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Attenuation by *Nardostachys jatamansi* of 6-hydroxydopamine-induced parkinsonism in rats: behavioral, neurochemical, and immunohistochemical studies

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Abstract

Parkinson's disease (PD) is one of the commonest neurodegenerative diseases, and oxidative stress has been evidenced to play a vital role in its causation. In the present study, we evaluated whether ethanolic extract of *Nardostachys jatamansi* roots (ENj), an antioxidant and enhancer of biogenic amines, can slow the neuronal injury in a 6-OHDA-rat model of Parkinson's. Rats were treated with 200, 400, and 600 mg/kg body weight of ENj for 3 weeks. On day 21, 2 μl of 6-OHDA (12 μg in 0.01% in ascorbic acid-saline) was infused into the right striatum, while the sham-operated group received 2 μl of vehicle. Three weeks after the 6-OHDA injection, the rats were tested for neurobehavioural activity and were sacrificed after 6 weeks for the estimation of lipid peroxidation, reduced glutathione content, the activities of glutathione-*S*-transferase, glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase, quantification of catecholamines, dopaminergic D_2 receptor binding and tyrosine hydroxylase expression. The increase in drug-induced rotations and deficits in locomotor activity and muscular coordination due to 6-OHDA injections were significantly and dose-dependently restored by ENj. Lesioning was followed by an increased lipid peroxidation and significant depletion of reduced glutathione content in the substantia nigra, which was prevented with ENj pretreatment. The activities of glutathione-dependent enzymes, catalase and superoxide dismutase in striatum, which were reduced significantly by lesioning, were dose-dependently restored by ENj. A significant decrease in the level of dopamine and its metabolites and an increase in the number of dopaminergic D_2 receptors in striatum were observed after 6-OHDA injection, and both were significantly recovered following ENj treatment. All of these results were exhibited by an increased density of tyrosine hydroxylase immunoreactive (TH-IR) fibers in the ipsilateral striatum of the lesioned rats following treatment with ENj; 6-OHDA injection had induced almost a complete loss of TH-IR fibers. This study indicates that the extract of Jatamansi might be helpful in attenuating Parkinsonism.

Keywords: *Nardostachys jatamansi*; 6-hydroxydopamine; Oxidative stress; Dopamine; Parkinson's disease; Neuroprotection; Herbal drugs

Abbreviations: 6-OHDA, 6-hydroxydopamine; BSA, bovine serum albumin; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; DA, dopamine; DHBA, 3,4-dihydroxybenzylamine; DOPAC, 3,4-dihydroxyphenyl acetic acid; HVA, homovanilic acid; DTNB, 5-5′-dithio-bis-2-nitrobenzoic acid; EDTA, ethylene diamine tetra acetic acid; ENj, extract of *Nardostachys jatamansi*; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione reduced; GSSG, glutathione oxidized; GST, glutathione-*S*-transferase; HPLC, high performance liquid chromatography; HVA, homovalinic acid; TBARS, thiobarbituric acid reactive substance; NADPH, nicotinamide adenine dinucleotide phosphate reduced; NBT, nitroblue tetrazolium; PD, Parkinson's disease; PMS, post-mitochondrial supernatant; rpm, rotations per minute; SOD, superoxide dismutase; PBS, phosphate-buffered saline; TH, tyrosine hydroxylase; TH-IR, TH immunoreactivity.

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1. Introduction

6-Hydroxydopamine (6-OHDA) is a specific neurotoxin for catecholaminergic pathways (Perese et al., 1989; Sachs and Jonsson, 1975). Being structurally similar to the catecholamines, it uses the respective transport system to enter the neurons and destroys them. 6-OHDA has been reported to produce some of the behavioural, biochemical, and pathological changes that are encountered in Parkinson's disease (PD) (Bloem et al., 1990) and, because of established stereotactic techniques and relatively low maintenance costs, is currently the most commonly used animal model for the disease (Breese and Breese, 1998). These toxic effects of 6-OHDA are attributed to the formation of various oxidants and free radicals (Cohen, 1984), lipid peroxidation (Slater, 1984), protein damage, and amino acid modifications (Dean et al., 1985). In addition, studies have demonstrated that 6-OHDA leads to reduction in glutathione (GSH) content and superoxide dismutase (SOD) and catalase (CAT) activity, and an increase in lipid peroxidation (Perumal et al., 1992; Kumar et al., 1995; Zafar et al., 2003a,b; Ahmad et al., 2005a,b) in striatum.

PD is an age-related disorder, more common in the aged population than in the young. The disease is accompanied by the symptoms of rest tremor, bradykinesia, rigidity, stooped posture, and instability. 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). The disease progresses slowly and can ultimately produce complete akinesia. 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.

Nardostachys jatamansi Jones DC (commonly called as Jatamansi) belongs to the family Valirenaceae of plant taxa. Various sesquiterpenes (such as Jatamansic acid and Jatamansone), lignans, and neolignans have been reported to be present in the roots of the plant (Chatterji and Prakashi, 1997; Arora, 1965). The decoction of the roots is use in mental disorders, insomnia, and disorders of blood and the circulatory system (Uniyal and Issar, 1969). Ali et al. (2000) have demonstrated the protective effect of alcoholic extract of Jatamansi on the thiacetamide-induced liver damage in rats. The rhizomes of Jatamansi are used as a bitter tonic, stimulant, and antispasmodic, as well as to treat epilepsy, hysteria, corea, palpitations, and convulsions (Bagchi et al., 1991). Tripathi et al. (1996) reported anti-lipid peroxidative property of Jatamansi in iron-induced lipid peroxidation. ENj causes an overall increase in the levels of biogenic amines and inhibitory neurotransmitters in rat brain (Prabhu et al., 1994). Salim et al. (2003), from our laboratory, have reported protective effects of ENj in rat cerebral ischemia. These pharmacologic properties of jatamansi prompted us to evaluate its efficacy in 6-OHDA-induced parkinsonism.

2. Material and methods

2.1. Chemicals

6-OHDA, apomorphine hydrochloride, GSH, glutathione oxidized (GSSG), glutathione reductase (GR), nicotinamide adenine dinucleotide phosphate reduced (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), 5-5′-dithio-bis-2-nitrobenzoic acid (DTNB), nitroblue tetrazolium (NBT), dopamine (DA), 3,4-dihydroxyphenyl acetic acid (DOPAC), homovanilic acid (HVA), 3,4-dihydroxybenzylamine (DHBA), heptane sulphonic acid, bovine serum albumin (BSA), thiobarbituric acid, ethylene diamine tetra acetic acid (EDTA), monoclonal tyrosine hydroxylase antibody, anti-mouse IgG, diaminobenzidine, ^{3}H-spiperone (specific activity 30 Ci/mmol), and haloperidol were purchased from Sigma-Aldrich Foreign Holding Chemical Company (Bangalore, India). Other chemicals were analytical grade.

2.2. Animals and treatments

Male Wistar rats, 80–90 days old, weighing 200 ± 10 g, obtained from Central Animal House of Jamia Hamdard (Hamdard University) were used for this study. Rats were housed in groups of four animals per cage and had free access to food and water.

2.3. Preparation of extract

Roots of *Nardostachys jatamansi* De Jones (Valerianaceae) were purchased from an herbal market of Delhi and were identified and authenticated by the expert taxonomist of the Department of Environmental Botany at Jamia Hamdard, New Delhi. The specimen was kept in the Herbarium Department of Ilmul Advia (Faculty of Medicine) under voucher specimen No. JH/T-05/1. Clean roots were air-dried and powdered to prepare the alcoholic extract as earlier described by Prabhu et al. (1994). One kilogram of moderately powdered roots of Jatamansi was extracted by refluxing with 95% ethyl alcohol in Soxhlet extractor for 6–8 h. The extract was evaporated to dryness under reduced pressure and temperature using rotatory vacuum evaporator, and dried residue was stored at 4 °C. The yield of dry extract from crude powder of Jatamansi was 10%. The extract was suspended in *Gum acacia* (2% in water) and applied orally by gavage.

2.4. Lesioning

After 3 weeks of treatment with *Nardostachys jatamansi* extract (ENj), all animals in experimental and sham-operated groups were anaesthetized with 35 mg/kg sodium pentobarbitone intraperitoneally (i.p.). Each animal was mounted on a stereotaxic stand, 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 0.5 mm, lateral 2.5 mm, dorso-ventral 4.5 mm relative to bregma and ventral from dura) with the tooth bar set at 0 mm. Thereafter, all

animals in experimental groups were lesioned by injecting 12 μg 6-OHDA/2 μl in 0.1% ascorbic acid-saline into the right striatum, while the sham-operated group received 2.0 μl 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 1.0 μl/min, and the needle was kept in place for an additional 1 min before being slowly retracted. The experiments were performed in accordance with the guidelines of the Animal Ethics Committee of Jamia Hamdard (Hamdard University).

2.5. Experiment 1

Experiment 1 was conducted to evaluate the pretreatment effect of ENj [200, 400, and 600 mg/kg body weight (BW)], for 3 weeks on the content of TBARS and GSH in the substantia nigra, while striatum was used for the assays of enzymatic parameters. The rats were divided into 8 groups, each having 10 animals. Group 1: vehicle-treated, sham-operated control (S), received 2.0 μl of vehicle intracranially; Group 2: ENj (200 mg/kg BW)-treated, sham-operated (N1 + S); Group 3: ENj (400 mg/kg BW)-treated, sham-operated (N2 + S); Group 4: ENj (600 mg/kg BW)-treated, sham-operated (N3 + S); Group 5: vehicle-treated, lesioned group (L); Group 6: ENj (200 mg/kg BW)-treated, lesioned group (N1 + L); Group 7: ENj (400 mg/kg BW)-treated, lesioned group (N2 + L); Group 8: ENj (600 mg/kg BW)-treated, lesioned group (N3 + L).

2.6. Experiment 2

Experiment 2 was conducted to evaluate the pretreatment effect of ENj (200, 400, and 600 mg/kg BW) for 3 weeks on dopaminergic D_2 receptor binding density and content of dopamine and its metabolites, DOPAC, and HVA in the striatum. The rats were divided into 8 groups, as in Experiment 1, each having 10 animals.

2.7. Experiment 3

Experiment 3 was conducted to evaluate the pretreatment effect of ENj (200, 400, and 600 mg/kg BW) for 3 weeks on tyrosine hydroxylase expression in striatum. The rats were divided into 8 groups, as in Experiment 1, each having 5 animals.

Behavioral parameters were performed in all experiments, and only those animals that showed a threshold number of drug-induced rotations were included in the study. All experiments were performed separately for all 3 doses of ENj and repeated twice.

2.8. Postoperative care

Recovery of anaesthesia took approximately 4–5 h. 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.

2.9. Behavioral studies

All of the behavioral studies were performed at room temperature in a calm room without any outside interference. All of the experiments were performed between 9:00 am and 7:00 pm.

2.9.1. Rotational behavior

On day 22 of lesioning, the rats were tested for drug-induced rotations with a Video Path Analyzer (Coulbourn Instruments, Allentown, PA, USA), following the same procedure as used for monitoring locomotor activity. After administration of 5.0 mg/kg of D-amphetamine (in ascorbic acid-saline) i.p., ipsilateral rotations of animals were collected, and their rotational scores were monitored over a period of 90 min. One week after amphetamine challenge, the animals were given 0.5 mg/kg apomorphine (in ascorbic acid-saline) subcutaneously to monitor contralateral rotations; these scores were collected at 40-min intervals.

2.9.2. Locomotor activity

On day 36, all animals were tested for locomotor activity with a Video Path Analyzer. This computerized animal activity monitor consists of a chamber ($50 \times 50 \times 35\,cm^3$), a video camera fixed over the chamber by an adjacent rod, an activity monitor, a programmer/processor, and a printer. A single animal was placed in the chamber, and its locomotor activity was monitored by activating the camera and was viewed on the screen. The activity chamber was furnished with black paper to provide a good contrast on the screen. Each animal was assessed for locomotor activity for three sessions of 5 min each. The data was fed to the printer to print out the intervals (min), wall hugging (sec), locomotion (sec), rest (sec), rearing (sec), stereotypic behavior (number), rotations (clockwise and counterclockwise), and distance travelled (cm). After each animal, the activity chamber was swabbed with 10% alcohol to avoid any interference due to animal odors.

2.9.3. Rota rod (muscular coordination)

Omni Rotor (Omnitech Electronics, Inc., Columbus, OH, USA) was used to evaluate the muscular coordination on the 40th day. It consists of a rotating rod (75 mm diameter), which is divided into four parallel compartments, permitting testing of 4 rats at a time. The apparatus automatically records the time in 0.1 sec when the rats fall of the rotating shaft. The speed was set at 10 rpm, and cut-off time was 180 sec. The drug-naïve animals were trained on the rod, so that they could stay on it at least for the length of the cut-off time.

2.10. Biochemical studies

2.10.1. 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 0.01 M phosphate buffer (pH 7.0) and centrifuged at 10,500×*g* for 20 min at 4 °C to obtain post-mitochondrial supernatant (PMS), while homogenized substantia nigra 10% w/v was used for the estimation of TBARS and GSH.

2.10.2. 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, 0.2 ml 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 0.2 ml was pipetted in an Eppendorf tube and placed at 0 °C incubation. After 1 h of incubation, 0.4 ml 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 535 nm. The rate of lipid peroxidation expressed as nmol of thiobarbituric acid reactive substance formed/min/mg protein.

2.10.3. Assay for reduced glutathione content

Reduced GSH was determined by the method of Jollow et al. (1974). 0.2 ml of homogenate was precipitated with 0.2 ml of sulfosalicylic acid (4%). The sample was kept at 4°C for at least 1 h and then subjected to centrifugation at 1200×*g* for 15 min at 4 °C. The assay mixture contained 0.1 ml of filtered aliquot, 1.7 ml phosphate buffer (0.1 M, pH 7.4), and 0.2 ml DTNB (4 mg/1 ml of phosphate buffer, 0.1 M, pH 7.4) in a total volume of 2.0 ml. The yellow color developed and was read immediately at 412 nm. The results are expressed as nmol GSH formed/g tissue.

2.10.4. Determination of glutathione-S-transferase activity

Glutathione-*S*-transferase (GST) activity was measured by the method of Habig et al. (1974) as described by Athar et al. (1989). The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5), 1.0 mM reduced GSH, 1.0 mM CDNB, and 0.1 ml PMS in a final volume of 2.0 ml. The changes in absorbance were recorded at 340 nm, and the enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein.

2.10.5. Determination of glutathione reductase activity

GR activity was assayed by the method of Carlberg and Mannervik (1975), as modified by Mohandas et al. (1984). The assay system consisted of 0.1 M phosphate buffer (pH 7.6), 0.1 mM NADPH, 0.5 mM EDTA, 1.0 mM GSSG, and 0.1 ml PMS in a total volume of 2.0 ml. The enzyme activity was quantitated at room temperature by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein.

2.10.6. Determination of glutathione peroxidase activity

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

2.10.7. Determination of superoxide dismutase activity

SOD activity was measured by the method of Beauchamp and Fridovich (1971) as described by Shah and Vohora (2002). The reaction mixture of total volume of 1.0 ml consisted of 0.5 M phosphate buffer (pH 7.4), 0.1 ml PMS, 1.0 mM xanthine, and 57 μM NBT. It was incubated for 15 min at room temperature and reaction was initiated by the addition of 50 mU xanthine oxidase. The rate of reaction was measured by recording change in the absorbance of 550 nm due to the formation of formazan; a reduction product of NBT.

2.10.8. Determination of catalase activity

Catalase activity (CAT) was assayed by the method of Coliborne (1985). Briefly, the assay mixture consisted of 0.05 M phosphate buffer (pH 7.0), 0.019 M H_2O_2, and 0.05 ml PMS in a total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of nmol H_2O_2 consumed/min/mg protein.

2.11. Markers for parkinsonism

2.11.1. Quantification of dopamine and its metabolites

The method of DeVito and Wagner (1989) as described by us (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 0.4 N perchloric acid containing 100 ng/ml of the internal standard, 3,4-dihydroxybenzylamine, followed by centrifugation at 15,000×*g* for 10 min at 4 °C and the filtrate was injected manually through a 20-μl loop over the ODS-C_{18} column coupled with HPLC/Electrochemical detector (Waters, Milford, MA, USA) for separation and quantification. The mobile phase consisted of 0.1 M potassium phosphate (pH 4.0), 10% methanol, and 1.0 mM heptane sulphonic acid. Samples were separated on ODS-C_{18} column using a flow rate of 1.0 ml/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.

2.11.2. Determination of dopaminergic D_2 receptor binding

Animals were sacrificed by decapitation, brains were removed, and striatum of the right hemisphere of each brain were dissected out, weighed, and were then homogenized (5% w/v) in 40 mM Tris–HCl buffer (pH 7.4), followed by centrifugation at 20,000 rpm for 20 min at 4 °C. Supernatant was discarded, the pellet resuspended in the buffer and stored at −20 °C. The

binding assay was performed by the method of our collaborating group (Agrawal et al., 1995). Briefly, the incubation mixture of 1.0 ml consisted of synaptic membrane, along with 1.0 nM 1-phenyl-4-3*H*-spiperone in 40 mM Tris–HCl (pH 7.4). A parallel incubation was conducted in the presence of 1.0 μM haloperidol to ascertain non-specific binding. The assay was run in triplicate. Reaction mixture was incubated for 15 min at 37 °C, terminated by cooling at 4 °C, and filtered through glass fiber filters (GF/C, Whatmann), using the Millipore Filtration Assembly. The filter discs were washed rapidly with 2 × 5 ml cold Tris buffer (40 mM, pH 7.4), transferred to scintillation vials, and dried properly. After adding 10.0 ml scintillation cocktail to vials, the radioactivity was counted in a β-scintillation counter (WALLAC-1410), with an efficiency of 50% for tritium. Specific binding was calculated by subtracting non-specific binding from total binding obtained in absence of haloperidol. Results are expressed as pmol ^{3}H-spiperone bound/mg protein.

2.11.3. Determination of protein

Protein was determined by the method of Lowry et al. (1951).

2.11.4. 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 (35.0 mg/kg) and perfused transcardially through ascending aorta with 100.0 ml of 0.1 M phosphate buffer saline (PBS) at pH 7.5 followed by 300.0 ml of 4% paraformaldehyde in 0.1 M 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 24 h 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 100 mM, sodium chloride 0.5 M, Triton X-100, sodium azide) at pH 7.4 for 20 min. 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 20 μl of anti-tyrosine hydroxylase antibodies (2% in PBS) and incubated for 2 h 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 3 h 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.

2.11.5. 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 (Leica Qwin 500 image analysis software, Wetzlar, Germany) as described earlier by our collaborating group (Chaturvedi et al., 2003). 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 μm^2 per total field view (250 μm × 250 μm; 75,000 μm^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.

2.12. Statistics

Results are expressed as means ± S.E.M.; ANOVA followed by Tukey–Kramer's post-hoc analysis was used to analyze differences between the groups, with significance level set at $p < 0.05$.

3. Results

Figs. 1–6 show the effect of *Nardostachys jatamansi* on amphetamine-induced ipsilateral and apomorphine-induced contralateral body rotations in the sham (S) and lesioned (L) groups and the partial restoration afforded by N1, N2, and N3 doses of ENj. Lesioning caused a significant ($p < 0.001$) elevation in rotations (apomorphine, 384.84%; amphetamine, 271.42%), as compared to S, while this rotation was restored (apomorphine, 136.36%, 242.42%, 303.3%; amphetamine, 85.71%, 171.42%, 214.28%) significantly and dose-dependently in N1 + L, N2 + L, and N3 + L, as compared to L group. No significant difference was observed in N1 + S, N2 + S, and N3 + S groups.

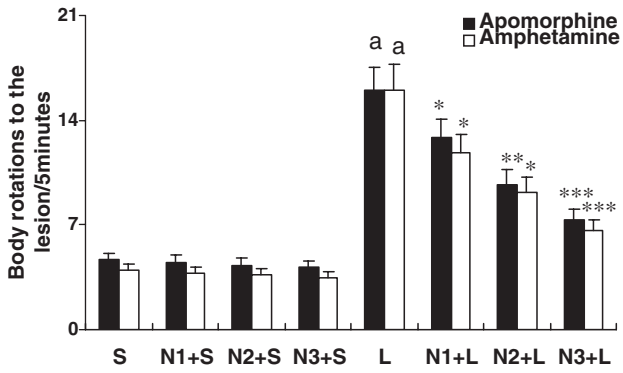

Fig. 1. The effect of *Nardostachys jatamansi* extract (200, 400 and 600 mg/kg) for 3 weeks pre-treatment on body rotations and in rats lesioned by a single injection of 12.0 μg 6-hydroxydopamine/2.0 μl in 0.1% ascorbic acid-saline and sham-received ascorbic acid-saline only (vehicle). Each bar represents the mean ± S.E.M. of six animals. $^{a}P < 0.001$ vs. S and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. L.

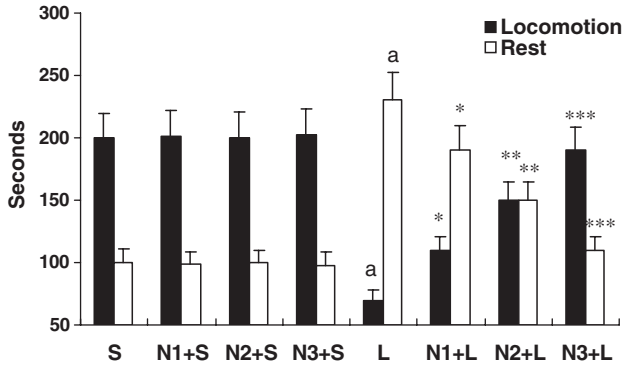

Fig. 2. The effect of *Nardostachys jatamansi* extract (200, 400 and 600 mg/kg) for 3 weeks pre-treatment on locomotor and rest time (sec) and in rats lesioned by a single injection of 12.0 μg 6-hydroxydopamine/2.0 μl in 0.1% ascorbic acid-saline and sham-received ascorbic acid-saline only (vehicle). Each bar represents the mean ± S.E.M. of six animals. [a]$P<0.001$ vs. S and *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L.

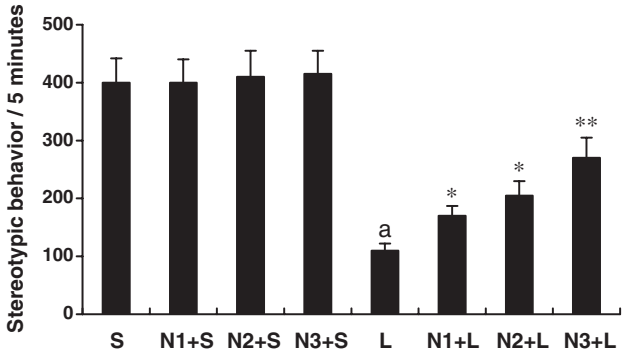

Fig. 4. The effect of *Nardostachys jatamansi* extract (200, 400 and 600 mg/kg) for 3 weeks pre-treatment on the number of stereotypic behavior and in rats lesioned by a single injection of 12.0 μg 6-hydroxydopamine/2.0 μl in 0.1% ascorbic acid-saline and sham-received ascorbic acid-saline only (vehicle). Each bar represents the mean ± S.E.M. of six animals. [a]$P<0.001$ vs. S and *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L.

A significant ($p<0.001$) reduction (65.21%) in locomotor time was observed in the L group, as compared to S group, while the reverse was the case for the rest time (214%). Treatment with ENj significantly and dose-dependently restored the locomotion (17.39%, 34.78%, 56.52%) and rest time (57.14%, 114.28%, 185.71%) in the N1 + L, N2 + L, and N3 + L groups, as compared to L, while drug sham showed no significant difference (Fig. 2). Distance traveled (cm) was significantly ($p<0.001$) low (71.42%) in the L group, as compared to the S group. In the N1 + L, N2 + L, and N3 + L groups, significant and dose-dependent restoration (21.42%, 35.71%, 57.14%) was noted in distance travelled, as compared to the L group; however, drug shams showed no significant effects (Fig. 3).

Fig. 4 shows the significant and dose-dependent recovery (15%, 37.5%, 57.5%) on the number of stereotypic behavior performed by the lesioned animals due to ENj treatment (N1 + L, N2 + L, and N3 + L), as compared to the L group. The stereotypic behavior performed was significantly ($p<0.001$) decreased (72.50%) in the L group, as compared to the S group. No significant difference was found in drug sham groups.

A significant and dose-dependent recovery (14.28%, 3.335, 47.61%) was observed on the rearing activity of the lesioned animals due to ENj treatment (N1 + L, N2 + L, N3 + L), as compared to the L group. The rearing activity was significantly ($p<0.001$) reduced (66.66%) in the L group, as compared to the S group (Fig. 5). No significant effects were observed in drug sham groups.

Fig. 6 shows the effect of lesioning on muscular coordination, which was significantly ($p<0.001$) low (68.18%) in the L group, as compared to the S group. Pretreatment of animals with different doses of ENj (N1 + L, N2 + L, N3 + L), afforded a significant and dose-dependent (18.18%, 36.63%, 50.00%) restoration in muscular coordination, as compared to the L group; however, no significant effects were observed in drug sham groups.

The content of TBARS in the substantia nigra was significantly elevated (273.74%; $p<0.001$) in the L group, as compared to the S group, and the increase in the generation of

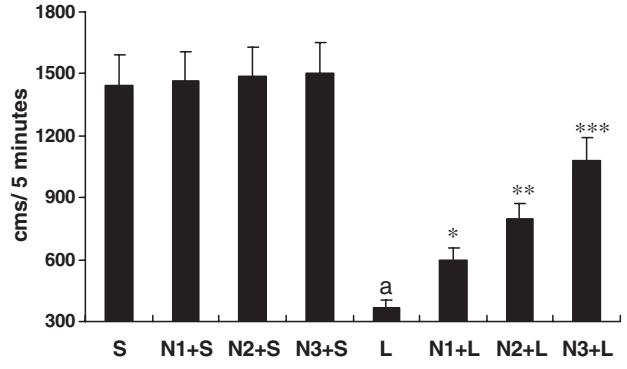

Fig. 3. The effect of *Nardostachys jatamansi* extract (200, 400 and 600 mg/kg) for 3 weeks pre-treatment on distance travelled (cm) and in rats lesioned by a single injection of 12.0 μg 6-hydroxydopamine/2.0 μl in 0.1% ascorbic acid-saline and sham-received ascorbic acid-saline only (vehicle). Each bar represents the mean ± S.E.M. of six animals. [a]$P<0.001$ vs. S and *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L.

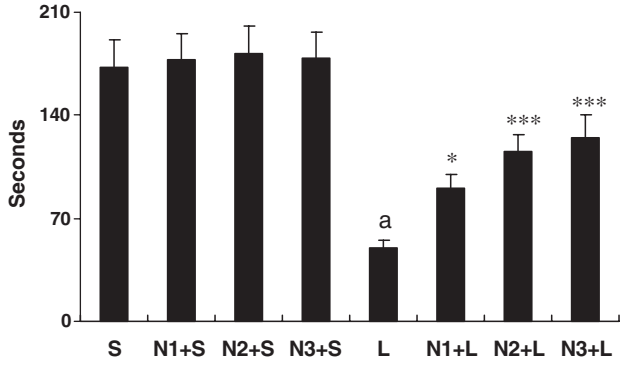

Fig. 5. The effect of *Nardostachys jatamansi* extract (200, 400 and 600 mg/kg) for 3 weeks pre-treatment on rearing and in rats lesioned by a single injection of 12.0 μg 6-hydroxydopamine/2.0 μl in 0.1% ascorbic acid-saline and sham-received ascorbic acid-saline only (vehicle). Each bar represents the mean ± S.E.M. of six animals. [a]$P<0.001$ vs. S and *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L.

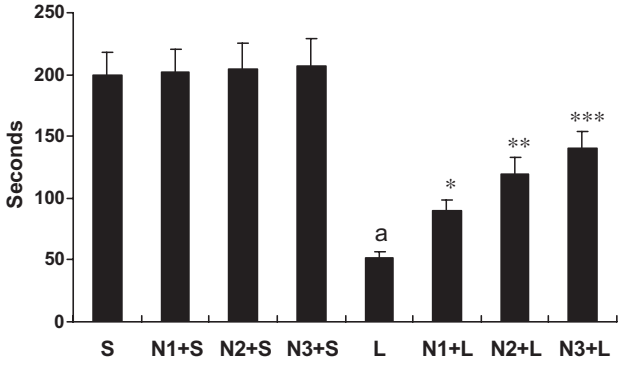

Fig. 6. The effect of *Nardostachys jatamansi* extract (200, 400 and 600 mg/kg) for 3 weeks pre-treatment on muscular coordination and in rats lesioned by a single injection of 12.0 μg 6-hydroxydopamine/2.0 μl in 0.1% ascorbic acid-saline and sham-received ascorbic acid-saline only (vehicle). Each bar represents the mean ± S.E.M. of six animals. [a]$P<0.001$ vs. S and *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L.

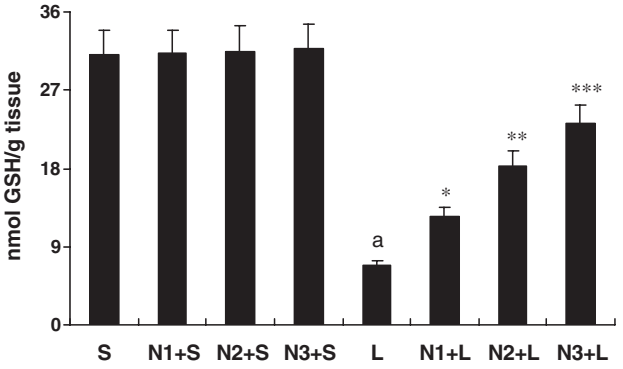

Fig. 8. Effect of extract of *Nardostachys jatamansi* extract (200, 400 and 600 mg/kg) for 3 weeks pre-treatment on reduced glutathione (GSH) content in substantia nigra in rats lesioned by a single injection of 12.0 μg 6-hydroxydopamine/2.0 μl in 0.1% ascorbic acid-saline and sham-received ascorbic acid-saline only (vehicle). Each bar represents the mean ± S.E.M. of six animals. [a]$P<0.001$ vs. S and *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L.

TBARS was significantly and dose-dependently reduced (55.53%, 129.40%, 201.00%) in the N1+L, N2+L, and N3+L groups, as compared to the L group. No significant change was observed in drug sham groups (Fig. 7).

The effect of ENj on GSH content in the substantia nigra is shown in Fig. 8. GSH content was significantly reduced by 83.58% ($p<0.001$) in the L group, as compared to the S group. The decrease in GSH content was significantly and dose-dependently restored (18.2%, 36.60%, 59.15%) in the N1+L, N2+L, and N3+L groups, as compared to the L group. No significant change was observed in drug shams.

Table 1 shows the activities of GST, GPx, and GR in striatum were significantly decreased ($p<0.001$) in the L group, as compared to the S group. Their activities were restored significantly in a dose-dependent manner in the N1+L, N2+L, and N3+L groups, as compared to the L group. No significant change was observed in drug sham groups.

Fig. 9 shows the effect of ENj on the activity of SOD activity in the striatum. The activity of SOD was significantly low (78.24%; $p<0.001$) in the L group, as compared to the S group. The decrease in SOD activity was restored significantly (24.43%, 43.06%, 61.95%) and dose-dependently in the N1+L, N2+L, and N3+L groups, as compared to the L group. No significant change was observed in drug shams. Activity of CAT in the striatum also showed the same trend, with a 77.10% decrease ($p<0.001$) in the L group, as compared to the S group. The decrease in CAT activity was significantly and dose-dependently restored (14.92%, 31.59%, 57.53%) in the N1+L, N2+L, and N3+L groups, as compared to the L group. No significant change was observed in drug sham groups (Fig. 10).

Dopaminergic D_2 receptor binding in the striatum increased manifold (300%; $p<0.001$) due to lesioning. Pretreatment of animals with ENj significantly and dose-dependently restored

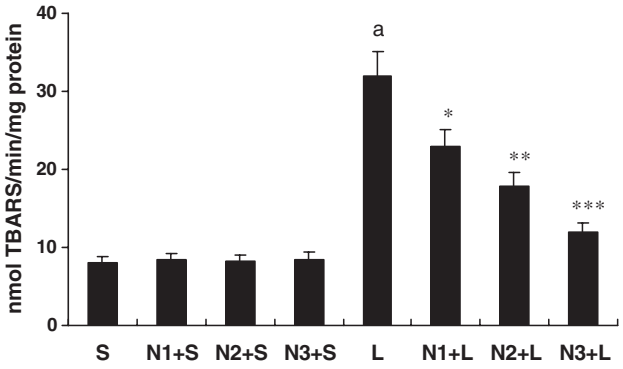

Fig. 7. The effect of *Nardostachys jatamansi* extract (200, 400 and 600 mg/kg) for 3 weeks pre-treatment on the *generation* of thiobarbituric acid reactive substance (TBARS) in substantia nigra in rats lesioned by a single injection of 12.0 μg 6-hydroxydopamine/2.0 μl in 0.1% ascorbic acid-saline and sham-received ascorbic acid-saline only (vehicle). Each bar represents the mean ± S.E.M. of six animals. [a]$P<0.001$ vs. S and *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L.

Table 1
Effect of *Nardostachys jatamansi* on the activities of GST, GR and GPx in striatum

Group	GST (CDNB conjugate formed/ min/mg protein)	GR (nmol NADPH oxidised/ min/mg protein)	GPx (nmol NADPH oxidised/ min/mg protein)
S	28.11±2.50	24.0±2.50	11.00±1.23
N1+S	28.36±2.80	24.35±2.51	11.00±1.02
N2+S	28.66±2.83	24.40±2.50	11.24±1.20
N3+S	29.13±3.05	24.45±2.02	11.32±1.04
L	6.83±0.76[a]	5.80±0.48[a]	2.71±0.25[a]
N1+L	10.15±1.03*	9.26±1.03*	4.61±0.56*
N2+L	14.15±1.51**	12.30±1.54**	5.65±0.65**
N3+L	17.2±1.52***	14.80±1.24***	7.20±0.68**

Rats were pre-treated with different doses of *Nardostachys jatamansi* extract (200, 400 and 600 mg/kg) for 3 weeks were injected once with 12.0 μg 6-OHDA/ 2.0 μl in 0.1% ascorbic acid-saline. Sham received ascorbic acid-saline only (vehicle). 6-OHDA-lesioned group differ significantly from sham group ([a]$P<0.001$) and 6-OHDA-lesioned groups + different doses of *Jatamansi*-treated groups i.e. groups N1+L to N3+L differ significantly (*$p<0.05$, **$p<0.01$, ***$p<0.001$) with 6-OHDA-lesioned group i.e. L group. There was no significant difference between sham and sham + drug-treated groups i.e. N1+S to N3+S. Each bar represents the mean ± S.E.M. of 6 animals.

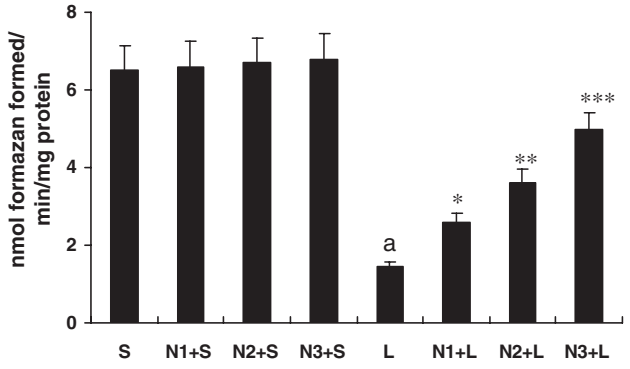

Fig. 9. Superoxide dismutase activity in striatum was protected by pre-treatment with 200, 400 and 600 mg/kg doses of *Nardostachys jatamansi* extract for 3 weeks. Rats were lesioned by a single injection of 12.0 µg 6-hydroxydopamine/2.0 µl in 0.1% ascorbic acid-saline and sham-received ascorbic acid-saline only (vehicle). Each bar represents the mean ± S.E.M. of six animals. [a]$P<0.001$ vs. S and *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L.

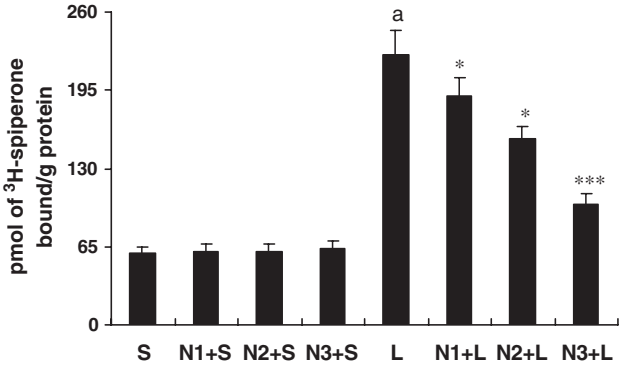

Fig. 11. Effect of *Nardostachys jatamansi* extract (200, 400 and 600 mg/kg) for 3 weeks pre-treatment on dopaminergic D_2 receptor binding in striatum in rats lesioned by a single injection of 12.0 µg 6-hydroxydopamine/2.0 µl in 0.1% ascorbic acid-saline and sham-received ascorbic acid-saline only (vehicle). Each bar represents the mean ± S.E.M. of six animals and the experiments were repeated twice. [a]$P<0.001$ vs. S and *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L.

(16%, 26%, 50%,) this dopaminergic D_2 binding in the N1 + L, N2 + L, and N3 + L groups, as compared to the L group. No significant change was observed in drug shams (Fig. 11).

Table 2 shows the effect of pretreatment with ENj on the content of DA and its metabolites DOPAC and HVA in the striatum. DA, DOPAC, and HVA were decreased significantly ($p<0.001$) in the L group, as compared to the S group, while ENj afforded a significant and dose-dependent restoration in their content in the N1 + L, N2 + L, and N3 + L groups, as compared to the L group. No significant change was observed in drug shams.

Immunohistochemical analysis showed a marked depletion in TH staining in the ipsilateral striata of the L group, as compared to the S group, and a pronounced restoration of TH staining in the ipsilateral striata of the ENj-treated, lesioned groups. We observed a significant and dose-dependent increase in the percentage of TH-IR fibers in the L + N1, L + N2, and L + N3 groups, as compared to the L group (Fig. 12). The number of TH-IR neurons in the ipsilateral side was analyzed as a percentage of the TH-IR neurons in the intact contralateral side (Fig. 13).

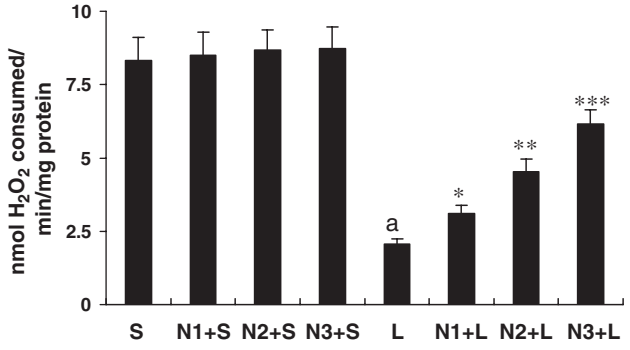

Fig. 10. Catalase activity in striatum was protected by pre-treatment with 200, 400 and 600 mg/kg doses of *Nardostachys jatamansi* extract for 3 weeks. Rats were lesioned by a single injection of 12.0 µg 6-hydroxydopamine/2.0 µl in 0.1% ascorbic acid-saline and sham-received ascorbic acid-saline only (vehicle). Each bar represents the mean ± S.E.M. of six animals. [a]$P<0.001$ vs. S and *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L.

4. Discussion

The present study demonstrates the neuroprotective effects of Jatamansi in a 6-OHDA model of Parkinson's disease. 6-OHDA caused a marked depletion of striatal dopamine content, which was significantly restored by pretreatment with Jatamansi, a result that is in accordance with the findings of Prabhu et al. (1994). Dopamine depletion is considered a cardinal feature in the causation of PD in humans or in animal models of the disease (Carder et al., 1989; Bloem et al., 1990). The enhancement of dopamine content by Jatamansi might have restored the alterations in locomotor activity and muscle coordination, which is supported by our earlier findings (Salim et al., 2003). Marked increase in amphetamine-induced

Table 2
Effect of *Nardostachys jatamansi* on the content of DA, DOPAC and HVA in striatum

Group	DA (ng/mg tissue)	DOPAC (ng/mg tissue)	HVA (ng/mg tissue)
S	9.14 ± 1.00	1.33 ± 0.15	1.15 ± 0.11
N1 + S	9.19 ± 0.91	1.35 ± 0.12	1.12 ± 0.10
N2 + S	9.30 ± 0.92	1.36 ± 0.14	1.20 ± 0.13
N3 + S	9.35 ± 0.65	1.50 ± 0.09	1.23 ± 0.09
L	2.0 ± 0.16[a]	0.35 ± 0.03[a]	0.31 ± 0.03[a]
N1 + L	3.30 ± 0.30*	0.56 ± 0.03*	0.43 ± 0.03*
N2 + L	5.50 ± 0.60**	0.75 ± 0.08***	0.65 ± 0.07**
N3 + L	7.80 ± 0.80***	0.82 ± 0.07***	0.78 ± 0.05**

Animals were pre-treated with different doses of *Nardostachys jatamansi* extract (200, 400 and 600 mg/kg) for 3 weeks were injected once with 12.0 µg 6-OHDA/ 2.0 µl in 0.1% ascorbic acid-saline. Sham received ascorbic acid-saline only (vehicle). Dopamine and their metabolites were analysed by HPLC (Waters). 6-OHDA-lesioned group differ significantly from sham group ([a]$P<0.001$) and 6-OHDA-lesioned groups + different doses of ENj-treated groups i.e. groups N1 + L to N3 + L differ significantly (*$p<0.05$, **$p<0.01$, ***$p<0.001$) with 6-OHDA-lesioned group i.e. L group. There was no significant difference between sham and sham + drug-treated groups i.e. N1 + S to N3 + S. Each bar represents the mean ± S.E.M. of 6 animals.

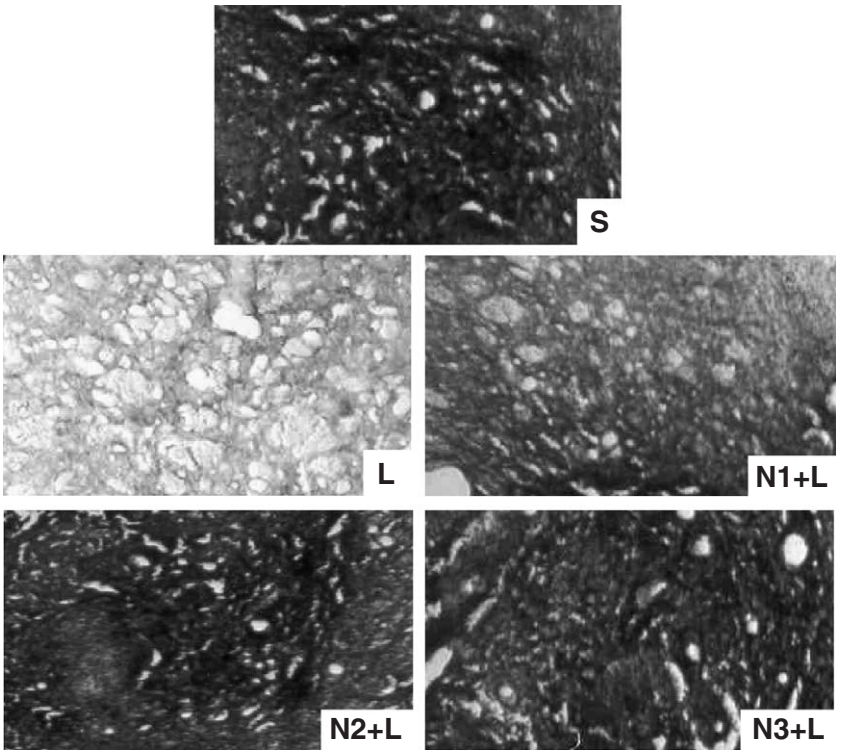

Fig. 12. Shows the effect of different doses of ENj on tyrosine hydroxylase staining in ipsilateral striatum. The expression of Tyrosine hydroxylase is almost negligible in L group as compared to S group while in groups N1+L to N3+L, there is dose dependent staining for tyrosine hydroxylase. However, in all the sham groups no discernible change in tyrosine hydroxylase staining was observed. Scale bar is 150 μm and magnification is 4×.

ipsilateral and apomorphine-induced contralateral rotation in 6-OHDA-lesioned rats is considered a very reliable marker for nigrostriatal dopamine depletion (Schwarting and Huston, 1996), which corroborates our findings. Further, Schwarting and Huston (1996) have shown that rotation might take place only when nigrostriatal neuron degeneration is more than 74%, while the partially lesioned rats do not rotate after such treatment. The restoration of drug-induced rotations by Jatamansi thereby confirms its protective role against 6-OHDA parkinsonism. The degeneration of dopaminergic neurons leads to an increase in the population of dopamine receptors (Schwarting and Huston, 1996) and we are of the opinion that it happens, as a compensatory mechanism to trap and utilize almost every available molecule of dopamine. In the present study, the increase in the D_2 receptor population in striatum due to 6-OHDA lesioning was significantly restored by pretreatment with Jatamansi.

Oxidative stress refers to the cytologic consequences of a mismatch between the production of free radicals and the ability of the cell to defend against them (Halliwell and Gutteridge, 1985; Freeman and Crapo, 1982). A defect in one or more of the naturally occurring antioxidant defenses could lead to neuro-degeneration in PD (Jenner and Olanow, 1996). Oxidative stress can thus occur when the production of free radicals increases, scavenging of free radicals or repair of oxidatively modified macromolecules decreases, or both. This imbalance results in a build-up of oxidatively modified molecules that can cause cellular dysfunction, and for neurons, it is lethal. Oxidant stress has been implicated in PD because of the coalition of the four biochemical features of the dopaminergic neurons in the substantia nigra, i.e., monoamine oxidase-B activity (Cohen, 1983; Oreland, 1991), autooxidation of dopamine (Graham, 1979; Halliwell and Gutteridge, 1985), accumulation of iron (Youdim and Lavie, 1994), and neuromelanin (Hirsch, 1993).

Our studies show an increase in lipid peroxidation and a decrease in GSH and the activities of related enzymes and of CAT and SOD, observations which are very well supported

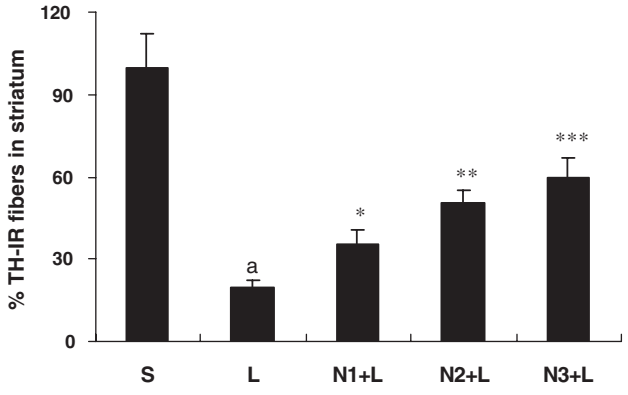

Fig. 13. Effect of *Nardostachys jatamansi* extract on the density of tyrosine hydroxylase-immunoreactive (TH-IR) fibers in ipsilateral striatum in 6-hydroxydopamine-lesioned animals (ratio of lesioned/intact side). Density of TH-IR fibers was calculated, averaging six sections per rat and three rats per group. $^{a}P<0.001$ vs. S and $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. L.

by our earlier findings (Ahmad et al., 2005a,b; Zafar et al., 2003a,b) and those of many authors (Perumal et al., 1992; Kumar et al., 1995). A reduction in GSH might impair H_2O_2 clearance and promote $OH^{\bullet}$ formation and, hence, oxidative stress. In that all antioxidant defenses are interrelated (Sun, 1990), the disturbance in one might derange the balance in all. The depletion in GSH content and enhancement of lipid peroxidation leads to the degeneration of nigrostriatal neurons and, consequently, leads to reduction in the content of catecholamines. The marked restoration of lipid peroxidation and enhancement of GSH content and antioxidant enzyme activities by pretreatment with Jatamansi is supported by our recent findings (Salim et al., 2003) and that of Tripathi et al. (1996).

6-OHDA produces grave behavioral, biochemical, and pathological changes typical of PD (Sachs and Jonsson, 1975; Bloem et al., 1990). These toxic effects are related to the formation of various oxidants and free radicals (Cohen et al., 1976) and lipid peroxidation (Slater, 1984). These toxic effects might be related to the low levels of GSH and diminished activities of antioxidant enzymes, as is clearly shown by the present study. GSH is the primary low-molecular weight thiol in the cytoplasm and is a major reserve for cysteine. GSH, in conjunction with the reductant NADPH, can reduce lipid peroxides, free radicals, and H_2O_2. GSH is converted to oxidized glutathione, which is reconverted to GSH by GR, while GST biotransforms the xenobiotics via the mercapturic acid pathway. Because GSH is involved in the detoxification of H_2O_2, reductions in GSH content could result from increased concentrations of H_2O_2 and, in the presence of metals, the highly reactive hydroxy radical. Mytilineou et al. (1998) demonstrated that buthionine sulphoximine (BSO), which is a selective inhibitor of α-glutamylcysteine synthetase (a key enzyme in the synthesis of GSH), induces a reduction in GSH content and is toxic to cultured dopaminergic neurons. GSH depletion enhances the neurodegeneration that is observed when rodents are treated with other toxins, such as 6-OHDA (Pileblad et al., 1989; Wüllner et al., 1996). Most H_2O_2 in the brain is removed by GPx, which uses it to oxidize GSH (Beckman et al., 1990). Our present study, shows that a restoration in the content of GSH and in the activities of its dependent enzymes might be due to the GSH-enhancing or antioxidant effects of Jatamansi, which are very well supported by the previous findings of Salim et al. (2003) and Tripathi et al. (1996).

SOD and H_2O_2-removing enzymes protect the cell against reduced intermediates of oxygen produced during normal aerobic metabolism, but they seem unable to cope when production of O_2 and H_2O_2 is excessive. SOD converts O_2 to H_2O_2 (Freeman and Crapo, 1982; Fridovich, 1975). Catalase, which is found at very low levels in the brain, also removes H_2O_2. Restoration of the activities of SOD and CAT due to pretreatment with Jatamansi further demonstrates the protective role of Jatamansi in 6-OHDA toxicity and is supported by various studies (Perumal et al., 1992; Zafar et al., 2003a,b; Ahmad et al., 2005a,b), in which parkinsonism was partially protected by the application of antioxidants. All of these effects are further evidenced by the increasing TH-IR fiber density in the ipsilateral striata after lesioning due to pretreatment with ENj, clearly signifying the dose-dependent increase in the number of surviving neurons and confirming the anti-Parkinson effects of ENj.

Antioxidants are a key to prevention and control of PD; many have reported that Parkinsonism was partially protected by the application of antioxidants (Cadet et al., 1989; Perumal et al., 1992; Zafar et al., 2003a,b; Ahmad et al., 2005a,b). Drugs that enhance the availability of dopamine or prevent its breakdown afford protection against PD in humans or in animal models (Parkinson Study Group, 1989; Myllyla et al., 1992). In our study, Jatamansi extract, which is reported to have both dopamine-enhancing properties (Prabhu et al., 1994) and antioxidant potential (Tripathi et al., 1996; Salim et al., 2003), might have afforded protection in the same way.

In conclusion, we anticipate that *Nardostachys jatamansi*, having shown anti-parkinsonian properties in our studies, can be used as a favored remedy for Parkinson's disease, pending further studies to elucidate proper mechanisms.

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