body (Badruddeen *et al*., 2012). Stress is the fundamental phenomenon of life that is experienced by every individual. It is a normal human response to pressure when faced with challenging dangerous situations. When stress become extreme it is destructive for the body, and hence to be treated. It includes number of diseases like psychiatric disorders such as immunosuppression, depression and anxiety, endocrines disorders including diabetes, peptic ulcer, ulcerative colitis, increased heart beat, hypertension, blood pressure and metabolic rate, all proposed to increase the overall performance and ability of body to overcome challenge (Agarwal and Marshall, 2001).

Butea frondosa Koen. Ex Roxb of the family Fabaceae, is a medium sized tree, growing to 15m tall and widely distributed throughout India. The different extracts of the plant is found to posses antidiabetic (Sujith *et al*., 2011), antiobessity (Dixit *et al*., 2012), hepatoprotective (Sharma and Shukla, 2011), antidiarrhoeal (Banji *et al.*, 2010), Aphrodisiac (Ramachandran *et al.*, 2004), anticonvulsant (Kasture *et al.*, 2002), antifilarial (Deshmukh *et al*., 2014), anti-inflammatory (Shahavi and Desai, 2008) antihelmintic (Borkar *et al*., 2010) and Antimicrobial (Londonkar and Ranirukmini, 2010). It is traditionally used for the treatment of piles, Conjunctivitis, bleeding nose, urinary tract infection. Leaves have astringent, carminative, tonic, diuretic, appetizer and aphrodisiac properties. They are also used to treat stomach disorders, diabetes, sore throat. Leaves have Glucoside, Kino-oil containing oleic, palmitic, linoleic acid lignoceric acid (Mishra *et al.*, 2012).

There is no report on the neuroprotective and effect on immunity of the ethanolic extract of BF, therefore, the present study was undertaken to evaluate the changes occur in a behavioural, immunological and neuropharmacological responses in normal and stress induced rats.

MATERIALS AND METHODS

Collection and authentication of the plant material

The leaves of *Butea frondosa* were obtained from Musa Bagh, Hardoi Road, Lucknow. It was confirmed and authenticated by National Botanical Research Institute (NBRI) Lucknow, Uttar Pradesh, India (Authentication No.CIF-RB-3-269).

Preparation of plant extract

The leaves were dried in shade and coarsely powdered. The powder was subjected in soxhlet apparatus for 72h with ethanol. The extract was evaporated under reduced pressure using rotary evaporator till all the ethanol had been removed to give an extract sample with a yield of

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5.06% w/w. The extract was stored at 4°C. It was freshly prepared in carboxy methyl cellulose immediately before its administration to the animals.

Animals

Sprague Dawley (SD) male rats (Body weight: 150-200 g), were purchased from the Central Animal House Facility, Central Drug Research Institute, Lucknow, India. Rats were housed under environmental condition of controlled temperature (25±2ºC), standard pellet diet and water *ad libitum* for at least 1 week before the experiments. The experiments were performed between 9:00 and 16.00h. The animal experiments were performed according to the rules and regulations of the CPCSEA and the proposed work was approved by Integral University's Institutional Animal Ethics Committee (IAEC), Lucknow, India (Approval No.IU/Pharm/PhD/IAEC/14/16).

Acute toxicity study

The SD male rats (150-250g weight) were divided into 4 groups of 3 rats each. Fixed doses of ethanolic extract of BF (5mg/kg, 50mg/kg, 300mg/kg, 2000 mg/kg p.o). were administered . They were observed for 24h. There was no mortality observed during 14 days after treatment. Thus three doses (100,200,400mg/kg) were employed for further pharmacological studies.

Antigenic materials

For the present study, the antigenic material used was sheep RBCs (SRBC). Fresh blood was collected from sheep sacrificed in the local slaughter house. It was mixed with Alsever's solution in 1:1 proportion and was stored at 4°C in refrigerator. During the experimentation, adequate amount of blood was taken from the above stock solution (i.e. SRBCs, stored in Alsever's solution) and was allowed to stand at room temperature. It was rinsed three times with pyrogen free normal saline (0.9% w/v NaCl). The RBC count of this suspension was estimated by hemocytometer using Neubauer Chamber. The known amount of RBCs (5×10^8) cells/mL was injected intraperitoneally to the rat as an antigenic challenge. (Wahab *et al.*, 2014)

Dosing schedule

The animals were divided randomly into five groups of 5 rats each (n=5). Group I: Normal control (NC) received carboxy methyl cellulose as a vehicle. Group II: Stress Control (SC) group induced stress by allowing them to swim in cold water (temperature-10±5°C) maximum for 120 min/day, with light/dark cycle for three consecutive weeks, with slight modification (Badruddeen *et al.*, 2012, Corror *et al*., 1997) Group III: BF 1 group received 100mg/kg extract and stress. Group IV: BF 2 group received 200mg/kg extract and stress. Group V: BF 3 group received 400mg/kg extract and stress.

The BF ethanolic extract at doses 100, 200mg/kg and 400mg/kg with carboxymethyl cellulose as a vehicle, were administered orally by using animal feeding intubation needles for 21 days and the control animals received an appropriate amount of vehicle for the same number of treatment days. 30 minutes after the treatment of the drug or the vehicle, the rats were subjected to stress once a day, for a period of 21 days, except for the non-stress group. All the behavioral and the biochemical tests were assessed 2 1/2 hours after the treatment of the last dose of drug/vehicle.

Behavioural assessment

Passive avoidance test

The activities of the rats on the avoidance chamber (passive paradigm) were used for the passive avoidance test, which was used as a short term memory task. The transfer latency of the animal was noted, which was associated to the retention of the memory task. The step-through passive avoidance apparatus (a shuttle box) was used for the assessment of the memory retention deficit in rodents. The apparatus consists of equal sized (30cm long x 30cm wide x 40cm high) light and dark compartment which were separated by a central wall. This wall has an opening, through which the animal can go by to both the compartments. The floor comprises of a metal grid which was connected to a shock scrambler. The test comprises of an acquisition and a retention trial. On day 20 after the treatment, the rat was placed in the light chamber. A trap-door which divided the chamber was open and the latency in entering the dark compartment was measured in seconds. Immediately after the rat got into the dark chamber, the trap-door was closed and an electric shock (1mA) was delivered for three secs. 5 seconds later, the rat was removed from the dark compartment and returned to its home cage. The retention test was executed 24 hours later in the same way, as in the acquisition trial and it was termed as the retention latency. Without giving the foot shock, the latency time was recorded to a maximum of 3 minutes (Badruddeen *et al.*, 2012).

Test for exploratory activity (Elevated plus maze)

The apparatus used in the present study comprised of two open arms (50×10cm) crossed with two closed arms of the same dimensions with walls 40cm high. Arms were joined by central area of (5×5cm) to give the apparatus a plus sign appearance. The apparatus was elevated 60cm above the floor. To monitor the activity, rats were individually placed in the central square in front of an enclosed arm and the time spent and the number of entries in a closed arm by the rat was noted. (Kulkarni, 2013)

Immunologoical assessment

Haemaglutination titre

Blood collected from retro-orbital puncture of the rats was centrifuged to separate the serum. Each well of the 96-well micro titration plate was filled with 50±1µl of normal saline. 50±1µl of serum was added to first cup and mixed. In this way serial two fold dilutions of serum was prepared. 50±1µl of 1% (v/v) SRBC was added to each

cup and then, the plate was incubated at 37°C for 1 hour. Afterwards, it was observed for agglutination. Positive haemagglutination reaction was visualized as a mat formation at the bottom whereas button formation indicated negative haemagglutination reaction (Hafeez *et al.*, 2001).

Cellular immune response

Cell mediated immune response was measured by footpad reaction test. Paw volume induced by an injection of sheep RBC (5×10^8 cells/ml in normal saline) in the subplater region of right hind paw. The mean percent increase in paw volume was considered as an index of cell mediated immunity. (Mohammad *et al*., 2013)

Biochemical assessment

Total leukocyte count

Blood samples were collected and TLC was assessed by routine hematological method using Neubauer's Chamber with haemocytometer. (Joharapurkar *et al.*, 2003)

Blood glucose

Blood samples were collected, and the blood glucose levels were estimated by using the GOD-POD method (Mishra *et al.*, 2010)

Estimation of acetylcholinestrase activity (AchE)

Animals were sacrificed by decapitation, immediately after behavioral evaluation for the biochemical analysis. The brains were dissected out, washed and weighed. The brain homogenate was prepared in phosphate buffered saline (pH 7.4). The homogenate was centrifuged (Decibel, India) and aliquots of supernatant was separated and used for biochemical estimation. AchE is a marker of extensive loss of cholinergic neurons in the forebrain. The AchE activity was assessed by Ellman method, with slight modification. The principle of the method is the calculation of the rate of production of thiocholine as acetylthiocholine is hydrolyzed. The change in absorbance was noted for 2 min at 30 s interval at 412 nm using UV visible spectrophotometer (Pharmaspec-1700, SHIMADZU). (Rehman *et al.*, 2011)

Relative organ weight determination

Rats of all groups were sacrificed 24h after the last dose. Body weight gain and relative organ weight (organ weight/100 g of body weight) of kidney, liver and spleen were determined for each animal.

STATISTICAL ANALYSIS

The data were expressed as mean ± SEM (Standard Error of the Mean) and the results were analyzed by ANOVA, followed by the Dunnett's t-multi comparison test. A value of $p<0.05$ was considered significant.

RESULTS

Acute toxicity

The acute toxicity study showed that all the doses (100, 200, 400 mg/kg) of the BF extract were non-toxic.

Effect of BF extract on behavioural study

The acquisition transfer latency (ATL) of the SC was found to be increased ($p<0.01$) and the retention transfer latency (RTL) was found to be decreased ($p<0.01$) significantly, when compared to the NC. ATL was significantly decreased ($p<0.05$) and ($p<0.01$) when compared with BF1 and BF2 respectively and the RTL was increased BF1 ($p<0.05$) and BF2 ($p<0.01$) when compared SC (table 1). The time spent in a closed arm was found to be significantly decreased ($p<0.01$), as compared to the NC, and it was found to be significantly increased ($p<0.05$) ($p<0.01$) when compared with BF 2, BF 3 respectively (fig. 1).

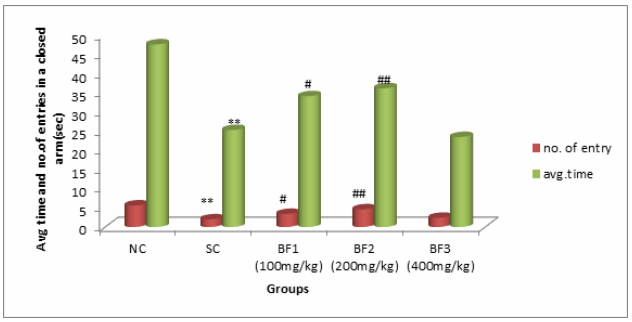

Fig. 1: Effect of *B. frondosa* extract on exploratory activity

Hemagglutinating antibody (HA) titre

The HA titre was used to assess humoral immune response. The humoral immunity of SC was found to be significantly decreased ($p<0.01$) as compared NC, means net formation in the cup number significantly disappeared with dilution and it was found to be highly significant ($p<0.01$), when compared with BF 2, and less significant ($p<0.05$) when compared with BF 1 , and it was found to be non significant when compared with BF 3 (table 2).

Foot pad reaction and total leucocytes counts

TLCs and the total paw edema of the SC were found to be significantly ($p<0.01$), as compared to the NC. TLC were found to be highly significant ($p<0.01$) with BF2, less significantly ($p<0.05$) with BF1 and it was non significant with BF 3 as compared to the SC. (table 2)

Effect on biochemical estimations

The blood glucose level of the SC was found to be significantly increased ($p<0.01$), as compared to the NC and it was found to be significantly decreased with all the doses highly significant ($p<0.01$) with BF1 and BF2 and less significant with BF 3($p<0.05$) as compared to the SC (table 3). The AChE activity in SC was found to be

Table 1: Effect of *B. frondosa* extracts on Acquisition and the Retention Latency (mean ± SEM)

Groups	Treatment	Acquisition Transfer latency (sec)	Retention Transfer latency (sec)
I	NC(CMC) 1ml/kg	12.2±0.86	131.4±0.74
II	SC+CMC	19.4±0.50**	98.2±3.4**
III	BF1(100mg/kg)+St	15.8±1.15#	108.8±2.2#
IV	BF 2(200mg/kg) +St	14.4±0.50##	122.6±1.69##
V	BF 3(400mg/kg)+St	19.0±0.77n.s	106±2.8n.s

Table 2: Effect of *B. frondosa* extracts on Humoral immunity and Cellular immunity (mean ± SEM)

Groups	Treatment	Mean antibody titer a (in terms of rank of cups in titre plate)	Total leucocyte count cells/mm^3	Mean of right foot pad thickness (mm)
I	NC(CMC) 1ml/kg	10.6±0.24	11500±223.61	0.28±0.016
II	SC+CMC	4.2±0.66**	6620 ±174.36**	0.15±0.009**
III	BF1(100mg/kg)+St	6.4±0.40#	8960±902.5#	0.13±0.012n.s
IV	BF2(200mg/kg)+St	8.2±0.66##	10220± 1001.7##	0.20±0.009#
V	BF3(400mg/kg)+St	5.0±0.31n.s	7540±156.84n.s	0.14±0.007n.s

Table 3: Effect of *B. frondosa* extracts on Biochemical estimation (mean ± SEM)

Groups	Treatment	Glucose level (mg/dl)	AchE (µ moles/min/mg protein)
I	NC(CMC) 1ml/kg	72.4±1.28	0.27±0.022
II	SC+CMC	185.4±2.2**	0.49±0.023**
III	BF1(100mg/kg)+St	130.2±1.46##	0.38±0.022#
IV	BF2(200mg/kg)+St	119.2±2.2##	0.27±0.022##
V	BF3(400mg/kg)+St	177±1.41#	0.44±0.047n.s

Table 4: Effect of *B. frondosa* on relative organ weight

Groups	Treatment	Relative organ weight (mean ± SEM)		
		Kidney	Liver	Spleen
I	NC (CMC) 1ml/kg	1.37±0.01	4.45±0.30	0.67±0.03
II	SC+CMC	1.72±0.03**	6.01±0.24*	0.45±0.03**
III	BF1(100mg/kg)+St	1.60±0.04#	4.58±0.38#	0.62±0.03#
IV	BF2 (200mg/kg)+St	1.50±0.03##	4.26±0.49##	0.64±0.05##
V	BF3 (400mg/kg)+St	1.63±0.01n.s	6.42±0.13n.s	0.51±0.02n.s

Values are expressed as Mean ±SEM (n=5), ** p<0.01significant when compared with Normal control. # p<0.05, ## p<0.01 significant, n.s = non significant when compared with stress control group

significantly increased (p<0.01), as compared to the NC, it was found to be significantly decreased (p<0.05) and (p<0.01) with BF 1 and BF 2 respectively (table 3).

Effect on relative organ weight

The sizes of the kidney and the liver of the SC were found to be increased (p<0.01) and (p<0.05) respectively and the size of the spleen was found to be decreased (p<0.01) significantly, as compared to those of the NC. The sizes of the kidney and the liver were decreased significantly (p<0.01) with BF2, and was found to be non significant with BF3 as compared with SC. On the other hand the size of the spleen was found to be significantly increased (p<0.05) with BF 1 and BF 2 when compared with SC (table 3).

DISCUSSION

Herbs today are being increasing used to cure all kinds of disorders. From mild cases like common cold to serious diseases like cancer, there is an always-growing need for genuine and well tested information regarding herbal cures. Research worldwide has advanced constantly, demonstrating the pharmacological effectiveness of different plant species in a variety of animal models. It has been reported that Stress triggers the immune function through emotional or behavioral manifestations. (Joshi *et al*., 2012)

In the present study, it is seen that induction of stress to rats leads to decrease ability of exploratory, learning memory estimated by passive avoidance tesr. Stress

results in the production of human diseased states, related to suppression of the immune response. Both humoral and cell-mediated immune responses are affected, indicating that stress may have a poor effect on normal immune surveillance. Ayurveda records that the *rasayanas* have the ability to protect the body against outside factors that induce disease. This implies that resistance against disease may represent the modern concept of immunity .BF has been shown to have an immunomodulatory action, improving immune reactivity. When rats were sensitized with sheep RBCs the humoral immune response was clearly suppressed in stress control group BF2 ($p<0.01$) highly significant, prevented the anticipatory fall in antibody titres comparable to stress control group. This may be due to the presence of flavonoids which augment the humoral response, by accelerating the macrophage and B-lymphocyte subsets involved in antibody synthesis (Makare *et al.,* 2001). In Foot pad test response pretreatment of animals with the extract shows inhibitory effect on stress-induced alterations in stress control group. In response to stress, the Adrenocorticotropic hormone (ACTH) is released, which acts on the adrenal cortex to stimulate the synthesis and release of cortisol. Increase in plasma cortisol influences the mobilization of stored fat and carbohydrate reserves, which in turn increase the blood glucose level (Nagaraja *et al.,* 1999) Treatment with the BF extracts significantly ameliorated the stress-induced changes in the elevated level of glucose. Increased weight of kidney and liver after forced swim stress supports the earlier reports by (Chang *et al*, 1995) who observed an increase in the kidney and liver weight after exposing the animals to exercise stress. Forced swimming might have increased the work load on the kidney as there was every chance for the animals to have more water intake during swimming. Changes in the homeostatic mechanism such as increase in cardiac output and blood pressure during stress might have contributed to increase in kidney weight in stress.

Increased weight of the liver during stress may be due to the increased secretion of the stress hormones, which are known to increase the metabolic activities and output m RNA levels in the hepatic cells. Oral administration of BF extracts for 21 days decrease the size of both the organs and significantly reversed the effect of stress. It was reported that stress will results in decrease in the weight of spleen (Krishnan *et al*., 2015). However in the present study significant increase in weight of spleen was observed after the administration of BF extract for 21 days, when compared with the stress induced group. The brain acetylcholine is associated with the consolidation of memory, and it is hydrolyzed by the brain cholinesterase. A significant inhibition of AChE activity has been found in the rats treated with 100 and 200mg/kg b.w. for 21 days, thus the plant extract is found to inhibit the rise in AChE activity. This result affirms the estimations, which might be due to a reduction in gene transcription, translation and enhance cholinergic activity thereby improving cognitive function. Phytochemical screening of the ethanolic extract revealed the presence of secondary metabolites such as sterols, tannins, flavonoids, carbohydrates, amino acids, glycosides, phenolic compounds, saponins and alkaloids supports the earlier reports (Rizvi *et al.,* 2014). Phenolic compounds present in the ethanolic extract of leaves of *Butea frondosa* so it might be responsible to prevent the stress-induced alterations in the behavior, immunity, and biochemical estimations indicating the protective effect against stress.

Although the mechanisms underlying these effects are still unknown and require more pharmacological, neurochemical and pharmacokinetic research to found any therapeutic advantage. However the use of BF might be suggested for the prevention of cognitive decrease during aging and neurodegenerative diseases.

CONCLUSIONS

This study concluded that *Butea frondosa* leaves extract showed a protective action against the stress induced cognitive impairement, functions related with immunity, and the brain acetylcholinestrase inhibition. Further studies are requires to be carried out in isolating the potential chemical constituents present in ethanolic extract of *B frondosa* leaves and to find its mechanism of action in curing neurogenerative disorders.

ACKNOWLEDGEMENT

The authors offer their sincere thanks to Dean Research & Development, Integral University for providing technical support and assigning Communication no. IU/R&D/2017-MCN000110.

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