INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-4, Issue-4, Oct-Dec-2013 Coden : IJABPT Copyrights@2013

ISSN : 0976-4550

Received: 14th August-2013 *Revised: 20th August-2013* *Accepted: 28th August-2013*

Research article

STUDIES ON BEHAVIORAL, BIOCHEMICAL, IMMUNOHISTOCHEMICAL AND QUANTIFICATION OF DOPAMINE AND ITS METABOLITES IN THE STRIATUM OF 6-HYDROXY DOPAMINE INDUCED PARKINSONISM IN RATS - ATTENUATION BY BACOSIDE-A, A MAJOR PHYTOCONSTITUENT OF *BACOPA MONNIERA*.

Chandrasekar Shobana and Thangarajan Sumathi *

Department of Medical Biochemistry, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai – 600 113, Tamil Nadu, India. Telephone +91- 44- 24547086; Fax: +91- 44- 24540709.

E-mail: sumsthangarajan@gmail.com

ABSTRACT: Bacoside-A, a major constituent isolated from *Bacopa monniera* is held in high repute as a potent nerve tonic. Rats were pretreated with Bacoside - A (10mg/kg and 20mg/kg body weight) for 21 days. A Parkinsonian model in rats was developed by giving 6-hydroxy dopamine (12µg/2µl in 0.1% ascorbic acid- saline) in the right striatum on 22nd day. A significant protection on lipid peroxidation, glutathione, glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase was observed in the striatum of lesioned group animals pretreated with 10 mg/kg body weight of Bacoside-A for 21 days as compared to lesion group animals. We tested the behavioral response at different time points after injection of 6-hydroxy dopamine to evaluate the onset and progression of behavioral abnormalities. Also quantification of dopamine and its metabolites, DOPAC and HVA was done using HPLC coupled with electrochemical detector. The results showed a reduction in the levels of dopamine and its metabolites, increase in the locomotor activity, increase in "depression"-like behavior and a marked change in the social behavior in the 6 - hydroxy dopamine induced group whereas learning and memory abilities were not affected. Finally, all of these results were exhibited by an increase in the density of TH-IR fibers in the ipsilateral substantia nigra of the lesioned group following treatment with Bacoside-A. This study indicates that Bacoside-A, an active compound from *Bacopa monniera*, is helpful in attenuating the changes caused by 6-hydroxy dopamine induced lesions and has therapeutic potential in fighting against Parkinson's disease.

Keywords: Parkinson's disease, Dopamine, Metabolites, Bacoside-A, Striatum, Neuroprotection.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a preferential loss of dopaminergic neurons of the substantia nigra pars compacta and the loss of their axon terminals in the striatum. Dopaminergic cell loss is associated with the presence of an eosinophilic intraneuronal inclusion, called Lewy bodies. The presence of a Lewy body is not restricted to the central nervous system. They are also observed in the peripheral nervous system of a Parkinson patient (Vanderhaeghen et al., 1970). Access of free radicals is also responsible for neurodegeneration or its oxidation to reactive metabolites (Orth and Schapira, 2002). 6-Hydroxy dopamine (6-OHDA) is used as a parkinsonian model in animals. It caused a loss of dopaminergic neurons by the formation of various oxidants and free radicals. It is widely used to investigate the pathogenesis of PD in rats (Breese and Breese, 1998; Ahmad et al., 2005a, b). The neuropathology of the disease is based on the depigmentation and cell loss in the dopaminergic nigrostriatal tract of the brain (Fearnley and Lees, 1991), with a corresponding decrease in striatal dopamine content.

Exact cause of this disease still remains a mystery despite the known role of oxidative stress, free radical formation (Jenner and Olanow, 1996), genetic susceptibility (Bandmann et al., 1998), programmed cell death (Ziv et al., 1998) and another unknown factor, which might be endogenous or exogenous (Calne and Langston, 1983). Parkinson's disease symptoms originate from the degeneration of the neural connection between the substantia nigra (SN) and the striatum, two brain nuclei essential for normal motor function (Graybiel, 2000; Graybiel et al., 2000). The striatum receives its dopaminergic (DAergic) inputs from neurons of SN (Smith and Bolam, 1990). Striatal dopamine (DA) deficiency, and the resultant changes in motor circuitry, is believed to underlie many of the clinical manifestations of Parkinson's disease (Albin et al., 1989; Crossman, 1989; Greenamyre, 1993). The loss of the DA-mediated control of striatal neuronal activity is therefore generally considered as the functional substrate of the motor symptoms of Parkinson's disease. The striatum is also considered to be the main site of action of the substitutive pharmacological agent levodopa (L-dopa), the action of which is directed towards a restoration of physiological concentrations of DA in this brain area (Papa et al., 1995; Calabresi et al., 2000; Chase and Oh, 2000; Picconi et al., 2002). L-DOPA therapy is still considered the gold standard in the treatment of Parkinson's disease. Hence, L-DOPA is used as the reference drug in the present study. Bacoside-A (Bacoside-A) is a dammarane type triterpenoid saponin isolated from the plant *Bacopa monniera* (Garay et al., 1996), which is held in high repute as a potent nerve tonic (Chopra et al., 1956). The structure of Bacoside-A was initially deduced as 3-(a-L arabinopyranosyl)–O-b-D-glucopyranosid-10, 20-dihydroxy-16-keto-dammarene- (24) mainly by analyzing the acid hydrolysis products of B-A (Chatterji et al., 1965). Preliminary studies with 13C-NMR indicated that Bacoside-A was a mixture of saponins in accordance with an earlier report (Kawai and Shibata, 1978) and not a single chemical entity as had been proposed (Chatterji et al., 1965). HPLC studies on Bacoside-A however showed it to be a mixture of bacoside A3, bacopaside II, jujubogenin isomer of bacopasaponin C and bacopasaponin C (Deepak et al., 2005). Bacoside-A usually co-occurs with Bacoside-B, the latter differing only in optical rotation and probably an isomer of B-A and its structure has not so far been elucidated (Rastogi, 1990). The nootropic activity of the extract has been attributed to the presence of two saponins, namely bacoside A and bacoside B, of which the former is the more important (Singh and Dhawan, 1997). Bacoside A and B themselves have been studied for anti-stress activities (Chowdhuri et al., 2002). Besides, several agronomy related studies on Bacoside-A content in *Bacopa monniera* have been published (Mathur et al., 2001). Bacoside-A also protects the brain from cigarette smoking induced membrane damage (Anbarasi et al., 2006). It also exhibits hepatoprotective (Sumathi et al., 2008) and wound healing (Sharath et al., 2010). Earlier we reported the neuroprotective effects of the alcoholic extract of *Bacopa monniera* on 6-OHDA lesioning in rats (Shobana et al., 2012).

On the basis of our earlier findings (Shobana et al., 2012), we isolated Bacoside-A in *Bacopa monniera* extract in our lab and checked its efficacy against 6-hydroxy dopamine induced lesioning and found that Bacoside-A could be used in preventing 6- hydroxy dopamine induced changes. Our results have shown that Bacoside-A effectively and significantly protected the brain from 6-OHDA toxicity in terms of behavioral, biochemical and neurochemical studies.

MATERIALS AND METHODS

Chemicals

6-hydroxy dopamine (6-OHDA), 3, 4-dihydroxy phenyl acetic acid (DOPAC), Homovannilic acid (HVA) were purchased from Sigma Aldrich. All other chemicals used were of analytical grade.

Animals and treatments

Male Albino rats, 5 months old, weighing 250 – 300g were obtained from Central Animal House, Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai - 113, Tamil Nadu, India. Rats were housed separately in polypropylene cages and fed standard pellet diet, kept under hygienic conditions. Rats were kept on a 12hr light and dark cycles with free access to water *ad libitum*. All experiments and protocols described in the present study (IEAC No. 02/07/2011) were approved by the Institutional review committee for the use of human or animal subjects (Institutional Animal Ethics Committee (IEAC) of Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai - 113, Tamil Nadu, India). Rats were divided into six experimental groups of 6 animals each. Group I: Vehicle treated, sham operated control received 2µl of vehicle (0.1% ascorbic acid-saline) intracranially (S).

Group II: Vehicle treated, lesioned with 6 hydroxy dopamine on 22nd day (L). Group III: Rats pretreated with Bacoside-A (10mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC1+L). Group IV: Rats pretreated with Bacoside-A (20mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC2+L). Group V: Rats pretreated with L-DOPA (10mg/kg) i.p. for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (L-DOPA+L). Group VI: Rats administered with Bacoside-A (20mg/kg) alone orally for 21 days, sham – operated (BAC2+S).

Isolation of Bacoside-A (Bacoside-A)

The plant material *Bacopa monniera* (Linn) was collected at Chennai, Tamil Nadu and was authenticated by Dr. A. Sasikala, Captain Srinivasa Murti Drug Research institute for Ayurveda, Arumbakkam, Chennai, Tamil Nadu. The dammarene type triterpenoid saponin Bacoside-A was isolated from the plant by the procedure carried out by Deepak *et al* (2005). The activity-guided isolation of Bacoside A was carried out using the brine shrimp lethality assay to direct the fractionation of a methanolic proportion of methanol. The fractions eluted with chloroform:methanol 85:15 were combined and concentrated under vacuum (7 g). This fraction was subjected to flash chromatography on silica gel (100–200 mesh) and the fractions eluted with chloroform:methanol 85:15 were combined, concentrated under vacuum and crystallised from 70% methanol in water to obtain Bacoside A (2.5 g). Aqueous suspension of Bacoside-A was given orally to the animals at a dosage of 10 mg/kg, b.w/day (Anbarasi et al., 2006) and 20 mg/kg, b.w/day. HPLC-Finger Print analysis of *Bacopa monniera* was performed using the conditions given in Table 1.

Table 1: HPLC Finger print analysis of *Bacopa monniera*

HPLC system	Shimadzu HT2010 Chromatographic system with in combination with Class LC 10A software & UV detection		
Column	RP C-18 Luna phenomenex (250 x 4.6 mm)		
Column oven temperature	25^{O}C		
Mobile phase	A - 0.25% Orthophosphoric acid in water		
	B - Acetonitrile		
Flow rate	1.5 ml		
Injection volume	25.0µl		
Gradient	Time	Concentration of A	Concentration of B
	0	75	25
	25	60	40
	35	40	60
	38	75	25
	45	75	25
Detection wavelength	205 nm		
Run Time	45 min		
Sample preparation	Weigh accurately 500mg Extract to a 100ml volumetric flask dissolve in 50ml methanol, sonicate for 10- 15 min. Cool then make up to 100ml with methanol. Filter through 0.45 microns membrane filter paper.		

Lesioning

After 3 weeks of treatment with Bacoside-A, all animals in experimental and sham-operated groups were anaesthetized with ketamine and xylazine intraperitoneally (i.p.). Each animal was mounted on a stereotaxic stand (Instruments and Chemicals, Ambala, New Delhi), the skin overlying the skull was cut to expose it, and the coordinates for the striatum (Paxinos and Watson, 1982) were measured accurately (antero-posterior 0.5mm, lateral 2.5mm, dorso-ventral 4.5mm relative to bregma and ventral from dura) with the tooth bar set at 0mm.

Thereafter, all animals in experimental groups were lesioned by injecting 12µg 6-OHDA/2µl in 0.1% ascorbic acid-saline into the right striatum, while the sham-operated group received 2.0µl of the vehicle. The injections were made manually, with the help of a Hamilton syringe, through the burr holes made on the skull surface in both groups. The injection rate was 1.0µl/min, and the needle was kept in place for an additional 1min before being slowly retracted. The experiments were performed in accordance with the guidelines of Institutional Animal Ethics Committee (IEAC) of Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai - 113, Tamil Nadu, India.

Post-operative care

Recovery of anesthesia took approximately 4–5h. The rats were kept in a well-ventilated room at 25±3°C in individual cages until they gained full consciousness; they were then housed together in groups of 4 animals per cage. Food and water was kept inside the cages for the first week, allowing animals' easy access, without physical trauma due to overhead injury. Animals were then treated normally; food, water, and the bedding of the cages were changed twice per week, as usual.

Behavioral studies

All the behavioral studies were performed at room temperature in a calm room without any outside interference. All the experiments were performed between 10.00 am and 6.00 pm.

Narrow Beam Maze

After 21 days of lesioning, the animals were tested for the balance and motor coordination on a narrow beam maze (NBM). This has a smooth wooden narrow beam of 105 cm long, 4 cm in width and thickness of 3 cm. The beam was elevated from the ground by 1m with additional supports. It has a start platform of 20 cm in dimension from the start of the beam and an end platform of 20 cm dimension at the end of 105 cm on the NBM. There was food on the end platform for the reward of the animals. The animals were trained on NBM for 10 trials per day with a 1 min interval. The rats were allowed to explore the NBM for 10 trials with a 1 min interval on 22 days of lesioning. The journey time between start and end goal was measured (Allbutt and Henderson, 2007).

Morriz Water Maze

The animals were tested on a morriz water maze test 8 weeks after the 6-OHDA injection. The test was preceded by 2 days of habituation to the apparatus filled with water (e.g., the experimental subject was placed in the water and let swim for 2min), to decrease stress associated with novelty that reportedly affects learning and memory performance (McGaugh and Roozendaal, 2002).

At each time point, two different water maze protocols were exploited to investigate spatial and procedural learning and memory. The water maze apparatus consisted of a black plexiglass circular pool 140cm in diameter and 33cm in height was placed in the middle of the experimental room. The pool was filled with water kept at a temperature of 26°C ± 1°C. A transparent plastic platform (15cm in diameter) was placed 1.5cm below the water surface and 15cm from the edge of the pool. The platform was not visible to the experimental subjects.

For both spatial and procedural protocols, the entire procedure consisted of five trials (four acquisition plus one probe trial) carried out on a single day. To avoid visual orientation prior to release, rats were transferred from their cage into the pool in a non transparent plastic cup, from which they glide into the water facing the pool wall. Animals that did not find the platform were trained in locating the platform for 10sec at the end of the trial.

In the spatial version of the task, the platform position remained fixed for the first four trials. This version of the task requires rats to learn the position of the platform on the basis of spatial location. To allow spatial learning, the walls surrounding the pool were provided with visual cues. During the last trial of the day (probe trial), the platform was removed from the pool and each rat was tested for a 60 sec period.

In the procedural version of the task, a visual cue was always located 20cm above the water level and the platform position was changed in each trial. Therefore, this task could be acquired by learning an approach response to the visual cue. During the last trial of the day (probe trial), the platform was removed from the pool and each rat was tested for a 60 sec period, and the visual cue was placed in a new position.

Platform finding was defined as staying on the platform for atleast 3sec. Once the platform has been found, the rat was given the opportunity to climb on a wire-mesh grid and placed back in the cage kept in a warm environment. Intertrial duration was about 20min. During the test, the experimenter was in a position not to be viewed by the experimental subjects.

The swim path of the rats was recorded by means of a video. The variables recorded were length of swim path (total distance moved), latency to reach the platform (escape latency), mean swimming speed and relative turn angle. For the probe phase, the variables recorded were time spent in each quadrant, number of platform crossing, mean swimming speed and relative turn angle.
The procedure was conducted on 2 subsequent days (five trials per day). On day 1, half of the subjects were tested in the spatial version of the task, and the other half performed the test in the procedural task. On day 2, the order was reversed.

Social Interaction Test

After 7 weeks from 6-OHDA injection, the animals underwent a social interaction test (Alleva, 1993; File and Seth, 2003; Branchi et al., 2006a) consisting of rats being placed for 30min in a clean Plexi glass box (42 x 17 x 14 cm), of a size different from the home cage, with an unfamiliar conspecific of the same weight, sex, and age. All tests were carried out between 10.00am and 4.00 pm i.e., during the white light period, and animals were transferred to the experimental room at least 30 min before the test in order to let them acclimatize to the test environment. During the social interaction, behavior was video recorded using a digital video camera. The behavioral categories and elements were as follows.
Social activity: Investigate elements: social sniff (sniffing the body or other specific area of the body of the partner), follow (moves directly behind other animal), mutual circle (partners are mutually sniffing each other's anogenital region, while describing tight circles with their reciprocal following movements); Affliative elements: social rest (while the animals show immobility their body are in contact), social investigation (the experimental animal goes into contact with explores the partner), allogrooming (grooming the partner); play soliciting: push under (head and forepart of body pushed beneath other animal), push past (animals come into close lateral contact when moving in opposite directions), rough and tumble play (the two rats exhibit sudden darting movements, which are associate with pouncing on the partner back, chasing, wrestling and pinning). The play bout is often anticipated by a prolonged and intense bout of allogrooming, often accompanied by gross movements of the whole body of the performer and by vigorous pulling of the fur of the partner. The bout is considered concluded when both participants either turn their attention away from each other or shift to amicable nonplayful interaction.
Non-social activity: Activity and exploration/escape behavior: locomotion (distance moved), jump (jumps up vertically to the wall, often repeatedly, with the snout directed upward), dig (animal digs the sawdust with the forelimbs, often kicking it away with the hindlimbs), shaking behavior (a rapid rhythmic shaking of the head in a radial motion); Maintenance activity: self grooming (animal licks and mouths its own fur, sometimes helping itself with its forepaws).
Aggressive behavior. Attack (fighting episode), aggressive grooming (violent grooming of the animal on the back of the partner), offensive upright posture (the animal stands on its hindlimbs facing the opponent aggressively), defensive upright posture (the animal stands on its hindlimbs and pushes the aggressive opponent with its forepaws), crouched posture (the animal lies on its ventrum, the head flat on the cage floor. This posture is often observed as an answer to aggressive grooming performed by the partner), submissive upright posture (the animal stands on its hindlimbs, the head pulled far back and its body rigid), freezing (animal reacts to the physical movements of the partner by remaining motionless), flee (animal moves very rapidly away from the partner).

Sucrose Preference Test

Animals received a sucrose preference test 7 weeks after the 6-OHDA injection. The test consisted of training the animals to drink a 2% sucrose solution, by exposing them to the solution in place of water for 48hrs. Immediately afterward, the animals were deprived of any liquid to drink for 16hr and then were presented simultaneously two bottles, the one containing the sucrose solution (2%), and the other water. During the test, both bottles were removed, weighed and replaced (with the position of two bottles reversed) each 30min for 3 times. At the end of the test, the animals were returned to their home cages. Reduced amount of sucrose solution drunk is suggestive of anedonia and thus of depression like behavior.

Biochemical Studies

Tissue preparation for antioxidant enzymes and GSH assays
After 6 weeks, the animals were sacrificed and their brains were removed quickly for harvesting striatum and substantia nigra by cutting coronal sections of 1.0-mm thickness, using a rat brain matrix according to the rat brain atlas (Paxinos and Watson, 1982). For enzymatic assays, striatum was homogenized (10% w/v) in 0.01M phosphate buffer (pH 7.0) and centrifuged at 10,500×g for 20min at 4°C to obtain postmitochondrial supernatant (PMS), while homogenized substantia nigra 10% w/v was used for the estimation of TBARS and GSH.

Assay for thiobarbituric acid reactive substance, a marker of lipid peroxidation

The method of Utley et al. (1967) was modified for the estimation of lipid peroxidation. Briefly, 0.2ml homogenate was pipetted in Eppendorf tube and incubated at 37±1°C in a metabolic water bath shaker for 60 min at 120 strokes up and down; another 0.2ml was pipetted in an Eppendorf tube and placed at 0°C incubation. After 1h of incubation, 0.4ml of 5% TCA and 0.4 ml of 0.67% TBA was added in both samples (i.e., 0°C and 37°C). The reaction mixture from the vial was transferred to the tube and centrifuged at 3500×g for 15 min. The supernatant was transferred to another tube and placed in a boiling water bath for 10 min. Thereafter, the test tubes were cooled and the absorbance of the color was read at 535nm. The rate of lipid peroxidation expressed as nmol of thiobarbituric acid reactive substance formed/min/mg protein.

Assay for reduced glutathione content

Reduced GSH was determined by the method of Moron et al. (1979). 0.2ml of homogenate was precipitated with 0.2ml of sulfosalicylic acid (4%). The sample was kept at 4°C for atleast 1h and then subjected to centrifugation at 1200×g for 15min at 4°C. The assay mixture contained 0.1ml of filtered aliquot, 1.7ml phosphate buffer (0.1M, pH 7.4), and 0.2ml DTNB (4mg/1ml of phosphate buffer, 0.1M, pH 7.4) in a total volume of 2.0ml. The yellow color developed and was read immediately at 412nm. The results are expressed as nmol GSH formed/g tissue.

Determination of glutathione-S-transferase activity

Glutathione-S-transferase (GST) activity was measured by the method of Habig et al. (1974). The reaction mixture consisted of 0.1M phosphate buffer (pH 6.5), 1.0mM reduced GSH, 1.0mM CDNB, and 0.1ml PMS in a final volume of 2.0ml. The changes in absorbance were recorded at 340nm, and the enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein.

Determination of glutathione reductase activity

GR activity was assayed by the method of Carlberg and Mannervik (1985). The assay system consisted of 0.1M phosphate buffer (pH 7.6),0.1mM NADPH, 0.5mM EDTA, 1.0mM GSSG, and 0.1ml PMS in a total volume of 2.0ml. The enzyme activity was quantitated at room temperature by measuring the disappearance of NADPH at 340nm and was calculated as nmol NADPH oxidized/min/mg protein.

Determination of glutathione peroxidase activity

GPx activity was measured according to the procedure of Mohandas et al. (1984). The reaction mixture consisted of 0.05M phosphate buffer (pH 7.0), 1.0mM EDTA, 1.0mM sodium azide, 1.4U of 0.1ml GR, 1.0mM GSH, 0.2mM NADPH, 0.25mM H_2O_2, and 0.1ml of PMS in a final volume of 2.0ml. The disappearance of NADPH at 340nm was recorded at room temperature. The enzyme activity was calculated as nmol NADPH oxidized/min/mg protein.

Determination of superoxide dismutase activity

Superoxide dismutase (EC 1.15.1.1) activity was measured according to the method described by Marklund and Marklund (1974), with some minor modifications. This method is based on the ability of SOD to inhibit the auto oxidation of pyrogallol at alkaline pH (8.2). Enzyme activity was expressed as units/mg protein. One unit is equivalent to the amount of SOD required to inhibit 50% of pyrogallol autooxidation.

Determination of catalase activity

Catalase activity (CAT) was assayed by the method of Aebi (1984). Briefly, the assay mixture consisted of 0.05M phosphate buffer (pH 7.0), 0.019M H_2O_2, and 0.05ml PMS in a total volume of 3.0ml. Changes in absorbance were recorded at 240nm. Catalase activity was calculated in terms of nmol H_2O_2 consumed/min/mg protein.

Markers for Parkinsonism

Quantification of dopamine and its metabolites

The method of DeVito and Wagner (1989) as described by Zafar et al., 2003a, b was used for the estimation of dopamine and its metabolites, DOPAC and HVA. The striatum (20% w/v) was sonicated in 0.4N perchloric acid containing 100ng/ml of the internal standard, 3, 4- dihydroxy benzylamine, followed by centrifugation at 15,000×g for 10min at 4°C and the filtrate was injected manually through a 20-µl loop over the ODS-C18 column coupled with HPLC/Electrochemical detector for separation and quantification. The mobile phase consisted of 0.1M potassium phosphate (pH 4.0), 10% methanol, and 1.0mM heptane sulphonic acid. Samples were separated on ODS-C18 column using a flow rate of 1.0ml/min. The concentrations of dopamine and its metabolites were calculated using a standard curve generated by determining the ratio between three known amounts of the amine or its metabolites and a constant amount of internal standard, and they are represented as ng/mg of tissue.

Tyrosine hydroxylase immunohistochemistry

Tyrosine hydroxylase immunohistochemistry was performed according to the method of Ahmad et al. (2005a). The animals were deeply anaesthetized by sodium pentobarbitone (35.0mg/kg) and perfused transcardially through ascending aorta with 100.0ml of 0.1M phosphate buffer saline (PBS) at pH 7.5 followed by 300.0ml of 4% paraformaldehyde in 0.1M phosphate buffer. Brains were immediately removed, and tissue blocks, including the striata, were dissected out and further immersed in the same fixative for an additional 24h at 4°C. Furthermore, the tissues were preserved in 10%, 20%, and 30% sucrose solution (in phosphate buffer) until they sank. The tissues were then kept in final sucrose solution until sectioning. The fixed tissues were embedded in OCT compound (polyvinyl glycol, polyvinyl alcohol, and water) and frozen at −20°C. Coronal sections of 25-µm thickness were cut on a freezing cryostat (Leica), collected in PBS, and stored at 4°C. The sections were then transferred to gelatin-coated slides and immersed in wash buffer (sodium phosphate 100mM, sodium chloride 0.5M, Triton X-100, sodium azide) at pH 7.4 for 20min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and 10% methanol in PBS and incubated for half an hour at room temperature. Thereafter, the slides were washed with PBS, and the sections were overlaid with 20µl of anti-tyrosine hydroxylase antibodies (2% in PBS) and incubated for 2h in a humid chamber at room temperature. The slides were washed again to remove the unbound antibodies and incubated with a 20-µl solution of biotinylated anti-mouse IgG (2% in PBS) for 3h at 4°C in the humid chamber. Then the slides were exposed to streptavidin-peroxidase and the labeled sites were visualized with a solution of diaminobenzidine and hydrogen peroxide. Finally the sections were dehydrated, cover-slipped, and viewed under microscope, and photomicrographs were
taken.

Image analysis of tyrosine hydroxylase immunohistochemistry

The density of tyrosine hydroxylase (TH)-immunoreactive (IR) fibers in the striatum was determined using a computerized image analysis system as described by Chaturvedi et al., 2006. The unbiased stereological method was employed, where a person unknown to the experimental design performed the image analysis. Computerized analysis enabled the assessment of the percent area of a selected field that was occupied by TH-IR fibers. This area was expressed as µm2 per total field view (250µm×250µm; 75,000µm^2). The density of TH-IR fibers was measured in the striatum in all groups in the ipsilateral, as well as contralateral, side. Analyzed values obtained in the ipsilateral side are expressed as a percentage of those on the intact contralateral side.

Statistical analysis

Data represents mean ± S.D. Statistical comparisons were performed by one way analysis of variance (ANOVA) followed by student't' test using SPSS 10 version. If ANOVA analysis indicated significant differences, Tukey's post-hoc test was performed to compare mean values between treatment groups and control. A value of $p<0.01$ was considered as statistically significant.

RESULTS

HPLC – chromatogram of Bacopa monniera extract

HPLC – chromatogram of *Bacopa monniera* extract, showing *Bacosides* and the other flavonoids (Luteolin and apigenin) is given in Fig 1.

Behavioral Study

All the behavioral studies were performed at room temperature in a calm room without any outside interference. All the experiments were performed between 10.00 am and 6.00 pm.

Effect of Bacoside-A on narrow beam maze

The narrow beam maze was performed to test the balance and working efficiency of 6-OHDA induced rats and its protection with Bacoside-A (Fig 2). The crossing time on narrow beam was increased significantly in 6-OHDA induced group (L) as compared to control (S) rats. L-DOPA treated group significantly improved the locomotor time ability. The Bacoside-A pretreated and lesioned group (BAC1+L) has significantly ($p<0.01$) improved the locomotor time ability than BAC2+L ($p<0.05$) group as compared to L group. BAC1+L pretreated group was found to be similar to that of the L-DOPA pretreated group. No significant changes were observed in the Bacoside-A alone treated rats.

 Coden : IJABPT ISSN : 0976-4550

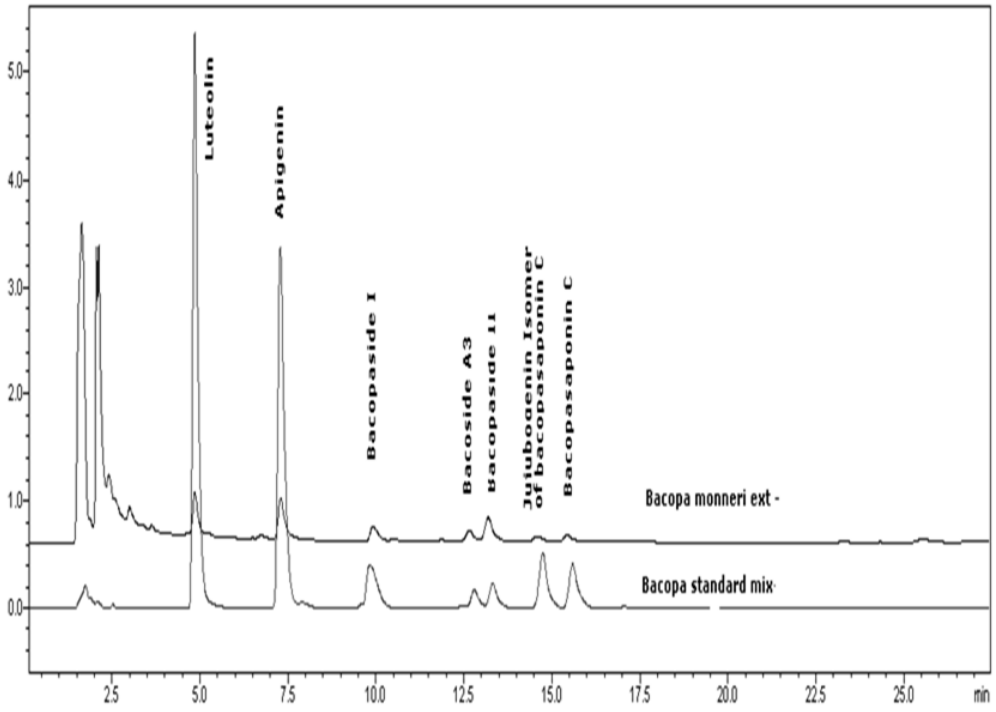

Fig. 1. HPLC – chromatogram of *Bacopa monniera* extract

HPLC – chromatogram of *Bacopa monniera* extract, showing Bacosides and the other flavonoids (Luteolin and apigenin).

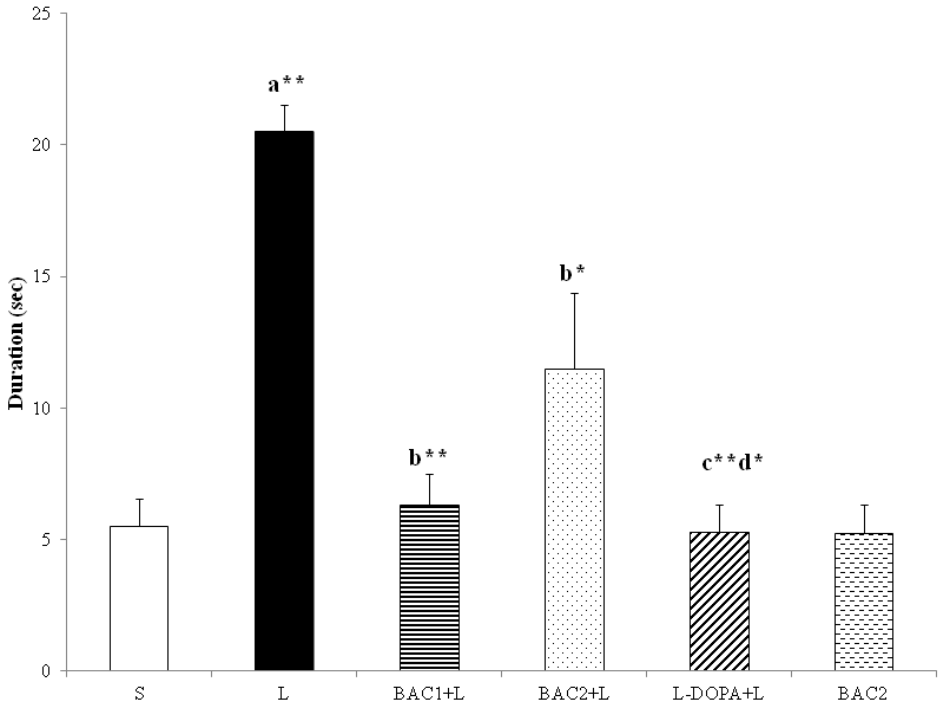

Fig. 2. Effect of Bacoside-A on narrow beam maze in rats induced with 6-OHDA.

Data represents mean ± S.D (n = 6 in each group). Group I: Vehicle treated, sham operated control received 2µl of vehicle (0.1% ascorbic acid-saline) intracranially (S). Group II: Vehicle treated, lesioned with 6 hydroxy dopamine on 22nd day (L). Group III: Rats pretreated with Bacoside-A (10mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC1+L). Group IV: Rats pretreated with Bacoside-A (20mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC2+L). Group V: Rats pretreated with L-DOPA (10mg/kg) i.p. for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (L-DOPA+L). Group VI: Rats administered with Bacoside-A (20mg/kg) alone orally for 21 days, sham – operated (BAC2+S).** $p<0.01$; *$p<0.05$; L group compared with S (a); BAC1+L, BAC2+L compared with L (b); L-DOPA+L compared with BAC1+L (c) and BAC2+L (d) by one way ANOVA with Tukey's post hoc test.

Effect of Bacoside-A on Morriz water maze

The morriz water maze was performed using two different protocols to investigate both spatial and procedural learning and memory abilities. However, no difference between 6-OHDA and sham-operated animals was found at any point (data not shown) suggesting that behavioral competences required to accomplish these tasks successfully are not affected by the lesioning protocol used in the present work. All experimental subjects improved their learning performances 4 weeks after 6-OHDA injection, decreasing trial after trial the latency to find the platform during either the spatial or procedural version. In both spatial and procedural probes, experimental subjects showed a clear quadrant preference spending more time in the quadrant where the platform was located compared with the opposite quadrant.

3.5. Social Interaction Test

After 4 weeks of lesioning, all the experimental subjects were tested for social interaction activity. 6-OHDA lesioned groups showed less offensive behavior (Fig 3), increased propensity to interact socially (Fig 4) and decreased aggressive grooming (Fig 5) when compared with control rats. However, L-DOPA treatment showed a better improvement and Bacoside-A pretreatment showed a dose dependent improvement in all the above social interaction tests and was found to be similar to that of control group. BAC1+L group showed a significant ($p<0.01$) improvement as that of L-DOPA treatment. Bacoside-A alone treated rats did not show any such changes in their behavior.

Sucrose Preference Test

Unilateral 6-OHDA lesioning in the striatum affected the amount of sucrose solution drunk by rats (Fig 6). In particular, the lesioned group drank less amount of sucrose solution when compared with control rats. Rats pretreated with Bacoside-A drank more amount of sucrose solution when compared with that of L group. BAC1+L group showed a significant ($p<0.01$) improvement as that of L-DOPA treatment. Bacoside-A alone treated group showed no significant changes and resembled control rats.

Biochemical Studies

Effect of Bacoside - A on the contents of TBARS and glutathione (GSH)

The content of TBARS in substantia nigra was elevated significantly ($p < 0.01$) in the L group as compared to the S group (Fig.7). The increased TBARS level was significantly and dose-dependently restored in the BAC1+L and BAC2+L groups as compared to the L group. No significant change was observed in the S + BAC2 group as compared to the S group. On the other hand, the content of GSH in substantia nigra was depleted significantly ($p < 0.01$) in the L group as compared to the S group and its depleted level was restored significantly and dose-dependently in the BAC1+L and BAC2+L groups as compared to the L group. BAC1+L group showed a significant ($p<0.01$) improvement as that of L-DOPA treatment. No significant change was observed in the S+BAC2 group as compared to the S group.

Effect of Bacoside- A on the activity of antioxidant enzymes (GPx, GR, GST, CAT, SOD)

Figure 8 shows the activities of GST, GPx, and GR in striatum. The activity of all the three enzymes were significantly decreased ($p<0.01$) in the L group, as compared to the S group. Their activities were significantly increased in a dose-dependent manner in the BAC1+L ($p<0.01$) and BAC2+L ($p<0.05$) groups, as compared to the L group. BAC1+L group showed a significant ($p<0.01$) improvement as that of L-DOPA treatment. No significant change was observed in drug sham groups.

Fig. 9 shows the effect of BACOSIDE-A on the activities of SOD and CAT in the striatum. The activity of SOD and CAT was found to be significantly reduced ($p<0.01$) in the L group, as compared to the S group. The decrease in SOD activity was significantly and dose-dependently brought back to normalcy in the BAC1+L ($p<0.01$) and BAC2+L ($p<0.05$) groups, as compared to the L group. BAC1+L group showed a significant ($p<0.01$) improvement as that of L-DOPA treatment. No significant change was observed in drug sham groups.

3.8. Modulatory effect of Bacoside-A on the content of DA, DOPAC and HVA in the striatum of rat brain

Fig 10, 11 and 12 shows that the content of DA, DOPAC and HVA in the striatum of rat brain. All these levels were significantly ($p<0.01$) decreased in the L group when compared with the control group (S). However treatment with Bacoside-A significantly increased the level of these contents BAC1+L ($p<0.01$), BAC2+L ($p<0.05$) in the striatum of rat brain when compared with L group. BAC1+L group showed a significant ($p<0.01$) improvement as that of L-DOPA treatment. No significant changes observed in Bacoside-A alone administered groups.

Effect of Parkinsonism on the immunohistochemical analysis and protection by Bac-A

Fig 13A shows the normal TH staining. Immunohistochemical analysis of the ipsilateral striata of L group revealed that there was depletion in TH staining when compared to control group (Fig 13B). This depletion in the striata was significantly restored by treatment with Bac-A at a dose of 10mg/kg b.w (Fig 13C) than 20mg/kg b.w. (Fig 13D). There was also a significant decrease in the TH-IR fibres in the L group which was found to be increased after Bac-A pretreatments. The number of TH-IR neurons in the ipsilateral side was analyzed as a percentage of neurons in the intact contralateral side. The percentage of TH-immunopositive neurons was decreased in L group when compared to control groups (Figure 14). BAC1+A administration protected the loss of the number of TH-immunopositive neurons by 6-OHDA lesioning compared to the 6-OHDA-lesioned rats. No significant difference was observed in the groups administered with BAC2 alone as compared to sham groups.

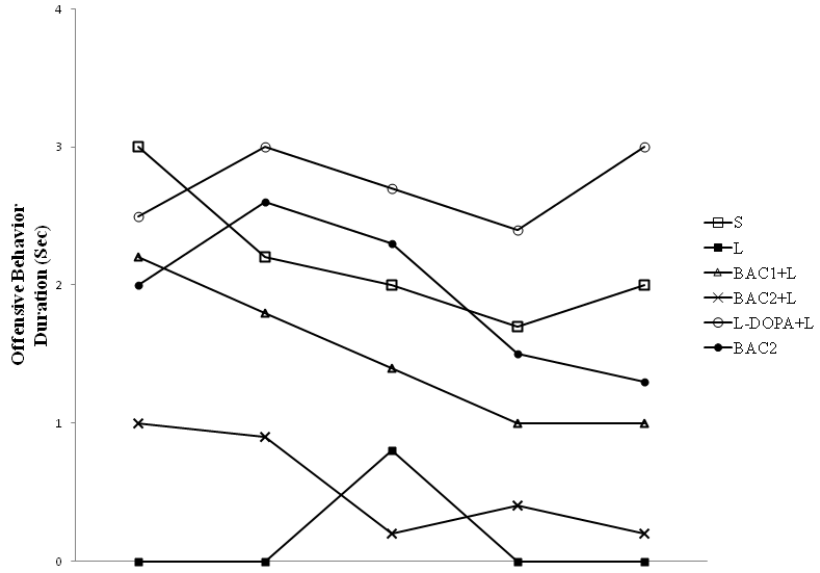

Fig. 3. Effect of Bacoside-A on offensive behavior in rats induced with 6-OHDA.

Data represents mean ± S.D (n = 6 in each group). Group I: Vehicle treated, sham operated control received 2µl of vehicle (0.1% ascorbic acid-saline) intracranially (S). Group II: Vehicle treated, lesioned with 6 hydroxy dopamine on 22nd day (L). Group III: Rats pretreated with Bacoside-A (10mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC1+L). Group IV: Rats pretreated with Bacoside-A (20mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC2+L). Group V: Rats pretreated with L-DOPA (10mg/kg) i.p. for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (L-DOPA+L). Group VI: Rats administered with Bacoside-A (20mg/kg) alone orally for 21 days, sham – operated (BAC2+S).** $p<0.01$; *$p<0.05$; L group compared with S (a); BAC1+L, BAC2+L compared with L (b); L-DOPA+L compared with BAC1+L (c) and BAC2+L (d) by one way ANOVA with Tukey's post hoc test.

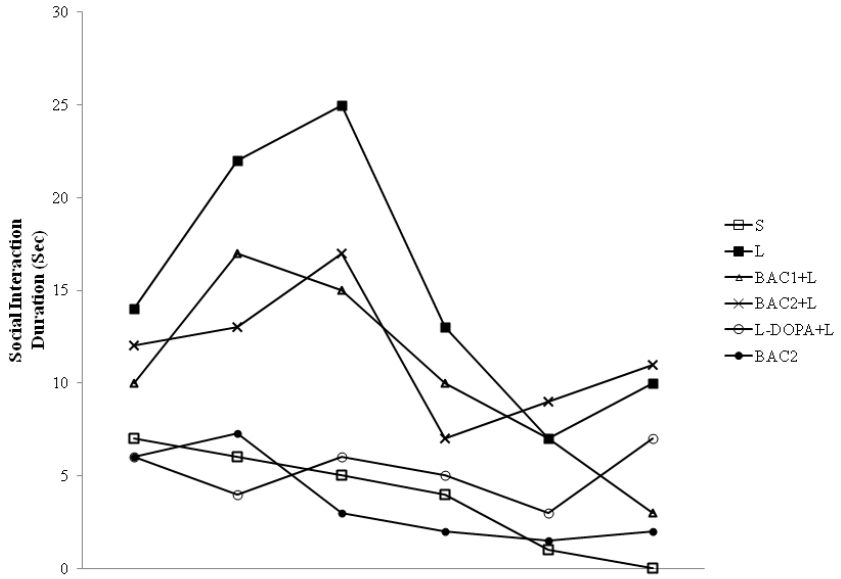

Fig. 4. Effect of Bacoside-A on social interaction in rats induced with 6-OHDA.

Data represents mean ± S.D (n = 6 in each group). Group I: Vehicle treated, sham operated control received 2µl of vehicle (0.1% ascorbic acid-saline) intracranially (S). Group II: Vehicle treated, lesioned with 6 hydroxy dopamine on 22nd day (L). Group III: Rats pretreated with Bacoside-A (10mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC1+L). Group IV: Rats pretreated with Bacoside-A (20mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC2+L). Group V: Rats pretreated with L-DOPA (10mg/kg) i.p. for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (L-DOPA+L). Group VI: Rats administered with Bacoside-A (20mg/kg) alone orally for 21 days, sham – operated (BAC2+S).** $p<0.01$; *$p<0.05$; L group compared with S (a); BAC1+L, BAC2+L compared with L (b); L-DOPA+L compared with BAC1+L (c) and BAC2+L (d) by one way ANOVA with Tukey's post hoc test.

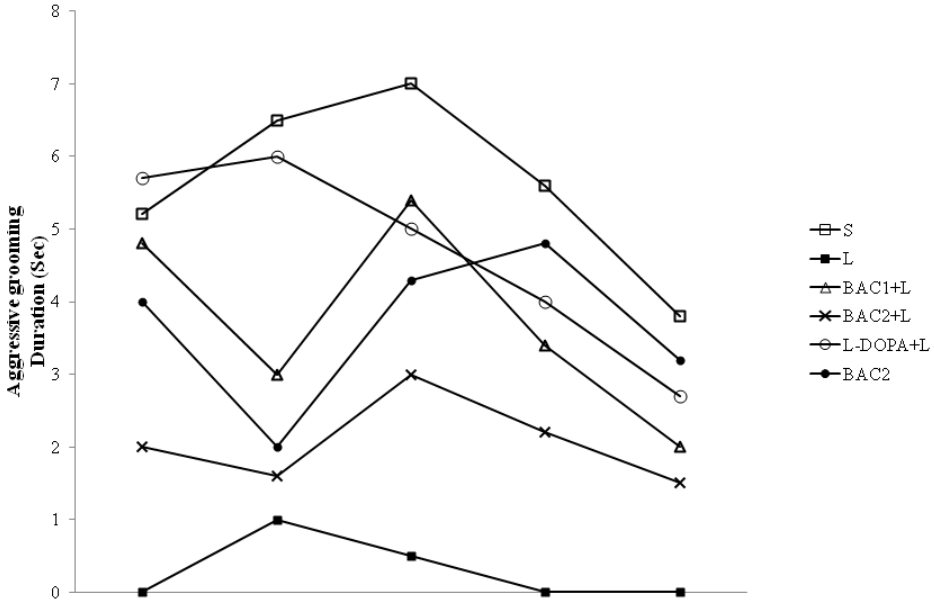

Fig. 5. Effect of Bacoside-A on aggressive grooming in rats induced with 6-OHDA.

Data represents mean ± S.D (n = 6 in each group). Group I: Vehicle treated, sham operated control received 2µl of vehicle (0.1% ascorbic acid-saline) intracranially (S). Group II: Vehicle treated, lesioned with 6 hydroxy dopamine on 22nd day (L). Group III: Rats pretreated with Bacoside-A (10mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC1+L). Group IV: Rats pretreated with Bacoside-A (20mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC2+L). Group V: Rats pretreated with L-DOPA (10mg/kg) i.p. for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (L-DOPA+L). Group VI: Rats administered with Bacoside-A (20mg/kg) alone orally for 21 days, sham – operated (BAC2+S).** p<0.01; *p<0.05; L group compared with S (a); BAC1+L, BAC2+L compared with L (b); L-DOPA+L compared with BAC1+L (c) and BAC2+L (d) by one way ANOVA with Tukey's post hoc test.

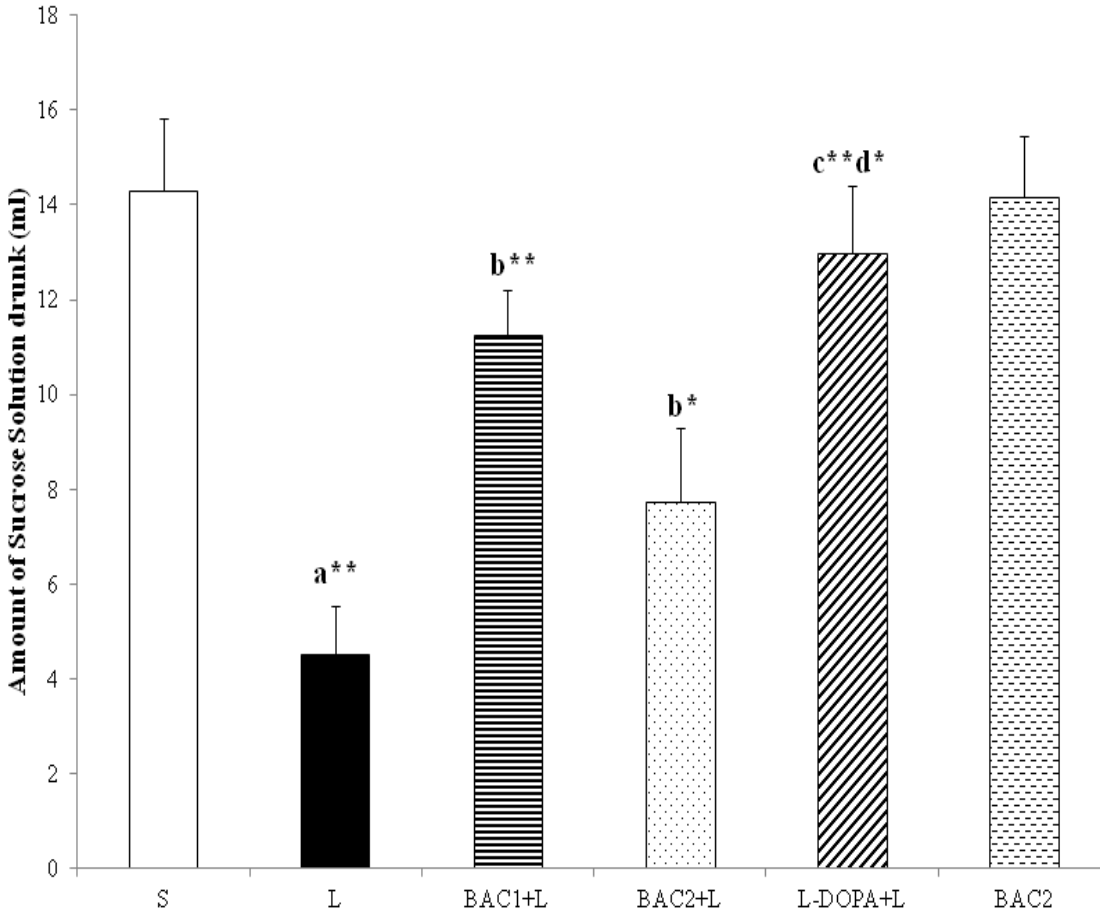

Fig. 6. Effect of Bacoside-A on sucrose preference test in rats induced with 6-OHDA.

Data represents mean ± S.D (n = 6 in each group). Group I: Vehicle treated, sham operated control received 2µl of vehicle (0.1% ascorbic acid-saline) intracranially (S). Group II: Vehicle treated, lesioned with 6 hydroxy dopamine on 22nd day (L). Group III: Rats pretreated with Bacoside-A (10mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC1+L). Group IV: Rats pretreated with Bacoside-A (20mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC2+L). Group V: Rats pretreated with L-DOPA (10mg/kg) i.p. for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (L-DOPA+L). Group VI: Rats administered with Bacoside-A (20mg/kg) alone orally for 21 days, sham – operated (BAC2+S).** p<0.01; *p<0.05; L group compared with S (a); BAC1+L, BAC2+L compared with L (b); L-DOPA+L compared with BAC1+L (c) and BAC2+L (d) by one way ANOVA with Tukey's post hoc test.

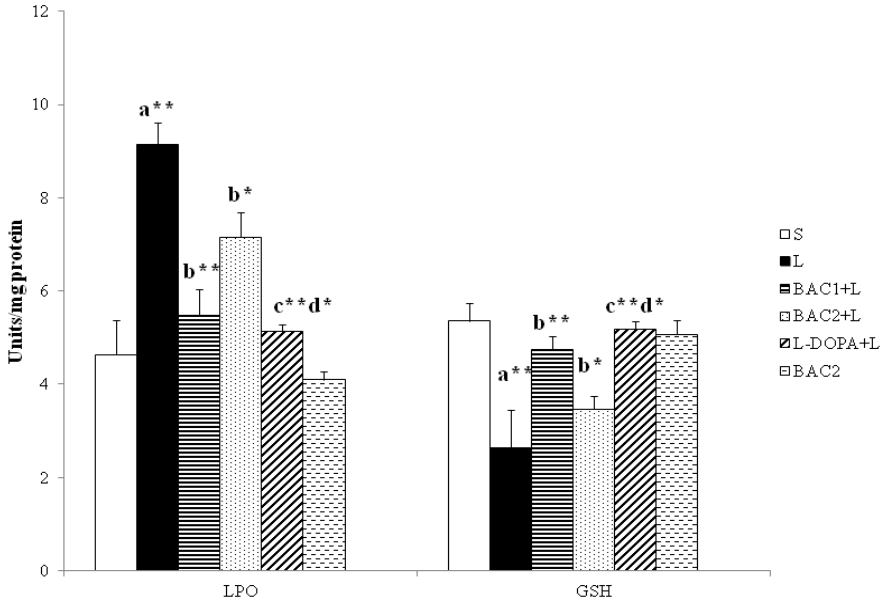

Fig.7. Protective effect of Bacoside-A against 6-OHDA induced TBARS and GSH levels in substantia nigra of rats. Data represents mean ± SD of 6 rats in each group. TBARS units expressed as µmoles of TBARS/mg protein. GSH units expressed as µM/mg protein. Group I: Vehicle treated, sham operated control received 2µl of vehicle (0.1% ascorbic acid-saline) intracranially (S). Group II: Vehicle treated, lesioned with 6 hydroxy dopamine on 22nd day (L). Group III: Rats pretreated with Bacoside-A (10mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC1+L). Group IV: Rats pretreated with Bacoside-A (20mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC2+L). Group V: Rats pretreated with L-DOPA (10mg/kg) i.p. for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (L-DOPA+L). Group VI: Rats administered with Bacoside-A (20mg/kg) alone orally for 21 days, sham – operated (BAC2+S).** $p<0.01$; *$p<0.05$; L group compared with S (a); BAC1+L, BAC2+L compared with L (b); L-DOPA+L compared with BAC1+L (c) and BAC2+L (d) by one way ANOVA with Tukey's post hoc test.TBARS – Thiobarbituric acid reactive substances; GSH –glutathione.

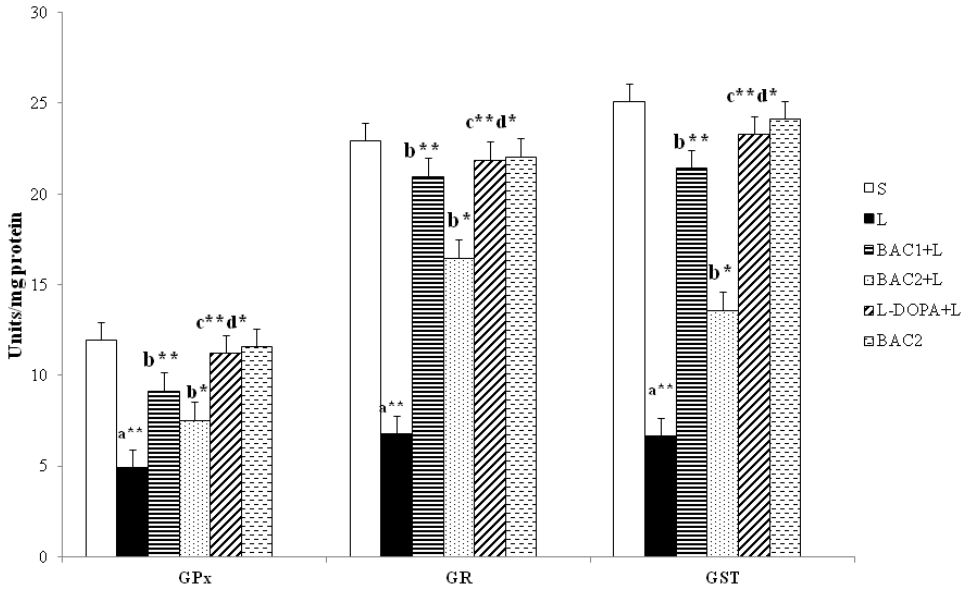

Fig. 8. Restoration of the activities of GPx, GR and GST in striatum by Bacoside-A among 6-OHDA administered rats.

Data represents mean ± SD of 6 rats in each group. GPx units expressed as µg/min/mg protein. GR units expressed as nmol NADPH oxidized/min/mg protein.GST units expressed as nmol CDNB conjugate formed/min/mg protein. Group I: Vehicle treated, sham operated control received 2µl of vehicle (0.1% ascorbic acid-saline) intracranially (S). Group II: Vehicle treated, lesioned with 6 hydroxy dopamine on 22nd day (L). Group III: Rats pretreated with Bacoside-A (10mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC1+L). Group IV: Rats pretreated with Bacoside-A (20mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC2+L). Group V: Rats pretreated with L-DOPA (10mg/kg) i.p. for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (L-DOPA+L). Group VI: Rats administered with Bacoside-A (20mg/kg) alone orally for 21 days, sham – operated (BAC2+S).** p<0.01; *p<0.05; L group compared with S (a); BAC1+L, BAC2+L compared with L (b); L-DOPA+L compared with BAC1+L (c) and BAC2+L (d) by one way ANOVA with Tukey's post hoc test. GPx – Glutathione peroxidase; GR – Glutathione reductase; GST - Glutathione S Transferase.

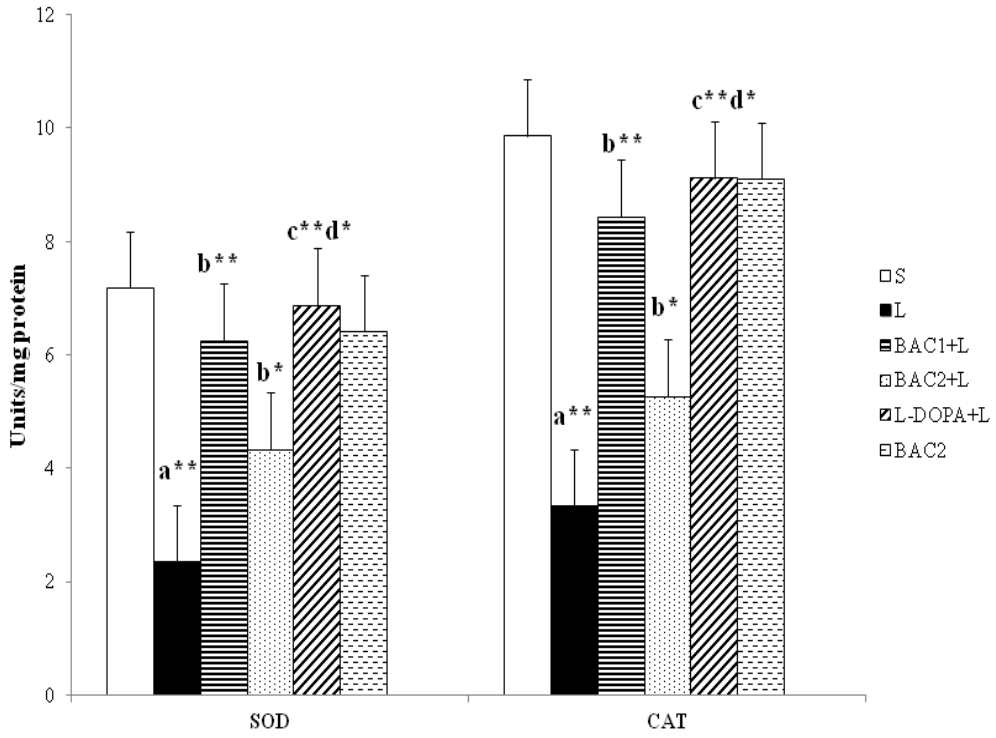

Fig.9. Status of SOD and CAT in the striatum of 6-OHDA lesioned rats and the modulatory effect of Bacoside-A pretreatment

Data represents mean ± SD of 6 rats in each group. SOD units expressed as enzyme activity to inhibit 50% of pyrogallol autooxidation. CAT units expressed as nmol of H_2O_2 reduced/min/mg protein. Group I: Vehicle treated, sham operated control received 2µl of vehicle (0.1% ascorbic acid-saline) intracranially (S). Group II: Vehicle treated, lesioned with 6 hydroxy dopamine on 22nd day (L). Group III: Rats pretreated with Bacoside-A (10mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC1+L). Group IV: Rats pretreated with Bacoside-A (20mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC2+L). Group V: Rats pretreated with L-DOPA (10mg/kg) i.p. for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (L-DOPA+L). Group VI: Rats administered with Bacoside-A (20mg/kg) alone orally for 21 days, sham – operated (BAC2+S).** p<0.01; *p<0.05; L group compared with S (a); BAC1+L, BAC2+L compared with L (b); L-DOPA+L compared with BAC1+L (c) and BAC2+L (d) by one way ANOVA with Tukey's post hoc test. SOD – Superoxide dismutase; CAT – Catalase.

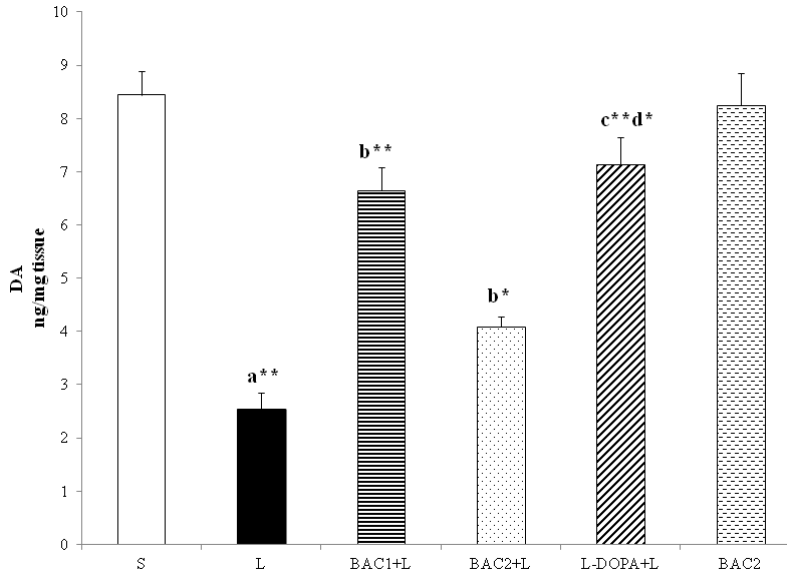

Fig. 10. Effect of Bacoside-A on the level of DA in the striatum of rat brain induced with 6-OHDA.

Data represents mean ± S.D (n = 6 in each group). Group I: Vehicle treated, sham operated control received 2µl of vehicle (0.1% ascorbic acid-saline) intracranially (S). Group II: Vehicle treated, lesioned with 6 hydroxy dopamine on 22nd day (L). Group III: Rats pretreated with Bacoside-A (10mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC1+L). Group IV: Rats pretreated with Bacoside-A (20mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC2+L). Group V: Rats pretreated with L-DOPA (10mg/kg) i.p. for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (L-DOPA+L). Group VI: Rats administered with Bacoside-A (20mg/kg) alone orally for 21 days, sham – operated (BAC2+S).** $p<0.01$; *$p<0.05$; L group compared with S (a); BAC1+L, BAC2+L compared with L (b); L-DOPA+L compared with BAC1+L (c) and BAC2+L (d) by one way ANOVA with Tukey's post hoc test.

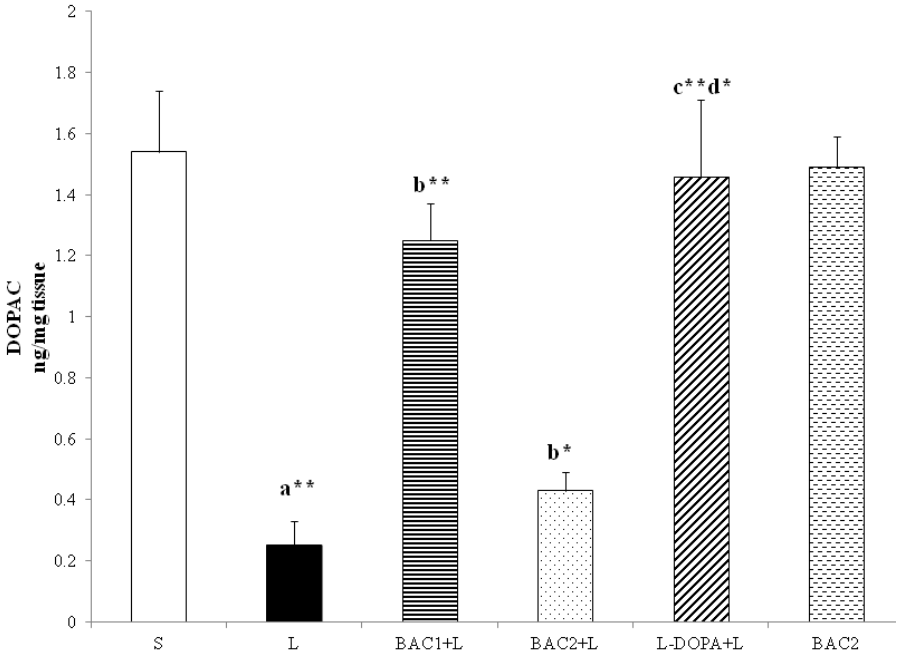

Fig. 11. Effect of Bacoside-A on the level of DOPAC in the striatum of rat brain induced with 6-OHDA.

Data represents mean ± S.D (n = 6 in each group). Group I: Vehicle treated, sham operated control received 2µl of vehicle (0.1% ascorbic acid-saline) intracranially (S). Group II: Vehicle treated, lesioned with 6 hydroxy dopamine on 22nd day (L). Group III: Rats pretreated with Bacoside-A (10mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC1+L). Group IV: Rats pretreated with Bacoside-A (20mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC2+L). Group V: Rats pretreated with L-DOPA (10mg/kg) i.p. for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (L-DOPA+L). Group VI: Rats administered with Bacoside-A (20mg/kg) alone orally for 21 days, sham – operated (BAC2+S).** p<0.01; *p<0.05; L group compared with S (a); BAC1+L, BAC2+L compared with L (b); L-DOPA+L compared with BAC1+L (c) and BAC2+L (d) by one way ANOVA with Tukey's post hoc test.

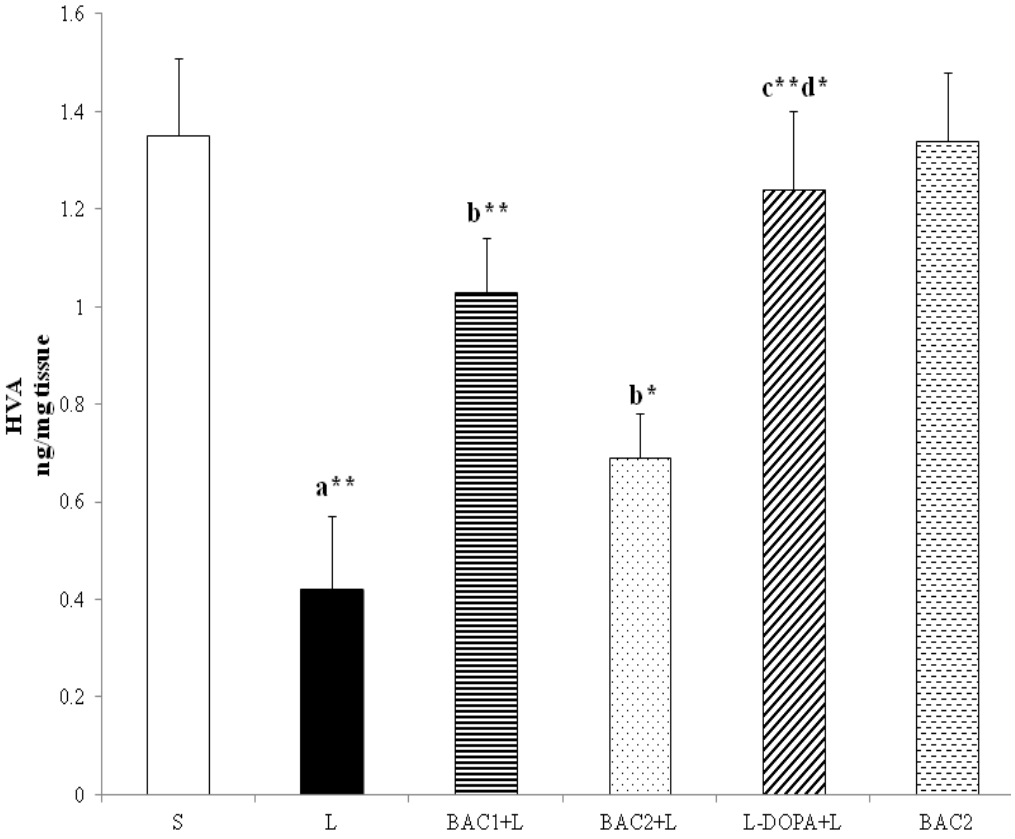

Fig. 12. Effect of Bacoside-A on the level of HVA in the striatum of rat brain induced with 6-OHDA.

Data represents mean ± S.D (n = 6 in each group). Group I: Vehicle treated, sham operated control received 2µl of vehicle (0.1% ascorbic acid-saline) intracranially (S). Group II: Vehicle treated, lesioned with 6 hydroxy dopamine on 22nd day (L). Group III: Rats pretreated with Bacoside-A (10mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC1+L). Group IV: Rats pretreated with Bacoside-A (20mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC2+L). Group V: Rats pretreated with L-DOPA (10mg/kg) i.p. for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (L-DOPA+L). Group VI: Rats administered with Bacoside-A (20mg/kg) alone orally for 21 days, sham – operated (BAC2+S).** p<0.01; *p<0.05; L group compared with S (a); BAC1+L, BAC2+L compared with L (b); L-DOPA+L compared with BAC1+L (c) and BAC2+L (d) by one way ANOVA with Tukey's post hoc test.

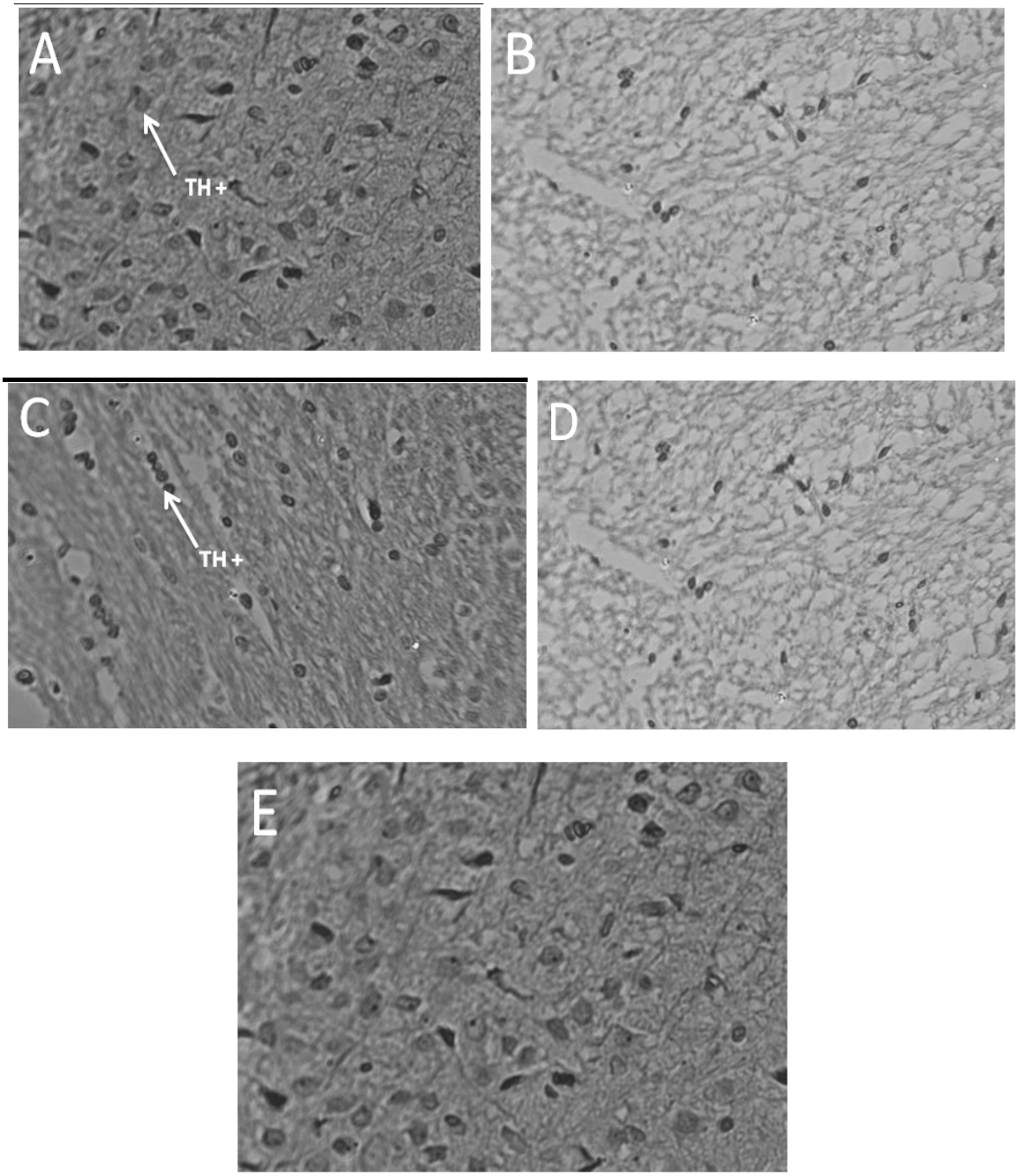

Fig. 13. – (A–E). Effect of 3 weeks of pretreatment with curcumin on tyrosine hydroxylase (TH) expression in the striatum of the rats when infused with 6-OHDA.

Fig 13A: Normal TH staining of the sham group (40x). Fig 13B: The expression of TH was almost negligible in the lesion group compared to the sham group (40x). Fig. 13C: The lesioned group pretreated with 10 mg/kg b.w of Bacoside-A has shown staining of TH (40x). Fig 13D: The lesioned group pretreated with 20mg/kg b.w of Bacoside-A has shown lesser staining of TH (40x). Fig 13E: However, Bacoside-A alone administered group has shown no discernible change in TH staining and was found to be similar to that of the control (40x).

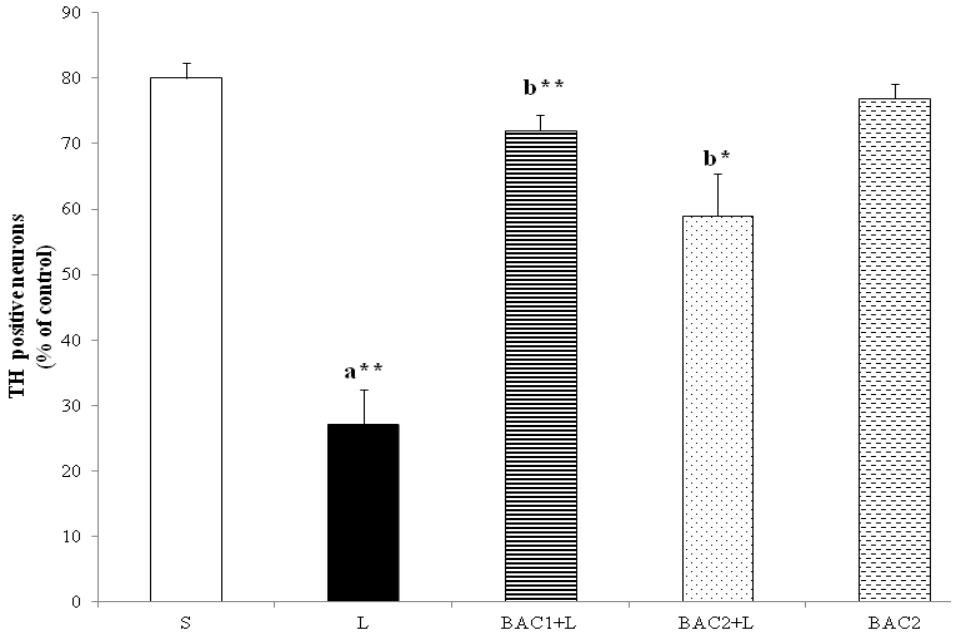

Fig. 14. The number of surviving TH-immunopositive cells in the ipsilateral substantia nigra analyzed as a percentage of that in the intact contralateral side.

Data represents mean ± S.D (n = 6 in each group). Group I: Vehicle treated, sham operated control received 2µl of vehicle (0.1% ascorbic acid-saline) intracranially (S). Group II: Vehicle treated, lesioned with 6 hydroxy dopamine on 22nd day (L). Group III: Rats pretreated with Bacoside-A (10mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC1+L). Group IV: Rats pretreated with Bacoside-A (20mg/kg) orally for 21 days; on 22nd day single dose of 6-hydroxy dopamine (12 µg of 6-OHDA/2µl in 0.1% ascorbic acid-saline) injected into right striatum (BAC2+L). Group V: Rats administered with Bacoside-A (20mg/kg) alone orally for 21 days, sham – operated (BAC2+S). ** $p<0.01$; *$p<0.05$; L group compared with S; BAC1+L, BAC2+L compared with L by one way ANOVA with Tukey's post hoc test

DISCUSSION

The present study demonstrates the neuroprotective effects of Bacoside-A in a 6-OHDA model of Parkinson's disease. The present work shows that the most prominent behavioral alterations such as changes in locomotor activity, marked changes in the social behavior whereas learning and memory abilities are spared. The locomotor activity was greatly affected by 6-OHDA injection. The study has shown that the mean time taken to cross a 105cm beam was more in 6-OHDA lesioned rats, but was decreased in the Bacoside-A pretreated groups indicating that the rats have more ability to coordinate their movement on a narrow beam maze and is in harmony with the studies of Gulrana et al., (2011). In the water maze test, in both spatial and procedural learning paradigms, no difference between the experimental groups emerged which was found in accordance with Branchi et al., (2008).

At 7 weeks after injection, in the social interaction test, 6-OHDA animals showed a drastic reduction in the aggressive behavior associated with a stronger propensity to interact socially compared with sham operated animals. The dopaminergic system has been widely reported to control social and aggressive behavior (Yudofsky et al., 1984; Gualtieri and Schroeder, 1990; Tiihonen et al., 1997; McDougle et al., 1998; van Erp and Miczek, 2000; Morgan et al., 2002; de Almeida et al., 2005). For instance, it has been shown that DA levels are associated with aggression and ability to manage social interactions in different mammal species (Grant et al., 1998; Soderstrom et al., 2001; de Almeida et al., 2005), including humans (Soderstrom et al., 2001). Accordingly, we found that 6-OHDA lesion produces a drastic reduction in the aggressive behavior. These results are in line with those showing that PD patients have histories of poor social skills and experience worsening social relationships (Bell et al., 1995; Macht et al., 2005). The poor social relationships were improved by treatment with Bacoside-A.

One of the universally accepted etiologies of PD is the imbalance between free radical formation and the maintenance of the neuronal integrity through the endogenous antioxidant defense system resulting in oxidative stress (Halliwell and Gutteridge, 1985). A surplus amount of free radical generation is thought to be the key module of neuronal damage in the brain. ROS threaten neuronal survival by their ability to propagate the initial attack on lipid rich membranes of the brain to cause lipid peroxidation (LPO) (Kale et al., 1999). However, cell damage can be prevented by detoxification of free radicals, which eventually prevent the progress of LPO. We have observed an elevated level of TBARS accompanied by a depleted GSH level in PD rat brain, which was in agreement with previous observations (Zafar et al., 2003; Ahmad et al., 2006; Chaturvedi et al., 2006). The results indicate that peroxidative stress takes its toll on the GSH content. However our experimental finding reveals that pre-treatment with Bacoside-A partially attenuates the elevated level of TBARS and the depleted level of GSH which is concomitant with the previous observations where antioxidants were used as a remedy in experimental PD models. Brain cells are continuously exposed to reactive oxygen species generated by oxidative metabolism, and in certain pathological conditions defense mechanisms against oxygen radicals may be weakened and/or overwhelmed which can be effectively protected by the use of various antioxidants (Osawa et al., 1990). Oxidative stress to dopaminergic neurons of SNpc is believed to be one of the leading causes of neurodegeneration in PD. Oxidative stress promotes lipid peroxidation and alters the antioxidant defense system in the brain. GSH is a tripeptide in which thiol residue plays a major role on membrane protection. Its significantly depleted level may trigger the formation of lipid peroxidation and consequently disrupt the hemostat. A reduction in GSH content may also impair H_2O_2 and promote •OH formation. In the present study, reversal of GSH induced by 6-OHDA was observed in the lesion group rats pretreated with Bacoside-A. All antioxidant defenses are interconnected (Sun, 1990); hence disruption of one would disrupt the whole microenvironment and eventually could lead to a catastrophe. GPx plays a predominant role in removing excess of free radicals and hydroperoxidases and is a major defense system against oxidative stress in the brain (Imam and Ali, 2000). Most of the H_2O_2 in the brain is removed by GPx, which uses it to oxidize GSH (Beckman et al., 1990). GR plays a major role in providing the pool for GSH which protects the membrane from toxification. The activity of these enzymes was decreased significantly in the 6-OHDA group due to which the toxicant level has increased significantly which caused severe toxicity and cell death. The curcumin has protected the activity of GPx and GR which play a major role in detoxifying oxidative stress and protect cell death. SOD converts superoxide into H_2O_2 (Freeman and Crapo, 1982). The catalase, which was found at a very low level of activity in the brain, detoxifies H_2O_2 to H_2O. In our findings, we also found that Bacoside-A significantly reduced the TBARS level along with the increased activity of antioxidant enzymes following 6-OHDA induction.

6-OHDA caused a marked depletion of striatal dopamine content which was significantly restored by pretreatment with Bacoside-A, a result that is in accordance with the findings of Gulrana et al., (2011) and Ahmad et al., (2006). Dopamine depletion is considered a cardinal feature in the causation of PD in human or in animal models of the disease (Carder et al., 1989; Bloem et al., 1990). The enhancement of dopamine content by Bacoside-A might have restored the alterations in the locomotor activity and muscular coordination, which is supported by our earlier findings (Shobana et al., 2012). The 6-OHDA model of nigral injury has been utilized for many years as a classical experimental model of Parkinsonism (Ungerstedt, 1968). The unilateral stereotaxic injection of 6-OHDA into the striatum of rats leads to a progressive death of the dopaminergic neurons. At the neurochemical level, the reduction in the striatal DA, DOPAC and HVA levels in the present study are also in accordance with the previous reports (Amalric and Koob, 1987; Amalric et al., 1995; Lee et al., 1996; Yuan et al., 2004; Ahmad et al., 2006; Branchi et al., 2008, Hyun et al., 2010). However, Bacoside-A treatments resulted in an improvement in the levels of DA, DOPAC and HVA, indicating that Bacoside-A administration protected the reduction of dopamine and its metabolites induced by 6-OHDA. Furthermore to strengthen the study, we have carried out tyrosine hydroxylase immunohistochemistry, a rate limiting enzyme in the biosynthesis of dopamine. The density of TH-IR fibres were decreased in 6-OHDA lesioned rats. The above changes was evidenced by the increasing TH-IR fibre density in the ipsilateral striata after lesioning due to pretreatment with Bac-A at a dose of 10mg/kg b.w., clearly signifying the increase in the number of surviving neurons and confirming the anti-parkinson's effects of Bac-A.

CONCLUSION

From this study, we can conclude that Bacoside-A can be used as the best tool to prevent 6-hydroxy dopamine induced Parkinsonism in rats. Further studies are underway to elucidate the molecular mechanism of Bacoside-A in treating 6-hydroxy dopamine induced lesioning in rats.

ACKNOWLEDGEMENT

The first author is thankful to University Grants Commission, New Delhi, India for the financial assistance rendered for this study in the form of Project Fellow. The authors thank the Department of Pathology and Central Research Facility, Sri Ramachandra University for their timely help in doing immunohistochemistry and HPLC studies.

REFERENCES

Aebi H. (1984); Catalase in vitro. Methods Enzymol. 105:121–126.

Ahmad M, Saleem S, Ahmad AS, Ansari MA, Yousuf S, Hoda MN, Islam F. (2005a). Neuroprotective effects of Withania somnifera on 6-hydroxydopamine induced Parkinsonism in rats. Hum Exp Toxicol;24:1–11.

Ahmad M, Saleem S, Ahmad AS, Yousuf S, Ansari MA, Khan MB, Ishrat T, Chaturvedi RK, Agrawal AK, Islam F. (2005b). Ginkgo biloba affords dose-dependent protection against 6-hydroxydopamine-induced parkinsonism in rats: neurobehavioural neurochemical and immunohistochemical evidences. J Neurochem;93:94–104.

Ahmad M, Yousuf S, Khan MB, Saleem S, Ishrat T, Hoda MN, Islam F. (2006). Protective effects of ethanolic extract of Delphinium denudatum in a rat model of Parkinson's disease. Human Expt Toxicol 25(7):361–368.

Albin RL, Young AB, Penney JB. (1989). The functional anatomy of basal ganglia disorders. Trends Neurosci;12:366-75.

Allbutt HN, Henderson JM. (2007). Use of the narrow beam test in the rat 6-hydroxydopamine model of Parkinson's disease. J Neurosci Methods;159:195–202.

Alleva E. (1983). Assessment of agressive behavior in rodents. In: Conn M.P. editor. Methods in neuroscience. Paradigms for the study of behavior. New York Academic Press.;111-137.

Amalric M, Koob GF. (1987). Depletion of dopamine in the caudate nucleus but not in nucleus accumbens impairs reaction-time performance in rats. J Neurosci;7:2129-2134.

Amalric M, Moukhles H, Nieoullon A, Daszuta A. (1995). Complex deficits on reaction time performance following bilateral intrastriatal 6-OHDA infusion in the rat. Eur J Neurosci;7:972-980.

Anbarasi K, Vani G, Balakrishna K, Shyamala Devi CS. (2006). ;Effect of Bacoside A on brain antioxidant status in cigarette smoke exposed rats. Life Sciences 78:1378 – 1384

Bandmann O, Marsden DC, Wood NW. (1998). Genetic aspects of Parkinson's disease. Mov Disord (Review);13:203–11.

Beckman JS, Beckman TW, Chen J. (1990). Apparent hydroxyl radical production by peroxynitrite implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA;87:1620–1624.

Bell IR, Amend D, Kaszniak AW, Schwartz GE, Peterson JM, Stini WA, Miller JW, Selhub J. (1995). Trait shyness in the elderly: evidence for an association with Parkinson's disease in family members and biochemical correlates. J Geriatr Psychiatry Neurol;8:16-22.

Bloem BR, Irwin I, Buruma OJS, Haan J, Roos RAC, Tetrud JW. (1990). The MPTP model: versatile contributions to the treatment of idiopathic Parkinson's disease. J Neurol Sci;97:273–93.

Branchi Igor, Ivana D Andrea, Monica Armida, Tommaso Cassano Antonella, Pezzola Rosa Luisa, Potenza Maria, Grazia Morgese, Patrizia Popoli, Enrico Alleva. (2008). Nonmotor symptoms in Parkinson's disease: investigating early phase onset of behavioral dysfunction in the 6-hydroxy dopamine lesioned rat model. Journal of Neuroscience Research.;86:2050-2061.

Branchi I, D'Andrea I, Fiore M, Di Fausto V, Aloe L, Alleva E. (2006). Early social enrichment shapes social behavior and nerve growth factor and brain derived neurotrophic factor levels in the adult mouse brain. Biol Psychiatry 60:690-696.

Breese CR, Breese GR. (1998). The use of neurotoxin to lesion catecholamine neurons to model clinical disorder. In: Rm Kostrzewa (Ed.) Highly Selective Neurotoxin: Basic and Clinical Application. Humana Press New Jersey 19–73.

Calabresi P, Centonze D, Bernardi G. (2000). Electrophysiology of dopamine in normal and denervated striatal neurons. Trends Neurosci 23(10):S57-63.

Calne DB, Langston JW. (1983). Aetiology of Parkinson's disease. Lancet II;1457–9.

Carder RK, Jackson D, Morris HJ, Lund RD, Zigmond MJ. Dopamine released from mesencephalic transplants restores modulation of striatal acetylcholine release after neonatal 6-OHDA: an in vitro analysis. Exp Neurol 1989;105:251–9.

Carlberg I, Mannervik B. (1985). Glutathione reductase. Methods Enzymol 113:484-490
Chase TN, Oh JD. (2000). Striatal dopamine- and glutamate-mediated dysregulation in experimental parkinsonism. Trends Neurosci 23(10):S86-91.
Chatterji N, Rastogi RP, Dhar ML. (1965). Chemical examination of Bacopa monniera Wettst.: parti-isolation of chemical constituents. Indian J Chem.;3:24–29.
Chaturvedi RK, Shukla S, Seth K, Chauhan S, Sinha C, Shukla Y, Agrawal AK. (2006). Neuroprotective and neurorescue effect of black tea extract in 6-hydroxydopamine-lesioned rat model of Parkinson's disease. Neurobiol Dis;22:421–434.
Chopra RN, Nayar SL, Chopra IC. (1956). ;Glossary of Indian Medicinal Plant. CSIR New Delhi 32.
Chowdhuri DK, Parmar D, Kakkar P, Shukla R, Seth PK, Srimal RC. (2002). Anti-stress effects of bacosides of Bacopa monnieri: modulation of HSP to expression SoI and cytochrome P450 activity in rat brain. Phytother Res.;16:639–645.
Crossman AR. (1989). Neural mechanisms in disorders of movement. Comp Biochem Physiol;93:141-9.
de Almeida RM, Ferrari PF, Parmigiani S, Miczek KA. (2005). Escalated aggressive behavior: dopamine serotonin and GABA. Eur J Pharmacol 526:51-64
Deepak M, Sangli GK, Arun PC, Amit A. (2005). Quantitative determination of the major saponin mixture Bacoside A in Bacopa monniera by HPLC. Phytochem Anal 16:24–29.
DeVito MJ, Wagner GC. (1989). Methamphetamine induced neuronal damage: a possible role for free radicals. Neuropharmacology;28:1145–50.
Fearnley JM, Lees A. (1991). Ageing and Parkinson's disease: substantia nigra regional selectivity. Brain;114:2283–301.
File SE, Seth P. (2003). A review of 25 years of the social interaction test. Eur J Pharmacol;463:35-53.
Freeman BA, Crapo JD. (1982). Biology of disease: free radicals and tissue injury. Lab Invest;47:412–426.
Garay S, Mahato SB, Ohtani K, Yamasaki K. (1996). Dammarane-type triterpenoid saponins from Bacopa monniera. Phytochemistry;42:815–820.
Grant KA, Shively CA, Nader MA, Ehrenkaufer RL, Line SW, Morton TE, Gage HD, Mach RH. (1998). Effect of social status on striatal dopamine D2 receptor binding characteristics in cynomolgus monkeys assessed with positron emission tomography. Synapse 29:80-83.
Graybiel AM, Canales JJ, Capper-Loup C. (2000). Levodopa-induced dyskinesias and dopamine-dependent stereotypies: a new hypothesis. Trends Neurosci.;23(10):S71-7.
Graybiel AM. (2000). The basal ganglia. Curr Biol;10:R509-11.
Greenamyre JT. (1993). Glutamate-dopamine interactions in the basal ganglia: relationship to Parkinson's disease. J Neural Transm Gen Sect 91:255-69.
Gualtieri CT, Schroeder SR. (1990). Pharmacotherapy for self-injurious behavior: preliminary tests of the D1 hypothesis. Prog Neuropsycho-pharmacol Biol Psychiatry;14:S81-S107.
Gulrana Khuwajaa, Mohd. Moshahid Khan, Tauheed Ishrat (2011). Neuroprotective effects of curcumin on 6-hydroxydopamine-induced Parkinsonism in rats: Behavioral neurochemical and immunohistochemical studies. Brain Research 1368:254-263.
Habig WH, Pabst MJ, Jokoby WB. (2011). Glutathione-S-transferase: the first enzymatic step in mercapturic acid formation. J Biol Chem;249:7130–9.
Halliwell B, Gutteridge JMC. (1985). Oxygen radicals and the nervous system. Trends Neurosci;8:22–29.
Hyun Sook Choi, Mi Sook Park, Seung Hwan Kim, Bang Yeon Hwang, Chong Kil Lee, Myung Koo Lee. (2010). Neuroprotective effects of herbal ethanol extracts from Gynostemma pentaphyllum in the 6-hydroxydopamine lesioned rat model of Parkinson's disease. Molecules;15:2814-2824.
Imam SZ, (2000). Ali SF. Selenium an antioxidant attenuates methamphetamine induced dopaminergic toxicity and peroxynitrile generation. Brain Res;855:186–191.
Jenner P, Olanow CW. (1996). Oxidative stress and the pathogenesis of Parkinson's disease. Neurology;47(6 Suppl. 3):161–70.
Kale M, Rathore N, John S, Bhatnagar D. (1999). Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes: a possible involvement of reactive oxygen species. Toxicol Lett;105:197–205.
Kawai KI, Shibata S. (1978). Pseudojujubogenin a new sapogenin from Bacopa monniera. Phytochemistry;17:287–289.
Lee CS, Sauer H, Bjorklund A. (1996). Dopamine neuronal degeneration and motor impairments following axon terminal lesion by intra-striatal 6-hydroxy dopamine in the rat. Neuroscience;72:641-653.

Macht M, Schwarz R, Ellgring H. (2005). Patterns of psychological problems in Parkinson's disease. Acta Neurol Scand;111:95-101.

Marklund S, Marklund G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. European Journal of Biochemistry;47:469–474

Mathur S, Gupta MM, Kumar S. (2001). Expression of growth and bacoside A in response to seasonal variation Bacopa monnieri accessions. J Med Aroma Plant Sci;22/4A–23/1A:320–326.

McDougle CJ, Holmes JP, Carlson DC, Pelton GH, Cohen DJ, Price LH. (1998). A double-blind placebo-controlled study of risperidone in adults with autistic disorder and other pervasive developmental disorders. Arch Gen Psychiatry;55:633-641.

McGaugh JL, Roozendaal B. (2002). Role of adrenal stress hormones in forming lasting memories in the brain. Curr Opin Neurobiol;12:205-210.

Mohandas J, Marshall JJ, Duggin GG, Horvath JS, Tiller D. (1984). Differential distribution of glutathione and glutathione related enzymes in rabbit kidneys: possible implication in analgesic neuropathy. Cancer Res;4:5086–91.

Morgan D, Grant KA, Gage HD, Mach RH, Kaplan JR, Prioleau O, Nader SH, Buchheimer N, Ehrenkaufer RL, Nader MA. (2002). Social dominance in monkeys: dopamine D-2 receptors and cocaine self administration. Nat Neurosci;5:169-174.

Moron M, Depierre JW, Mannervik BT. (1979). Levels of glutathione glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochimica Biophysica Acta;582:67–78.

Orth M, Schapira AH. Mitochondrial involvement in Parkinson's disease. Neurochem Int 2002;40(6):533–541.

Osawa T, Namiki M, Kawakishi S. (1990). Role of dietary antioxidants in protection against oxidative damage. Basic Life Sci;52:139–153.

Papa SM, Boldry RC, Engber TM, Kask AM, Chase TN. (1995). Reversal of levodopa-induced motor fluctuations in experimental parkinsonism by NMDA receptor blockade. Brain Res;701:13-8.

Paxinos G, Watson C. (1982). The Rat Brain in Sterotaxic coordinates. Academic Press Newyork

Picconi B, Pisani A, Centonze D, Battaglia G, Storto M, Nicoletti F. (1990). Striatal metabotropic glutamate receptor function following experimental parkinsonism and chronic levodopa treatment. Brain 2002;125:2635-45.

Rastogi RP. Compendium of Indian Medicinal Plants vol. 1. CSIR New Delhi;118–122.

Sharath R, Harish BG, Krishna V, Sathyanarayana BN, Kumara Swamy HM. (2010). Wound Healing and Protease Inhibition Activity of Bacoside-A Isolated from Bacopa monnieri Wettest Phytother Res;24:1217–1222.

Shobana C, Ramesh R, Sumathi T. (2012). Alcoholic Extract of Bacopa monniera Linn. Protects Against 6-Hydroxydopamine-Induced Changes in Behavioral and Biochemical Aspects: A Pilot Study. Cell Mol Neurobiol 32:1099-1112.

Singh HK, Dhawan BN. (1997). Neuropsychopharmacological effects of the Ayurvedic nootropic Bacopa monniera Linn (Brahmi). Indian J Pharmacol;29:S359–S365.

Smith AD, Bolam JP. (1990). The neural network of the basal ganglia as revealed by the study of synaptic connections of identified neurones. Trends Neurosci 13:259-65.

Soderstrom H, Blennow K, Manhem A, Forsman A.(2001). CSF studies in violent offenders 5-HIAA as a negative and HVA as a positive predictor of psychopathy. J Neural Transm;108:869-878.

Sumathi T, Nongbri A. (2008). Hepatoprotective effect of Bacoside-A a major constituent of Bacopa monniera linn. Phytomedicine;15:901-905.

Sun Y. (1990). Free radicals antioxidant enzymes and carcinogenesis. Free Radic Biol Med;8:583–599.

Tiihonen J, Kuikka J, Bergstrom K, Lepola U, Kopenen H, Leinonen E. (1997). Dopamine reuptake site densities in patients with social phobia. Am J Psychiatry;154:239-242.

Ungerstedt U. (19686). Hydroxy-dopamine induced degeneration of central dopamine neurons. Eur J Pharmacol;5:107–110.

Utley HC, Bernheim F, Hochslein P. (1967). Effect of sulfhydryl reagent on peroxidation in microsome. Arch Biochem Biophys;260:521–31

Van Erp AM, Miczek KA. (2000). Aggressive behavior increased accumbal dopamine and decreased cortical serotonin in rats. J Neurosci;20:9320-9325.

Vanderhaeghen JJ, Périer O, Sternon JE. (1970). Pathological findings in idiopathic orthostatic hypotension. Its relationship with Parkinson's disease. Arch Neurol;22(3):207–214.

Yuan H, Sarre S, Ebinger G, Michotte Y. (2004). Neuroprotective and neurotrophic effect of apomorphine in the striatal 6-OHDA lesion rat model of Parkinson's disease. Brain Res;1026:95-107.

Yudofsky SC, Stevens L, Silver J, Barsa J, Williams D. (1984). Propranolol in the treatment of rage and violent behavior associated with Korsakoff's psychosis. Am J Psychiatry;141:114-115.

Zafar KS, Sayeed I, Siddiqui A, Ahmad M, Salim S, Islam F. (2003a). Dose-dependent protective effect of selenium in partial lesion model of Parkinson's disease: neurobehavioral and neurochemical evidences. J Neurochem;84:438–46.

Zafar KS, Siddiqui A, Sayeed I, Ahmad M, Saleem S, Islam F. (2003b). Protective effect of adenosine in rat model of Parkinson's disease: neurobehavioral and neurochemical evidences. J Chem Neuroanat;26:143–51.

Ziv I, Melamed E, Nardi N. (1998). Role of apoptosis in the pathogenesis of Parkinson's disease: a novel therapeutic opportunity. Mov Disord;13:865–70.