

Biochemical and Behavioral Evidences for Neuromodulatory Properties of *Selaginella prophyllaxis* Against 3-Nitropropionic Acid in Mice

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ABSTRACT

AIM: Experimental evidence on the neuromodulatory properties of the Indian resurrection herb 'Selaginella' (a pteridophyte) is limited. Here, we examined the hypothesis that extract of *Selaginella delicatula* can render protection against 3-nitropropionic acid (3NP)-induced oxidative impairments and neurotoxicity.

MATERIALS AND METHODS: Mice provided with supplements *S. delicatula* aqueous extracts (SDAE) orally (400 mg/kg bw/d, 15d) were challenged with 3NP (50 mg/kg bw/d, i.p. from day 11- day 15).

RESULTS: Exposure to 3NP induced significant motor deficits, as evidenced by the reduced stride length, increased narrow beam latency and overt behavioral deficits in the open field apparatus. Interestingly, there was a significant delay in the onset and the intensity of neurobehavioral deficits among SDAE prophylactic mice. Biochemical investigation of striatum revealed that SDAE prophylaxis modulated the striatal redox status but also attenuated the 3NP-induced oxidative stress, activity of antioxidant enzymes and mitochondrial complex II. More importantly, AChE activity and

dopamine levels were nearly normal among SDAE-mice.

CONCLUSION: Collectively, these data suggest that neuroprotection rendered by SDAE prophylaxis may be primarily attributed to the restoration effect on antioxidant defence and mitochondrial function which lead to improved locomotor phenotype. We propose that *Selaginella* aqueous extracts may provide new perspectives for the development of therapeutic strategies in protecting the brain against oxidative stress-mediated neurodegenerative disorders.

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Key words: *Selaginella delicatula*; 3-nitropropionic acid; Huntington disease; Motor function; Oxidative stress; Mitochondria

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INTRODUCTION

Neurodegeneration is a broad term used to describe the progressive loss of structure/ function of neurons, subsequently followed by death of the same. Disorders arising from degeneration of mid brain regions resulting in hypokinesia, hyperkinesia and/or dyskinesia are classified as movement disorders. One such movement disorder is Huntington's disease (HD) characterized by involvement of the striatal neurodegeneration^[1,2]. Apart from various molecular mechanisms, key contribution in disease progression is by the mutation of huntingtin protein, resulting in hampered cellular homeostasis. Besides, the major contribution factors for the pathophysiology of the disease include excitotoxicity, dopamine toxicity, mitochondrial dysfunction, oxidative stress, apoptosis and autophagy. The process

of neurodegeneration can be mimicked *in vitro/ in vivo* by exposure to various toxins viz., Rotenone, 3-nitro propionic acid (3NP), Paraquat, MPTP, MPP+M 6-OHDA etc.^[3-5].

3NP, a mycotoxin is known to cause significant neurotoxicity predominantly affecting the neurons involved in locomotor behavior in humans and animals. Studies in laboratory animals, suggest that 3NP induces neuronal damage that mainly affects the striatum and mimics most of the histological and neurochemical features of HD^[4,6] and hence this phenotypic model is gaining attention as a valuable tool to develop new therapies for HD^[4]. The mechanism of 3NP induced neurotoxicity involves inhibition of succinate dehydrogenase (SDH), an enzyme that acts in mitochondrial tricarboxylic acid cycle and the electron transport chain at complex II^[7] along with increased oxidative stress, as demonstrated by recent studies leaving scope for use of phytochemicals to reverse the neuronal damage.

Although the treatment approaches are limited^[1,2], proposed treatment paradigms generally involve the use of natural antioxidants exhibiting their action by either or combination of speculated processes involving (i) suppression of oxidative/ nitrosative stress (ii) enhancement of mitochondrial function in brain regions and (iii) alleviation of motor deficits. Hence various potent phytochemicals are being explored for HD-therapy owing to their free radical scavenging ability and potency to alleviate oxidative insult *in vivo*^[8]. Ferns represent a group of plants with possible potent candidates for neuroprotective strategies while there is no such data despite the use by the local tribes for different ailments. *Selaginella* (family: Selaginellaceae), a lithophytic fern (with over 700 species worldwide), is used to promote blood circulation, treat cardiovascular diseases and amenorrhea in traditional Indian and Chinese medicine^[9]. Tribal methods suggest consumption of aqueous leachates (overnight soaking) for the treatment of jaundice and urinary problems^[9]. Extracts of *Selaginella* are reported to possess antioxidant and immunomodulatory properties in experimental models of cancer cell lines and rodents^[10,11]. Various biflavonoids (the characteristic flavonoid dimers) identified in *Selaginella spp.* are reported to possess anti-metastatic and anti-inflammatory properties^[10,12]. Hence, we set out to understand the neuroprotection rendered by *Selaginella* and selected to work with *S. delicatula* (Desv. Alston, Collection site: Kannur, partially situated in Western Ghats of India).

Previously we have demonstrated the neuroprotective propensity of SDAE against rotenone induced neurotoxicity/ motor aberrations employing *Drosophila* and mice models^[13,14]. Hence we hypothesized that SDAE is more likely to protect against HD-like neuro-motor phenotype and striatal oxidative stress in mice model of 3NP. Accordingly, in the present study, we have gathered evidence on the prophylactic propensity of oral supplements of SDAE against 3NP-induced motor dysfunction, striatal oxidative stress and mitochondrial dysfunction in a mice model.

MATERIALS AND METHODS

Chemicals: All fine chemicals were of analytical grade procured from m/s Sigma Chemical Co. St. Louis, USA and m/s Sisco Research Laboratory (SRL) chemicals, India. Primary antibodies were purchased from m/s Sigma Chemical Co. St. Louis, USA and secondary antibodies from m/s Merck-Genei, Bangalore, India

Collection of plant material and preparation of *S. delicatula* aqueous extracts (SDAE): The plants were collected from Kannur, Kerala, India; authenticated by Dr. Raju Antony, Pteridophyte Taxonomist, Tropical Botanic Garden and Research Institute

(TBGRI), Thiruvananthapuram, Kerala, India, as *Selaginella delicatula* (Desv.) Alston; and herbarium preserved in our laboratory. *Selaginella delicatula* aqueous extract (SDAE) was prepared as described earlier^[13].

Animals and care: Male CFT-albino mice (4wk old, 24.3 ± 1.5 g) obtained from the 'Institute Animal House Facility' were housed in controlled atmosphere with a 12h light/dark cycle and maintained on a commercial chow (M/s Sai Durga Animal Feeds, Maharashtra, India) and tap water ad libitum. All the animal experiments including gavage, injection and sacrifice were conducted strictly as per the guidelines of 'Institutional Animal Ethics Committee' regulated by the committee for the purpose of Control and Supervision of Experiments on animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (Registration number: 49/1999/ CPCSEA).

Neuromodulatory propensity of SDAE in mice

Treatment regimen: Male mice were randomly assigned to four groups ($n=6$). The lyophilized aqueous extract was dissolved in saline (25mg/ mL) and given as oral supplements daily. 3NP was prepared afresh daily (concentration -25mg/ ml in saline). The criterion of dosage and duration selection of 3NP was based on previous studies from our laboratory^[9]. The treatment lasted for total of 15 days as follows:

Group I: Control - 100µl saline/ animal/ d, p.o. for 15 days;

Group II: SDAE - 400mg/ kg bw/ d, p.o. for 15 days;

Group III: 3NP - 50mg/ kg bw, i.p. on days 11, 12, 13, 14, and 15;

Group IV: SDAE+3NP- 400mg SDAE/kg bw/d, p.o. (for 15 days) and 3NP 50mg/ kg bw/d, i.p. (on days 11, 12, 13, 14 and 15).

The food intake was monitored daily and body weights were recorded every alternate day. Open field test and motor function tests were conducted on mice of all groups prior to the treatment and on days 10, 11, 13 and 15. Terminally, mice of control and treatment groups were sacrificed, brain excised and washed in ice cold phosphate buffered saline (0.1M, pH 7.4, 0.15M NaCl). The brain regions were separated on ice and striata were used for further biochemical analysis.

Neurobehavioral analyses

Open field test: The open-field test was performed under light condition as described earlier^[15]. The open field consisted of a square wooden platform (45 × 45 cm) with sidewalls (20cm height), inner walls and floor painted white. A central square (25 × 25 cm) marked was subdivided into nine equal squares. The open field was illuminated using 20W CFL bulb during the analysis. The animals were habituated with the apparatus 2days earlier to the total treatment regimen and repeated earlier to 3NP exposure (day 10). For the analysis of spontaneous activity, the animal was individually placed at the central square and recorded using a video camera for 3min. The videos were later analyzed manually for (1) initial latency to leave centre; (2) number of quadrants entered; (3) rearing behavior; (4) total time idle; (5) total time spent in margin and center; and (6) total average distance travelled (data not presented).

Stride length (SL) analysis: The SL apparatus^[14] contained a brightly lit runway (42 cm long, 4.5 cm width) having borders of 12 cm height, leading to a dark box (20 × 17 × 10 cm). For SL measurement, the animals were habituated to the apparatus, three days prior to the beginning of the treatment. Briefly, the analysis involved wetting the forepaws of the animals with pencil ink, and letting them trot on a strip of paper (42 cm long, 4.5 cm width) laid on the brightly lit runway towards the dark box. Three longest

stride lengths were measured and expressed as the distance (in cm) between two consecutive prints of the same paw, leaving the 7cm each on either ends of the paper strip (to avoid initial acceleration and terminal deceleration by the animal). SL measurements were expressed as distance between the two paw prints in centimeters.

Landing foot spread distance (LFSD) analysis: LFSD was measured among both control and treated rats as described previously^[16] with minor modifications. Briefly, the apparatus consisted of a wooden open field (60L × 30h × 40w cm) with a semi-hard base. The hind paws of mice were wetted with ink and were dropped from a height of 30cm onto the soft base of the apparatus and immediately the distance between the extreme digits on left and right hind paw prints were recorded. Three such observations were made for each animal. LFSD measurements were expressed as the distance between the two paw prints in centimeters.

Narrow Beam Test (NBT): Narrow beam test^[17] was used to measure hind-limb impairments. The narrow beam (NB) apparatus contained a wooden beam (60 cm long, 2 cm diameter) placed at a height of 20cm. One end of the beam uncovered while the other end leads into a dark goal box (20 cm height, 8 cm width). The whole apparatus was painted black. For the analysis, the animals were habituated to the apparatus, three days prior to the treatment. The animals were placed on the free end of the beam and allowed to crawl to the dark goal box. The time taken to cross the 50 cm of the beam and latency to enter the dark goal box (even after crossing the beam successfully) were recorded manually and expressed as latency to cross the beam (in seconds) and to enter goal box respectively. Each animal was tested for three trials with an interval of 5 min.

Biochemical determinations in striatum

Preparation of cytosol and mitochondria: The striata were separated on ice and homogenized (10% w/v) in ice-cold Tris-HCl buffer (pH 7.4, with 0.25M sucrose) and subjected to differential centrifugation (3000 rpm and 10000 rpm, 10min each, 4°C) to separate mitochondria and cytosol from the post nuclear supernatant^[18]. The crude mitochondrial pellet was washed with HEPES buffer (pH7.4, in mM: HEPES-10, EDTA-0.1, Mannitol-200, Sucrose-70) and suspended with the same. Protein in the samples was estimated using BSA as a standard^[19].

Quantification of MDA, ROS generation and hydroperoxide levels: The extent of lipid peroxidation was measured in terms of MDA by estimating the thiobarbituric acid reactive substances and expressed as nmol MDA formed/ mg protein^[20]. Generation of Reactive oxygen species (ROS) was determined^[14] by following DCF fluorescence. The ROS generation was quantified from a DCF standard curve and represented as pmol DCF/ min/ mg protein. The levels of hydroperoxides (HP) were determined based on a recycled ferrous ion mediated oxidation of xylenol orange^[21]. The color developed was quantified at 560nm (€ -15/mM/cm) and expressed as nmol hydroperoxides (HP)/ mg protein.

Estimation of reduced glutathione (GSH) and total thiols (TSH): The levels of reduced glutathione were estimated by following o-phthalaldehyde (OPT) fluorescence^[22]. Expressed as μmol GSH/ mg protein. For total thiol (TSH) estimation, an aliquot of sample (0.02 mg protein) was added to 0.190 mL of Tris-HCl buffer (0.2 M, pH 8.2) containing di-thiobis-nitrobenzoic acid (DTNB, 10 mM) in a 96-well microtitre plate. Following incubation for 30 min at room temperature the absorbance of the supernatant was measured at 412 nm and expressed as nmol TSH/mg protein (€-13.6/mM/cm)^[14].

Measurement of protein carbonyls (PC): The extent of protein oxidative modifications in the test sample was quantified as protein

carbonyls^[23] following reaction of sample (0.5mg protein) with 2, 4-Dinitrophenyl hydrazine in an acidic medium (2N HCl) for 1hr. The protein precipitated (using trichloroacetic acid 20%) was washed twice with cold acetone and dissolved in 2mM Tris-HCl buffer (pH 7.4, containing 2% SDS). The absorbance was measured at 370 nm and expressed as nmol carbonyl/mg protein (€ -22/ mM/ cm).

Measurement of nitrated proteins (3NT): slot-blot protocol: The extent of protein nitration was estimated in terms of 3-nitrotyrosine (3NT) employing the most used method of slot-blot^[14]. Briefly, aliquots (15 μg protein/ sample) were spotted in triplicates onto a nitrocellulose membrane using a slot blot apparatus. The membranes blocked with 2% BSA in PBST (PBS, 0.1M, pH 7.4, containing 0.14M NaCl and 0.05% Tween-20) for 60min at room temperature were probed with antibodies against 3-NT (1: 500)/ β-actin (1: 500) for the next 60min followed by alkaline phosphatase-conjugated secondary anti rabbit IgG (1: 3000) for 60min. The membrane were developed employing BCIP/ NBT reaction and optical density was measured with ImageJ free Software (NIH, USA). The extent of protein nitration was expressed as arbitrary units of pixels (3NT/β-actin).

Western blot protocol: Briefly, aliquots (20 μg protein/ sample) were loaded on to a 10% polyacrylamide gel electrophoresis (100mV) and the proteins were transferred onto a nitro cellulose membrane (30mV, 2hrs). The membranes blocked with 2% BSA in PBST (PBS, 0.1M, pH 7.4, containing 0.14M NaCl and 0.05% Tween-20) for 60 min at room temperature were probed with antibodies against HSP-70 (1: 500)/ β-actin (1: 500) for the next 60 min followed by Alkaline phosphatase-conjugated secondary anti rabbit IgG (1: 3000) for 60min. The membrane were developed employing BCIP/ NBT reaction and optical density was measured with ImageJ free Software (NIH, USA). Result was expressed as arbitrary units of pixels (HSP-70/ β-actin).

Activity levels of antioxidant enzymes: Superoxide dismutase (SOD) activity was measured using an indirect assay that monitors the inhibition of Quercetin autooxidation^[24] and expressed as units/ mg protein. Thioredoxin reductase (TR) activity^[25] was measured by monitoring the reduction of DTNB (250μM) at 412nm, in a potassium phosphate buffer (0.1M, pH 7.0, containing in mM: EDTA-10, NADPH-0.2). The activity was expressed as nmol substrate reduced/min/mg protein (€-13.6/mM/cm). Glutathione peroxidase (GPx) activity was measured^[26] by following the oxidation of NADPH and activity expressed as nmol NADPH oxidized/ min/ mg protein (€-6.22/mM/cm). Glutathione-S-Transferase (GST) activity was estimated^[27] by monitoring the conjugation of glutathione to CDNB at 340nm. The activity was expressed as nmol conjugate formed/ min/ mg protein (€ -9.6/mM/cm).

Activity of acetylcholinesterase: AChE activity was estimated according to a standard method^[28]. Enzyme catalyzed decomposition of Acetylthiocholine iodide (ATCI, 1.95mM in Phosphate buffer pH 8.0) was monitored by using a colored substrate, DTNB (2.5mM) at 412 nm and the enzyme activity was expressed as nmol substrate hydrolyzed/ min/ mg protein (€ -13.6 /mM/cm). BChE activity was measured using butyrylthiocholine iodide (BTCl, 1.8mM) instead of ATCI.

Estimation of dopamine levels by HPLC analysis: Dopamine (DA) levels in the striatal regions were analyzed by ultraviolet detection in a HPLC^[14]. A known volume of ice cold 5% TCA was added to equi-volume of the cytosolic fraction and centrifuged (5000 rpm, 10 min). The clear protein free supernatant was assessed in a HPLC system (Shimadzu LC-10 Avp) embroidered with an ultraviolet detector (280 nm). Sample was injected into a C18

column ('Discovery' Supelco-Sigma-Aldrich; C-18, 25cm-4.6mm, 5 μ m) and eluted with a cocktail of 0.2% aqueous trifluoroacetic acid and methanol (70: 30 v/v) at the flow rate of 1 ml/min (room temperature). DA levels were calculated from an external standard and expressed as μ g DA/mg protein.

Mitochondrial function markers

Activity levels of mitochondrial enzymes: The activity of succinate-cytochrome C reductase (complex II) was assessed following the reduction of cytochrome C^[29]. The reaction was started by adding cytochrome 'C' (0.1mM) to the phosphate buffer (0.1M, pH 7.4) containing mitochondria (0.1mg protein), Sodium succinate (20mM) and potassium cyanide (1 mM). The increase in absorbance was monitored at 550 nm for 3 min and the activity expressed as nmol cytochrome 'C' reduced/min/mg protein (ϵ -19.6 mM/cm). Mitochondrial function marker enzyme citrate synthase (CS) was determined^[30] by incubating an aliquot of mitochondrial fraction (10 μ g protein) in a 96 -well micro titre plate in Tris HCl buffer (0.1M, pH 8.0 containing 0.1% Tween-20; 0.2 mM DTNB, 0.5 mM oxaloacetate). The reaction was initiated with the addition of acetyl coenzyme A (0.05 mM) and the difference in the optical density was monitored over 3 minutes at 412 nm. The enzyme activity was expressed as nmol thiol/ min/ mg protein (ϵ -13.6/ mM/ cm).

MTT reduction assay^[31] was performed by incubating the mitochondrial aliquot (0.015 mg protein) in the mitochondrial resuspension buffer (containing sodium succinate – 20 mM, MTT 75 mg/ml) for 60 min at 37°C. The resultant purple-blue formazan dissolved in SDS reagent (pH 4.5, 10% – SDS, 45% – DMF) was measured at 570nm and expressed as Optical density/mg protein.

Estimation of mitochondrial membrane potential: Mitochondrial membrane potential was determined by measuring mitochondrial uptake of Rh123^[13]. Briefly, freshly prepared mitochondrial fraction (0.2 mg protein) was incubated in phosphate buffered saline (0.1M, pH 7.4, containing sucrose -75mM, Rh123 -0.8 μ M) at 37°C for 60 min. Following centrifugation at 10,000 g for 10 min, the supernatant was analyzed for rh123 fluorescence (excitation -490, emission -520nm). Higher the fluorescence (inability of mitochondria to retain the dye) lower is the membrane potential, expressed as Rh123 fluorescence/ mg protein.

Activity levels of mitochondrial membrane atpases: Mitochondrial ATPases viz., total, Mg²⁺ and Na⁺K⁺ ATPases were determined following the hydrolysis of ATP and subsequent estimation of the inorganic phosphate liberated^[32]. The assay buffers for (A) total ATPase consisted of Tris-HCl (0.01M, pH 7.4, containing in mM: NaCl-140, KCl-14, MgCl₂-3, ATP-3, EDTA-0.05, (B) Mg²⁺ ATPases assay buffer had Tris-HCl buffer (0.01M, pH 7.4) containing ATP (3 mM), MgCl₂ (3 mM), CaCl₂ (0.5 mM), NaCl (140 mM) and Ouabain (0.1 mM). The reaction was initiated by adding mitochondria (25 μ g protein) to the assay buffer and incubated for 15 min at 37°C. The reaction was stopped by the addition of 10%TCA. The inorganic phosphate (Pi) liberated was determined using molybdic acid method^[33] by incubating the assay mixture with coloring reagent (0.5% ammonium molybdate and 2% ascorbic acid in 1N H₂SO₄) and absorbance measured at 820nm. The enzyme activity was expressed as μ g Pi liberated/mg protein. The Na⁺-K⁺ ATPase activity was calculated as the difference between Mg²⁺ and total ATPase^[34].

Statistical analysis

All values are expressed as mean \pm SEM (standard error of the

mean). Data were analyzed with non-parametric one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test ($P \leq 0.05$). All statistical analyses were performed using GraphPad (Free statistical software package) version 5.0.

RESULTS

Food intake and growth

Oral supplements of SDAE for 15 days did not alter the food intake pattern among mice. Further, administration of 3NP also had no effect on the feed intake. As a consequence, SDAE supplements caused no change in body weight of mice (Initial: 33.5 \pm 0.65g; Terminal: 34.8 \pm 0.75g). Likewise 3NP administration also did not alter the body weights (Initial: 33.8 \pm 0.80g; Terminal: -36.2 \pm 0.97g) irrespective of whether or not provided with SDAE supplements.

Effects of SDAE against 3np induced locomotor phenotypes

Stride length (SL), Landing foot spread distance (LFS) and Narrow beam test (NBT): 3NP administration caused significant perturbations in the walking abilities of mice. Significant (31-35%) reduction in the SL was evident among mice measured on day13 and day15, while mice given SDAE prophylaxis exhibited normal SL scores (Figure 1A). Further, 3NP caused a progressive increase in LFS (post first dose-30% and post fifth dose-(35%), and the extent of protection though was initially marginal, complete normalization was evident by day15 (Figure 1B) with SDAE prophylaxis.

Interestingly, mice exposed to 3NP exhibited progressively elevated latency to cross the narrow beam (day11- 1.3 folds; day13- 2.2 folds; day 15- 3.4 folds), while mice given SDAE prophylaxis displayed normal latency. In addition, 3NP exposure caused a robust increase in the goal box latency (day11-16.4 folds; day13- 25 folds; day15- 107 folds), while this was also significantly reduced among SDAE mice (Figure 1C). As anticipated, the narrow beam (NB) latency (2 folds) and goal box (GB) latency (7 folds) were reduced among control mice during the observation period of five days, though they were trained until concordant latency recordings (Figure 1D).

Open field test (OFT): A significant changes in the OFT behavior was observed among control mice during the days of analysis. As anticipated, 3NP mice manifested severe alterations in the OFT behavior. There was a progressive but significantly marked reduction in the total distance travelled by 3NP mice (same day values were compared) from day11 (23%) to day15 (90%) which was partially normalized among SDAE mice on day11 (31%) and day15 (68%) (Results not presented). There was a reduction in the total number of quadrants crossed by 3NP mice reaching the minimum number on day 15 (71%), however it was normalized in mice receiving SDAE supplements on days 11 (45%) and 13 (170%), while on day 15 normalization was only partial (20%) but significant (Figure 2A). 3NP exposure to mice caused a significant increase in the total time idle (day11: 3folds; day13: 15folds and day15: 5folds) and this was normalized in mice receiving SDAE, while SDAE mice behaved normally (Figure 2B). Interestingly, we observed significant reduction in grooming activity among 3NP mice (grooming + rearing), and in contrast, absolutely no grooming was evident among 3NP mice on day15 (Figure 2C). 3NP mice displayed elevated latency for their first move out of the central area (day 15: 5 folds increase), while SDAE supplements reduced the effect (Figure 2D).

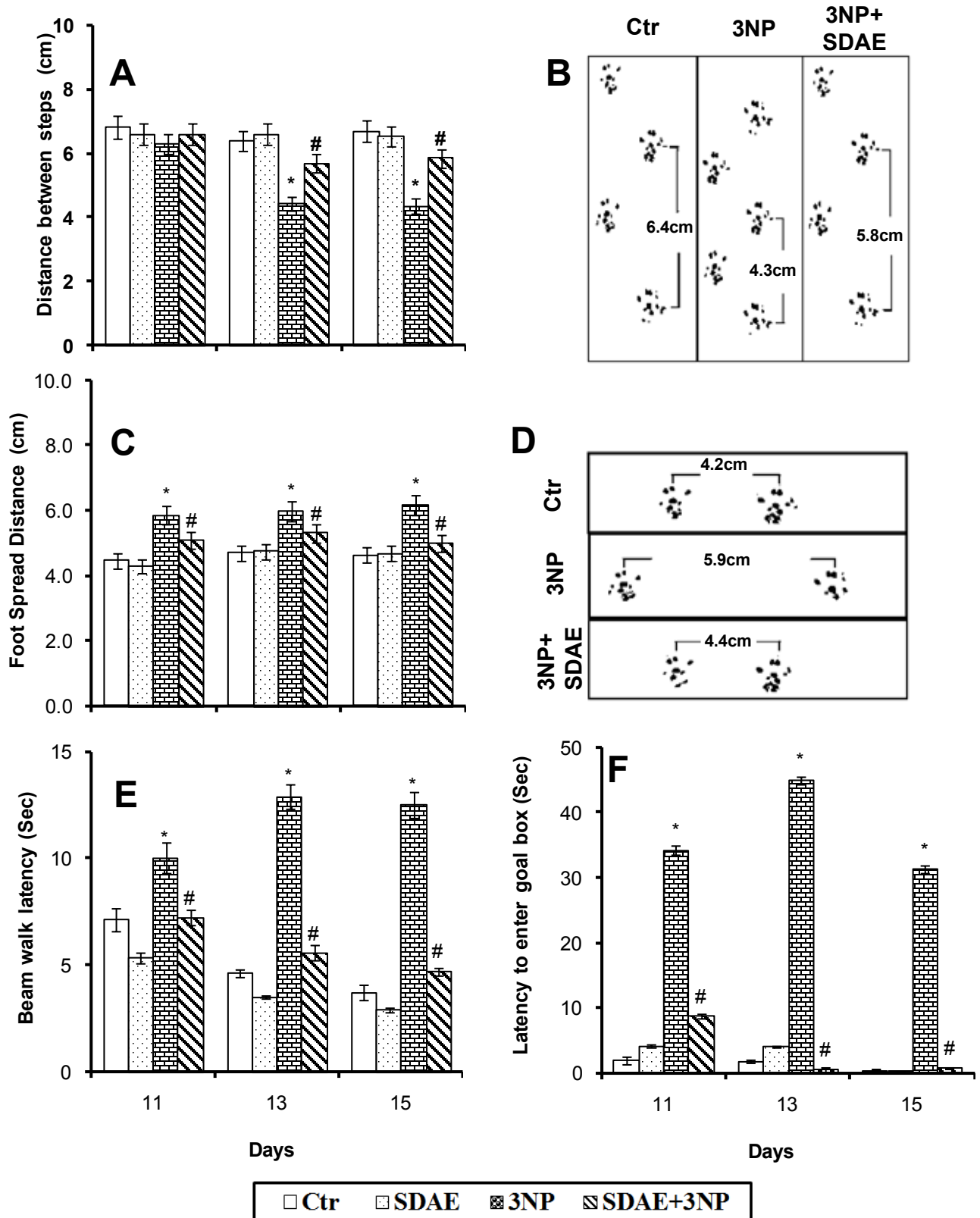


Figure 1 Modulatory efficacy of *S. delicatula* aqueous extracts (SDAE) against 3NP induced motor deficits as assessed on day 11, 13 and 15 (A) Stride length-SL graph and (B) representative imprint of the footsteps; (C) Landing foot spread distance-LFSD graph and representative imprint of the foot prints (D) ; (E) Narrow beam latency-NBL: time taken by the animal to travel across the beam; (F) Goal box latency: time taken to enter the goal box placed terminally on the beam. Values are mean \pm SEM (standard error of mean). Results analyzed by non-parametric one-way ANOVA followed by post hoc Tukey's test. CTR = control; SDAE = 400mg/ kg bw/d (p.o.) 15d; 3NP: 3-nitropropionic acid 50mg/ kg bw/d (i.p.) on last 5d. * compared to CTR ($p \leq 0.05$); # compared to 3NP ($p \leq 0.05$).

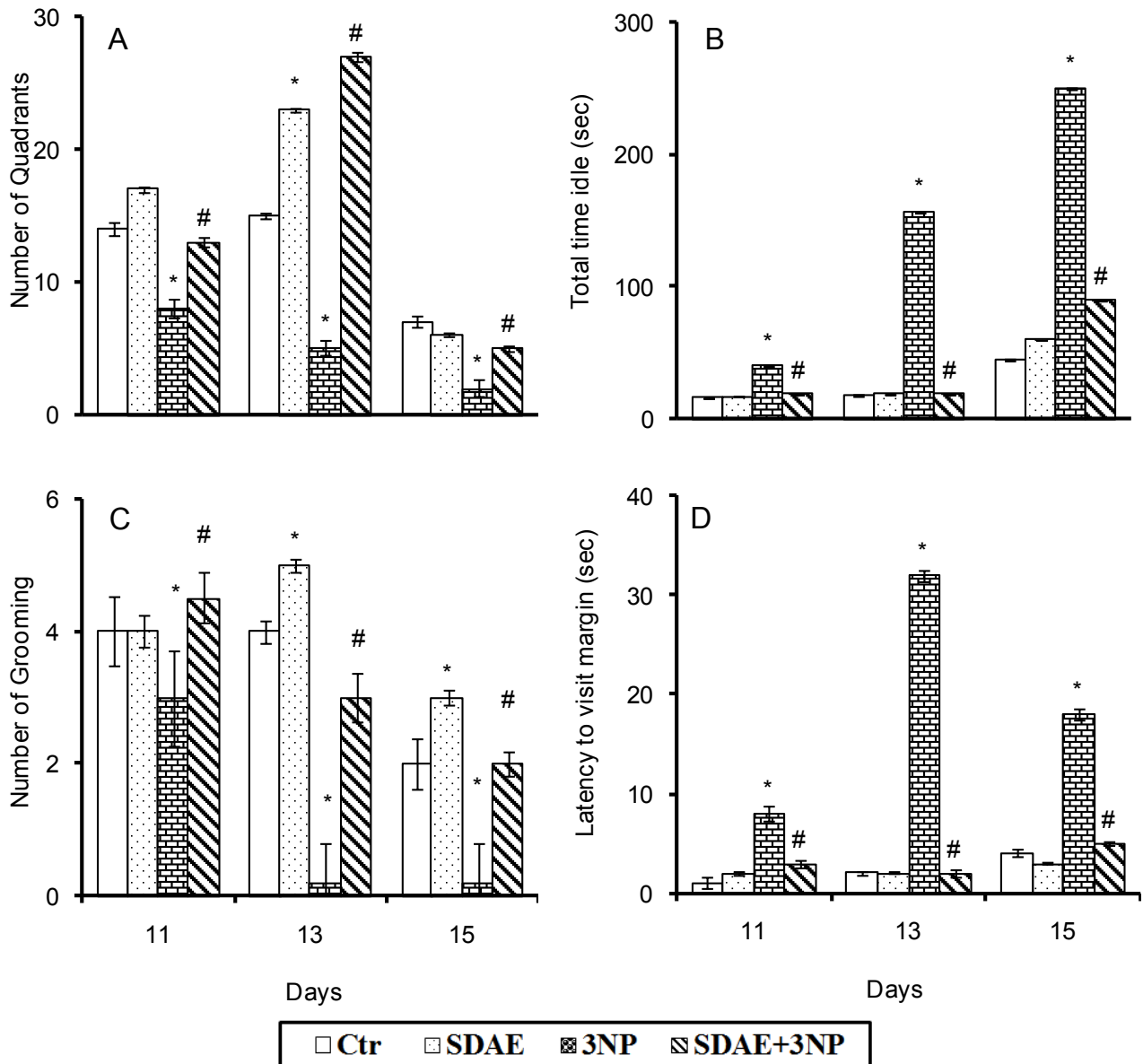


Figure 2 Modulatory efficacy of *S. delicatula* aqueous extracts (SDAE) against 3NP induced changes in open field behavior as assessed on day 11, 13 and 15 (A) No. of quadrants crossed; (B) Total time idle; (C) No. of grooming and (D) Latency for the first visit to the margin. Values are mean \pm SEM (standard error of mean). Results analyzed by non-parametric one-way ANOVA followed by post hoc Tukey's test. CTR: Control; SDAE: 400mg/ kg bw/d (p.o.) 15d; 3NP: 3-nitropropionic acid 50mg/ kg bw/d (i.p.) on last 5d. * compared to CTR ($p \leq 0.05$); # compared to 3NP ($p \leq 0.05$).

Effect of SDAE on 3np -induced striatal oxidative stress and enzymic antioxidants

Oxidative markers: 3NP exposure induced a marked elevation in striatal ROS levels (76%), while significant restoration was evident among mice provided with SDAE prophylaxis (Table 1). SDAE per se also caused an increase in ROS levels. Likewise, 3NP exposure significantly (37%) elevated the MDA and SDAE restored the MDA levels (Table 1). Further, mitochondrial HP levels markedly (38%) elevated with 3NP exposure were normalized in mice receiving SDAE supplements (Table 1). Interestingly, 3NP did not alter the MDA levels in mitochondria. Additionally, we found robust increase (70%) in cytosolic, NO levels among 3NP mice, while SDAE supplements normalized the levels (Table 1). Further, 3NP induced elevated (35%) NO levels in mitochondria were also restored with SDAE prophylaxis.

Reduced GSH levels: The reduced glutathione levels were markedly elevated (63%) in cytosol among SDAE mice while the levels were not altered in mice exposed to 3NP. While SDAE per se marginally increased (16%) the TSH levels in cytosol, 3NP did not affect the levels (Table 1). The mitochondrial levels of GSH and TSH were also unaffected (Data not presented).

Nitrated and oxidized protein level: While 3NP did not alter the nitrated protein levels in striatal cytosol, SDAE significantly reduced (22%) the degree of protein nitration (Figure 3A and B). Further, 3NP exposure caused a marginal increase (22%) in cytosolic PC levels which were restored with SDAE supplements (Figure 3C). SDAE per se significantly (28%) reduced the mitochondrial PC levels. 3NP caused a marked increase (45%) in mitochondrial PC levels, while SDAE not only normalized the 3NP effect but further reduced (25% below control levels).

There was a significant diminution in the activity of total ATPases (45%, Table 2) and Na⁺ K⁺ ATPases (56%) which were partially restored (respectively 37%; and 73%) with mice receiving SDAE supplements. There was no change in glutathione-s-transferase activity in striatum among different groups of animals. More interestingly the glutathione peroxidase activity levels were reduced significantly (26%) in SDAE mice exposed to 3NP. There was a marked elevation (44%) in the activity of thioredoxin reductase (Table 2).

Effect of SDAE on 3np -induced mitochondrial dysfunction

As anticipated, the activity levels of succinic dehydrogenase (complex II) were diminished (61%) in 3NP mice which were partially restored (29%) with SDAE supplements (Figure 4A). Interestingly, the reduction (26%) in the CS activity caused by 3NP was normalized with SDAE supplements (Figure 4B). Further, 3NP induced reduction (49%) in MTT assay of striatal mitochondria was also partially normalized (Figure 4C). 3NP exposure resulted in a significant (41%) loss of membrane potential (as estimated by the ability to retain Rh 123) and was normalized in mice receiving SDAE supplements (Figure 4D).

Modulatory effects on ache activity, da levels and hsp-70 levels

Furthermore, 3NP exposure caused a significant (23%) increase in the protein levels of HSP-70. Although SDAE per se marginally increased the HSP 70 levels, it diminished (46%) the levels among 3NP mice (Fig. 5). 3NP exposure in mice caused a significant depletion (44%) in the striatal DA levels, while the depletion was only 19% in mice receiving SDAE supplements suggesting a partial restoration (Figure 6A). Further, 3NP caused a significant (34%) increase in the AChE activity levels in striatum, which was partially normalized with SDAE supplements (Figure 6B).

Table 1 Modulatory efficacy of SDAE against 3NP induced changes in the redox markers in striatum.

	CTR	SDAE	3NP	SDAE + 3NP
Reactive oxygen species †				
Cyto	8.7 ± 0.52	11.1 ± 0.67	15.3 ± 0.92*	11.2 ± 0.67#
Mito	3.6 ± 0.22	3.9 ± 0.23	3.8 ± 0.23	4.1 ± 0.25
Hydroperoxides ‡				
Cyto	77.3 ± 4.64	73.5 ± 4.41	116.3 ± 6.98*	83 ± 4.98#
Mito	120 ± 7.2	119 ± 7.14	166 ± 9.96*	126.1 ± 7.57#
Malondialdehyde §				
Cyto	5.7 ± 0.16	4.7 ± 0.36	7.5 ± 0.22*	5.5 ± 0.15#
Mito	4.9 ± 0.26	3.7 ± 0.34*	4.7 ± 0.15	3.5 ± 0.25*
Nitric oxide ¶				
Cyto	3.4 ± 0.11	1.4 ± 0.13*	5.8 ± 0.17*	3.6 ± 0.21#
Mito	2.3 ± 0.15	2.4 ± 0.11	3.1 ± 0.16*	2.4 ± 0.19#
Reduced Glutathione §				
Cyto	20.69 ± 0.53	29.43 ± 0.87*	22.67 ± 0.63	24.51 ± 0.68*
Total thiols ¥				
Cyto	299.4 ± 14.97	346.7 ± 17.3*	325.2 ± 16.26	334.7 ± 16.74

CTR = control; SDAE = 400mg/ kg bw/d (p.o.) for 15d; 3NP = 3-nitro propionic acid 50mg/ kg bw/d (i.p.) on last 5d; Cyto-Cytosol and Mito-Mitochondria. Results analyzed by non-parametric one-way ANOVA followed by post hoc Tukey's test. * compared to CTR ($p \leq 0.05$); # compared to 3NP ($p \leq 0.05$).

† pmol DCF/ min/ mg protein

‡ nmol hydroperoxides/ mg protein

§ nmol malondialdehyde/ mg protein

¶ Optical density / mg protein

§ μmol glutathione/ mg protein

¥ nmol thiols/ mg protein

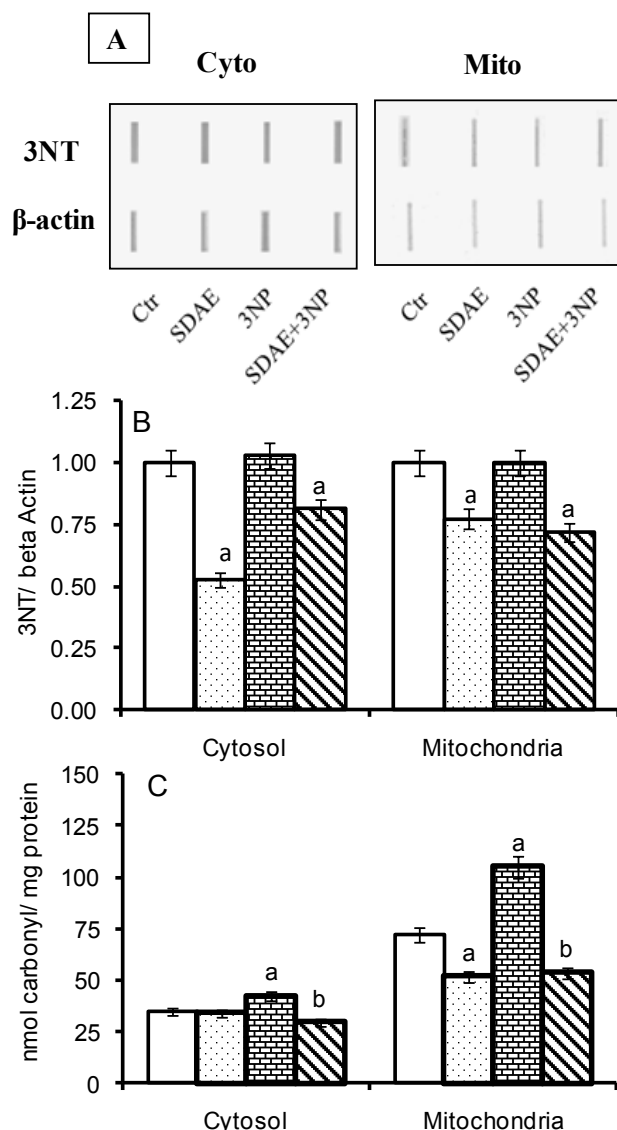


Figure 3 Modulatory efficacy of *S. delicatula* aqueous extracts (SDAE) against 3NP induced oxidative markers in cytosol and mitochondria of mice striatum: (A) 3-nitrotyrosine levels and (B) Protein carbonyls. Values are mean ± SEM (standard error of mean). Results analyzed by non-parametric one-way ANOVA followed by post hoc Tukey's test. CTR: Control; SDAE: 400mg/ kg bw/d (p.o.) 15d; 3NP: 3-nitropropionic acid 50mg/ kg bw/d (i.p.) on last 5d. * compared to CTR ($p \leq 0.05$); # compared to 3NP ($p \leq 0.05$).

Table 2 Modulatory effects of SDAE against 3NP-induced activity changes in antioxidant enzymes and mitochondrial ATPases.

	CTR	SDAE	3NP	SDAE + 3NP
Antioxidant enzymes				
SOD †	31.8 ± 1.64	29.0 ± 1.07	34.4 ± 1.28	39.8 ± 1.13*
TR ‡	12.69 ± 0.23	18.74 ± 0.34*	12.43 ± 0.56	13.02 ± 0.60
GPx §	58.33 ± 2.92	59.01 ± 1.95	39.57 ± 2.08*	52.40 ± 2.62#
ATPases				
Na ⁺ -K ⁺	0.337 ± 0.043	0.352 ± 0.041	0.166 ± 0.023*	0.287 ± 0.031#
ATPase ¶	0.245 ± 0.019	0.241 ± 0.019	0.108 ± 0.012*	0.209 ± 0.016#

CTR = control; SDAE = 400mg/ kg bw/d (p.o.) for 15d; 3NP = 3-nitro propionic acid 50mg/ kg bw/d (i.p.) on last 5d. Results analyzed by non-parametric one-way ANOVA followed by post hoc Tukey's test. * compared to CTR ($p \leq 0.05$); # compared to 3NP ($p \leq 0.05$).

† μg Pi/ mg protein

‡ Superoxide dismutase (units/ mg protein)

§ Thioredoxin reductase (nmol NADPH/ min/ mg protein)

¶ Glutathione peroxidase (nmol NADPH/ min/ mg protein)

¶ μg Pi/ mg protein

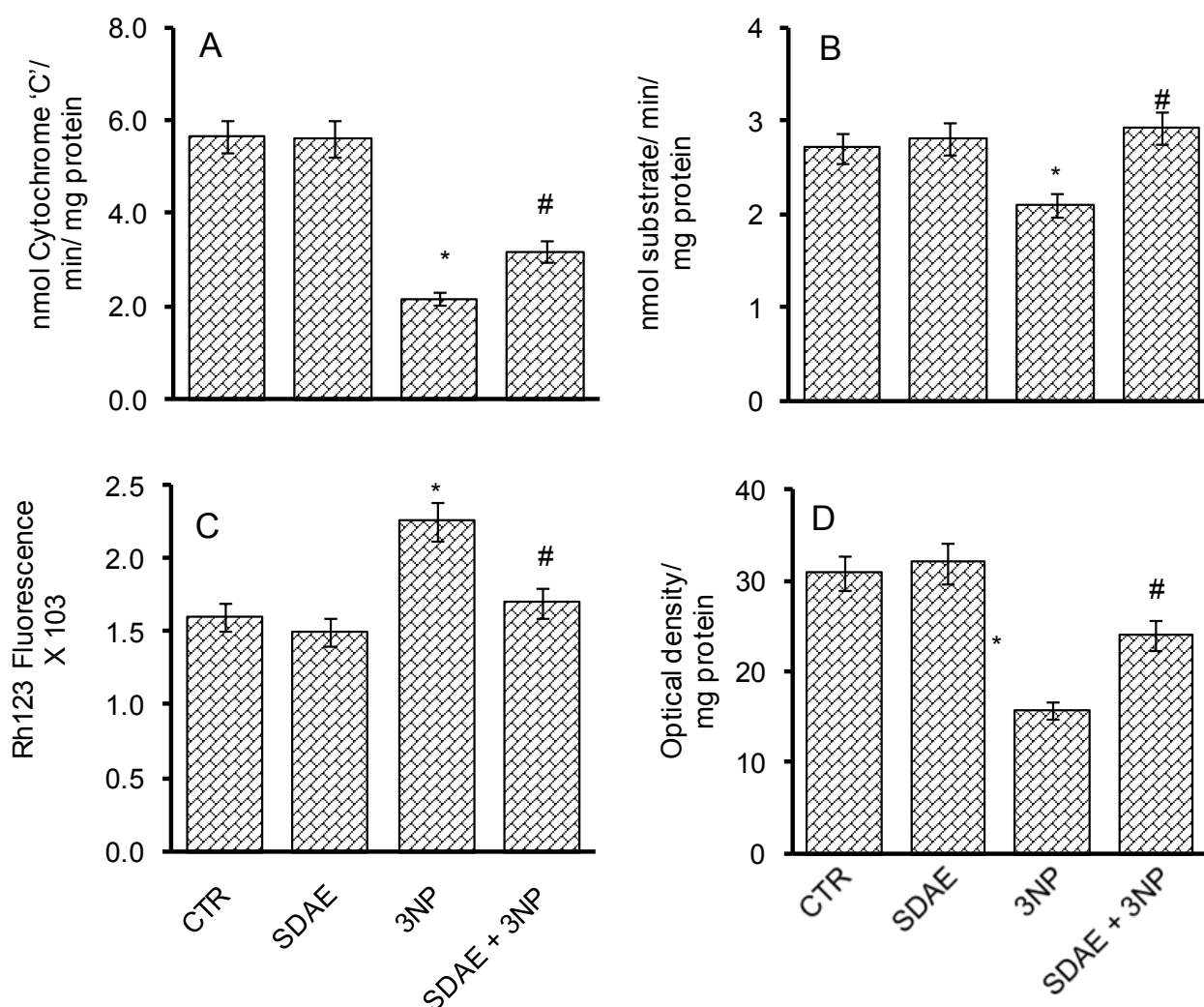


Figure 4 Modulatory efficacy of *S. delicatula* aqueous extracts (SDAE) against 3NP induced aberrations in the mitochondrial function markers in striatum of mice: (A) Complex II and (B) Citrate synthase; (C) Membrane potential and (D) MTT reduction assay. Values are mean \pm SEM (standard error of mean). Results analyzed by non-parametric one-way ANOVA followed by post hoc Tukey's test. CTR: Control; SDAE: 400mg/ kg bw/d (p.o.) 15d; 3NP: 3-nitropropionic acid 50mg/ kg bw/d (i.p.) on last 5d. * compared to CTR ($p \leq 0.05$); # compared to 3NP ($p \leq 0.05$).

DISCUSSION

The plant derived polyphenolic compounds not only exhibit strong antioxidant capacity *in vivo*, but also contain specific therapeutic targets that maintain the cellular stress response and key signaling cascades, which are involved in pathophysiology of neurodegenerative disorders^[35]. Although the major underlying event in the pathophysiology of NDD is disturbed cellular redox homeostasis in CNS regions, the current therapies provide only symptomatic relief with minimal emphasis on addressing the pathophysiology. Hence, there exists a constant need for the development of newer, efficient and safer approaches with specific cellular, physiological and biochemical targets, which are involved in progression of the disease. In this regard, we demonstrated the neuromodulatory effects of SDAE against Parkinson-like neurobehavioral symptoms in Rotenone model employing *Drosophila* and mice^[13,14]. In the present study, we examined the hypothesis whether SDAE prophylaxis possess the propensity to provide protection against a 3NP model of neurotoxicity in mice.

Previously, SDAE significantly inhibited the formation of lipid peroxides by 3NP in mice synaptosomes *in vitro*, (unpublished data).

As a proof of principle we further sought to validate the prophylactic efficacy of SDAE employing a mice model of 3NP neurotoxicity. Neurons are highly susceptible to the imbalance in the redox homeostasis and the moderate antioxidant defense may contribute to further damage. Hence our salient finding viz., dose dependent diminution in the endogenous levels of oxidative markers in mice striatum suggest the efficacy of SDAE to ameliorate the oxidative stress *in vivo* and corroborates our earlier reports^[13,14].

Since administration of 3NP in rodents produces a well characterized model of HD, characterized by selective degeneration of striatal neurons, oxidative damage and mitochondrial dysfunction, this phenotypic model has gained a great attention among pharmacologists for development of potent therapeutic strategies^[36-38]. Striatum has largely been the focus due to its substantial atrophy in post mortem tissues. Several molecular mechanisms underlie striatal degeneration, the major being the non-functional normal Huntingtin protein. In addition, excitotoxicity, metabolic impairment, dopamine toxicity, redox imbalance, mitochondrial dysfunction, and apoptosis have been involved in the progressive degeneration of striatal neurons. Though researchers are keen on studying the pathophysiology of HD, there are few treatment approaches

for the same^[1,2]. Midbrain neurons in particular are believed to exist in a persistent state of redox imbalance as dopamine per se undergoes auto-oxidation to form reactive quinones which in continuum maintains the autocatalytic cycle of oxidative stress and mitochondrial dysfunction. Since new drug therapy for a neurodegenerative condition must involve neuroprotective agents that are potent antioxidants *in vivo* capable of slowing down the disease progression by mitigating the endogenous oxidative stress. In the present model, oral prophylaxis with SDAE markedly reduced the levels of striatal oxidative markers ROS, PC and HP levels among both control and 3NP mice clearly suggesting its potent antioxidant property *in vivo* at the cellular level.

The key events with long term implication, resulting ultimately from excessive oxidative stress are protein oxidation and nitration. Accumulation of oxidatively/ nitrosatively modified proteins in striatum has been observed consistently in post mortem PD brain specimens and in animal models of HD. Oxidative stress with the impaired proteasomal activity leads to accumulation of oxidized/nitrated proteins in the cells as they resist normal proteolysis, form aggregates and also inhibit the action of proteases^[14]. In the present model, SDAE significantly elevated the reduced glutathione levels in striatum among both control and 3NP groups. This data corroborates our previous findings in both *Drosophila* and ROT-model of mice^[14]. Thus, SDAE elicited significant anti-oxidative effect as evident by the amelioration of 3NP-induced oxidative impairments, enhanced GSH levels, elevated thiols and restoration of antioxidant defenses. Interestingly, we found a specific effect on the activity levels of TR, a selenium dependent enzyme, which participates in the maintenance of cellular redox^[39]. SDAE increased the endogenous activity levels of TR activity in striatum suggesting its efficacy to modulate the non-

glutathione redox system *in vivo*. The glutathione and thioredoxin (Trx) systems complement mutually and provide reducing equivalents for various processes, which are directly linked to the regulation of the cellular redox.

We speculate that both enhanced GSH levels and elevated TR activity may represent a vital mechanism by which SDAE supplements render protection against 3NP-induced neurotoxicity. The major reactive nitrogen species (e.g. peroxynitrite) nitrate the tyrosine moieties into 3-nitrotyrosine (3-NT); accumulation of the latter in tissue proteins is associated with brain aging and NDD^[14,40]. In the present model, assessment of total protein nitration (by 3-nitrotyrosine slot blots) and oxidation (carbonyl levels) revealed that SDAE effectively attenuated not only the endogenous levels of 3NT and protein carbonyls, but also reduced the levels among 3NP mice, which may be partly facilitated by increased GSH levels and TR activity, which prevented protein oxidation/ nitration. Hence, our findings clearly suggest that SDAE possesses marked potential to counteract an oxidative insult *in vivo*.

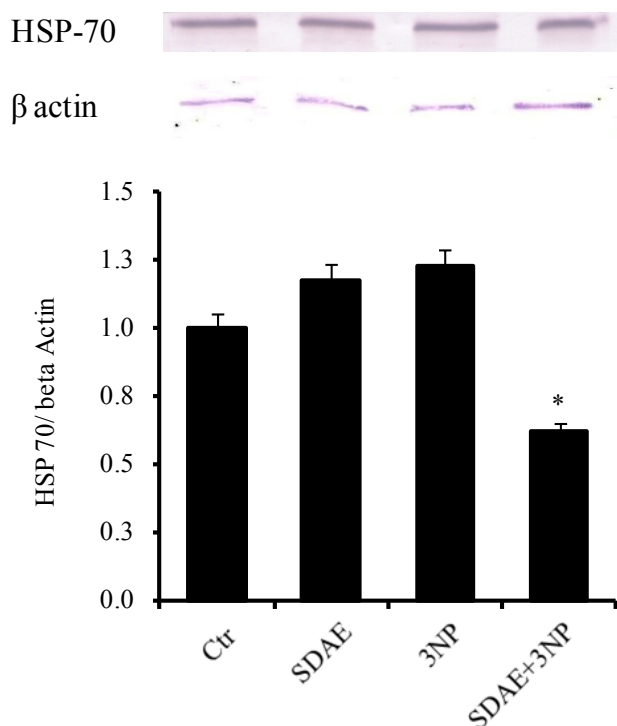


Figure 5 Modulatory efficacy of *S. delicatula* aqueous extracts (SDAE) against 3NP induced changes in HSP-70 levels in mice striatum. Values are arbitrary pixel units (HSP-70/ β -actin). CTR: Control; SDAE: 400mg/kg bw/d (p.o.) 15d; 3NP: 3-nitropropionic acid 50mg/kg bw/d (i.p.) on last 5d. * compared to CTR ($p \leq 0.05$); # compared to 3NP ($p \leq 0.05$).

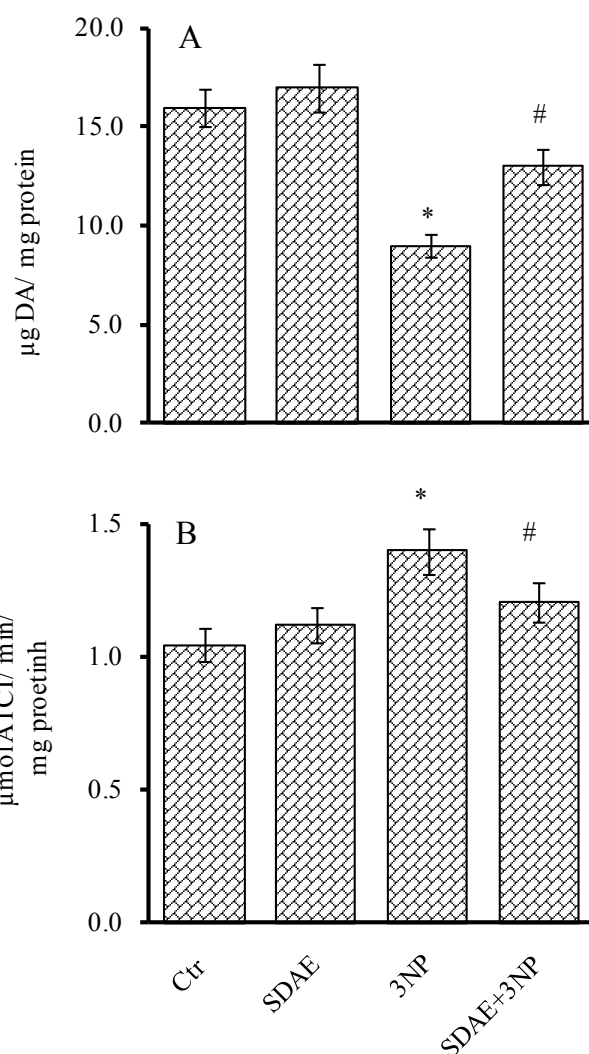


Figure 6 Modulatory efficacy of *S. delicatula* aqueous extracts (SDAE) against 3NP induced changes in neuronal markers in mice striatum: (A) Dopamine and (B) Acetylcholine esterase. Values are mean \pm SEM (standard error of mean). Results analyzed by non-parametric one-way ANOVA followed by post hoc Tukey's test. CTR: Control; SDAE: 400mg/kg bw/d (p.o.) 15d; 3NP: 3-nitropropionic acid 50mg/kg bw/d (i.p.) on last 5d. *compared to CTR ($p \leq 0.05$); # compared to 3NP ($p \leq 0.05$).

Amentoflavone is one of the major biflavonoid in the genus *Selaginella*. Previously the protective effects of *Selaginella* biflavonoids have been studied in few cell models including neuronal cells^[41-43]. However, studies describing the efficacy of these biflavonoids to modulate endogenous oxidative markers in cell models are rather limited. In a separate study amentoflavone treatment reduced 3NP induced oxidative stress in neuronal SH-SY5Y cells by reducing reactive oxygen species and increased GSH (data not presented). This suggests the neuroprotective effects of *Selaginella* extract may be attributed to amentoflavone however needs to be validated.

HSP70 is one of the vitagenes responsible for maintaining the cellular homeostasis during stress conditions^[39]. A few dietary flavonoids are reported to induce the HSP70 levels in experimental models, while preparing the cells to counteract the deleterious effects of endogenous oxidative stress or induced by a xenobiotic prooxidant. In the present model, the marginal induction of HSP 70 levels in SDAE per se group and markedly reduced levels among 3NP exposed mice possibly suggests the hermetic compensatory mechanism involved in the neuromodulation by SDAE. Further, chronic 3NP exposure significantly diminished the membrane function, particularly the activity levels of complex II and ATPases along with elevated HP levels in striatal mitochondria. The restorative effect of SDAE supplements on the activity levels of ATPases suggests its specific effect on the mitochondrial membrane stability. Electron transport chain defects and ROS production are key events in etiology of NDD. Interestingly, SDAE significantly restored the 3NP induced diminution in the activity levels of complex II. Na⁺K⁺ ATPases are trans-membrane proteins known to be extremely sensitive to lipid peroxidation as well as factors that alter the membrane lipid environment^[44,45]. In this study, elevated levels of ROS, MDA and HP were associated with reduced activity levels of ATPases, indicating the membrane dysfunction caused by 3NP. The amelioration of striatal mitochondrial dysfunctions by SDAE in 3NP mice demonstrates its effective mitochondrial modulation.

In the present model, SDAE prophylaxis significantly restored the activity levels of striatal AChE among 3NP administered mice, which is consistent with our previous findings in *Drosophila* and ROT-mice models^[13,14]. We also found that, SDAE inhibits AChE activity in cell extracts of brain regions as well as pure enzyme-substrate system in vitro (Unpublished findings). Although speculative, this finding may be of therapeutic relevance to neurotoxic property of accumulation of acetylcholine. A body of evidence suggests that natural products possessing inhibitory activity on AChE are of therapeutic importance as they are relatively safer than the regular AChEI^[46].

Further, gait disorders in general and reduction in stride length in particular are considered to be a characteristic of motor disorders in experimental animals and humans. SL, NBT and LFSD consistently reflect on the motor function and are reliable/reproducible parameters among different rodent species^[13]. In the present model, chronic 3NP exposure caused a significant and progressive reduction in stride length and narrow beam latency which was accompanied by increased LFSD^[16]. We found reduced stride length correlated with depleted levels of striatal DA clearly suggesting striatal neurodegeneration. We believe that normalization of motor function and marginally restored DA levels among 3NP mice evident with SDAE prophylaxis may be largely due to its specific effects in the striatum. The open field behavior of the SDAE mice displayed protection in terms of reduced anxiety like behavior. OFT, an excellent tool usually employed to assess the spontaneous activity/ hyperactivity of rodents allows a longer monitor period^[17].

The time spent at the centre or latency to move out of the centre was considered as a measure of avoidance behaviour while general activity was measured with number of quadrants visited and the total distance moved^[15,47]. Our results in OFT suggested the habituation related memory development as evidenced with reduced grooming activity and marginally increased latency to visit the margin among control mice. Interestingly, SDAE prophylaxis apparently acts as an excellent anti-stress agent as demonstrated by complete normalization of 3NP-induced stress like behavior as evidenced with markedly increased latency to visit the margin, total idle time and markedly reduced grooming activity. Similarly, 3NP affects cholinergic function in hippocampus (data not presented) which is consistent with the stress behavior in OFT and NBT^[48]. Cumulatively OFT results, the increased NBT-goal box latency as well as restoration of cholinergic function among 3NP mice, suggest a potent anti-stress and memory enhancing property SDAE supplements.

In conclusion, our findings in the 3NP model clearly display the neuroprotective efficacy of SDAE. Based on our earlier findings and the present data, it is proposed that *S. delicatula* aqueous extract may be utilized as a therapeutic agent/ adjuvant in protecting the CNS against oxidative stress-mediated NDD.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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