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# Original Research Article

# Effects of root extract of *Withania somnifera* in 3-Nitropropionic Acid-Induced Cognitive Dysfunction and Oxidative Damage in Rats

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## Abstract

***Purpose:*** *To investigate the possible modulatory role of root extract of Withania somnifera (WS) in 3-Nitroproiponic acid (3-NP)-induced cognitive impairment and altered level of oxidative defense in discrete areas of rat brain.*

***Methods****: 3-NP was administered in a dose of 10 mg/kg for 14 days where as WS root extract (100 and 200 mg/kg) was administered orally along with 3-NP. Cognitive dysfunctions were assessed in Morris water maze and plus-maze performance task paradigms. On 15$^{th}$ day the animals were scarified and reduced glutathione, total glutathione, oxidized glutathione (GSSG), glutathione-S-transferase (GST) and acetylcholinesterase enzyme levels were estimated in the striatum, cortex and hippocampus of the rat brian.*

***Results:*** *Chronic WS root extract (100, 200 mg/kg) treatment for a period of 14 days significantly improved 3-NP-induced cognitive impairment in Morris water and plus maze tests ($p<0.05$). Further, WS root extract treatment significantly restored GSH, total glutathione, oxidized glutathione, GST and attenuated acetylcholinesterase levels in striatum, cortex and hippocampus regions of brain.*

***Conclusion:*** *There is possible neuroprotective effect of WS root extract against a 3-NP- induced neurotoxicity in rats.*

***Key words****: Huntington's disease, 3-Nitropropionic acid, Oxidative stress, Withania somnifera*

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## Introduction

Huntington's disease (HD) is a fatal disease typically characterized by involuntary movements (chorea) and cognitive decline (dementia)[1]. HD specifically affects basal ganglia in brain that are responsible for important functions, such as movement and coordination [2]. Besides other brain structures responsible for thought, perception and memory are also affected, likely due to connections from the basal ganglia to the frontal lobes[1, 2]. Individuals suffering from HD are often poor in cognitive related tasks and several investigators have shown that memory recall is significantly more affected than memory[3,4]. The compound, 3-Nitro-proiponic acid (3-NP), causes neurotoxicity by impairing cellular energy generation resulting in oxidative damage to striatum[5,6,7]. The exact disease pathogenesis is yet to be understood but evidences suggest impairment of energy metabolism and oxidative stress[8,9]. Indeed, increasing evidences suggest that free radical generation and consequent oxidative damage causes macromolecular changes in vulnerable areas of brain leading to behavioral impairment and memory deficits[6,8]. The internal protective mechanisms that detoxify defense enzymes such as glutathione redox system play a crucial role in balancing the generation of reactive oxygen species and antioxidative defense system. Reduced glutathione at tissue level is a critical determinant for assessing the threshold of tissue injury. Glutathione has been shown to react directly with radicals in non-enzymatic fashion and acts as an electron donor in the reduction of peroxide. Alterations in the glutathione redox system have been implicated in 3-NP neurotoxicity.

Role of GABAergic in the pathogensis of HD has been well documented. *Withania somnifera (*WS*)* Dun. (Ashwa-gandha in Sanskrit), popularly known as Indian ginseng, has been used in Ayurvedic medicine to treat several neurological problems including cerebral functional deficits[11] and provide non-specific host defenses[12]. *Withania somnifera* has been well reported to act by GABAergic system. Recently, WS has been reported to be effective for treatment in 18 clinically diagnosed parkinsonian patients[13, 14]. *In vitro* and *in vivo* molecular pharmacological investigations have elucidated the association of secondary metabolites known as withanolides present in the plant with its therapeutic benefits [13, 15]. However, no study explored the possible role of *W. somnifera* in animal model of HD.

The present study was carried out to investigate the possible neuromodulatory effect of WS root extract against 3-NP-induced cognitive impairment and oxidative stress.

## Materials and Methods

### *Animals*

Male Wistar rats bred in Central Animal House facility of the Panjab University, Chandigarh and weighing between 250-300 g were used. The animals were kept under standard conditions of light and dark cycle with food and water *ad libitum* in groups of 2 in plastic cages with soft bedding. All the experiments were carried out between 09:00 and 15:00 hr. The protocol was approved by the Institutional Animal Ethics Committee and carried out in accordance with the Indian National Science Academy Guidelines for the use and care of animals.

### *Materials*

The following drugs were used in the present study: 3-NP (Loba Chem., New Delhi, India) was diluted with saline (adjust *p*H 7.4) and administered intraperitoneally to animals. WS root extract (Himalayan Drugs, India) was suspended in 0.05% carboxy methyl cellulose (CMC) solution and administered by per oral route in a constant volume of 0.5 ml per 100 g of body weight.

### *Study Design*

The animals were randomly divided into five groups of 10 animals in each. Group 1, vehicle treated control group, received vehicle for WS root extract (p.o.) and also normal saline (i.p.); Group 2 received 3-NP (10 mg/kg, i.p.) for 14 days, Group 3 received WS root extract (100 mg/kg) + 3-NP (10 mg/kg, i.p.) for 14 days; Group 4 received WS root extract (200 mg/kg) + 3-NP (10 mg/kg, i.p.) for 14 days; Group 5 received WS root extract (200 mg/kg) *per se* for 14 days. In groups 3 and 4, WS root extract was administered 1 hr prior to 3-NP administration and doses were selected on basis of previous studies conducted in laboratory[16, 17]. All the behavioral parameters were observed before and after drug treatment.

### *Morris Water Maze test*

The acquisition and retention of a spatial navigation task was examined using Morris Water Maze test[18]. In this test, the animals were trained to swim to a platform in a circular pool (180 cm diameter × 60 cm height) located in a test room. The pool was filled with water (28 ± 2 °C) to a depth of 40 cm. A movable circular platform 9 cm in diameter, mounted on a column, was placed in the pool 1 cm above the water level for maze acquisition test and another movable platform 9 cm in diameter, mounted on a column, was placed in the pool 1 cm below the water level for maze acquisition test. Animals received a training session consisting of 4 trials in a day for 4 days, starting from first day of 3-NP administration. In all 4 trials, the starting positions were different. The latency to find the escape platform was recorded up to a maximum of 2 min. The platform was fixed in the center of one of the 4 quadrants and remained in that location for the duration of experiment. Time taken by a rat to reach the platform on 5$^{th}$ day was recorded as initial acquisition latency (IAL). On 10$^{th}$ and 15$^{th}$ days, rats were randomly released at any one of the edges (N, S, E, W) facing the wall of the pool and tested for the retention of the memory. The time taken to reach the hidden platform on days 10$^{th}$ and 15$^{th}$ following initiation of 3-NP treatment was recorded.

### *Elevated Plus Maze test for spatial memory*

Elevated Plus Maze test was another paradigm to assess memory dysfunction. The test consists of two opposite open arms (50 × 10 cm), crossed with two closed arms of same dimensions with 40 cm high walls. The arms are connected with central square (10 × 10 cm). Acquisition of memory was assessed on day 14$^{th}$ after initiating 3-NP treatment. Rats were placed individually at one end of an open arm facing away from the central square. The time taken to move from open arm and enter into one of the closed arms was recorded as initial transfer latency (ITL). Animals were allowed to explore the maze for 30 sec after recording ITL and returned to its home cage. Retention latency was noted again on 15$^{th}$ day[7].

### *Dissection and Homogenization*

On day 15, after behavioral assessments, animals (n=10) were sacrificed by decapitation. The brains were removed, forebrain was dissected out and cerebellum was discarded and the remaining parts were stored on ice. The striatum, cortex and hippocampus were then separated and weighted. A 10% (weight/volume) tissue homogenate was prepared in 0.1 M phosphate buffer (*pH* 7.4). The homogenate was centrifuged at 10,000 *×g* for 15 min and aliquots of supernatant was separated and used for biochemical estimation.

### *Biochemical Estimation*

*Estimation of Glutathione levels*

GSH in striatum, cortex and hippocampus was estimated according to the method described by Ellman[19]. Results were calculated using molar extinction coefficient

of chromophore ($1.36 \times 10^4$ $Mm^{-1}$ $cm^{-1}$) and expressed as percentage of control. Total glutathione analysis was done by the method of Zahler and Cleland [20]. The method is based on the reduction with dithioerythritol and deter-mination of the resulting monothiols with 5, 5' dithiobis 2-nitrobenzoic acid (DTNB) (Ellman reagent) in the presence of arsenite. Oxidized glutathione were quantified by subtracting the value of glutathione reduced from total glutathione. GST was essayed by the method of Habig et al [21]. It catalyses the formation of the glutathione conjugates of 1- chloro-2,4-dinitrobenzene (CDNB) which absorb maximum at 340 nm and have an extinction coefficient of 9.6 $Mm^{-1}$ $cm^{-1}$

*Estimation of acetylcholinesterase (*AChE*) levels*

The quantitative measurement of acetyl-cholinesterase levels in brain was performed according to the method of Ellman [22]. The assay mixture contained 0.05 ml of supernatant, 3 ml of 0.01M sodium phosphate buffer (*pH* 8), 0.10 ml of acetylthiocholine iodide and 0.10 ml of DTNB. The change in absorbance was measured immediately at 412 nm using Perkin Ellman lambda 20 spectrophotometer. Results were calculated using molar extinction coefficient of chromophore ($1.36 \times 10^4$ $Mm^{-1}$ $cm^{-1}$) and expressed as percentage of control. Protein estimation was done by biuret method using bovine serum albumin as standard [23].

### *Statistical Analysis*

All the values are expressed as mean ± SEM. The data were analyzed by using analysis of variance (ANOVA) followed by Tukeys's test. At 95% confidence interval, p values less than 0.05 were considered to be significant.

## Results

### *Effect of WS root extract on spatial navigation task in 3-NP treated rats*

The cognitive function, assessed using Morris water maze test, indicated that mean escape latency for the trained rats decreased during the training session from 1st to 5th day in all the groups. When retention latency performance was assessed on 10th and 15th day in Morris water maze, 3-NP treated rats showed higher mean latency as compared to the control group ($p<0.05$). However, chronic WS root extract treatment (100 and 200 mg/kg, p.o.) starting before 3-NP admini-stration showed a significant improvement in memory performance on day 10th and 15th ($p<0.05$ when compared to 3-NP-treated rats) as illustrated in Figure 1. On the 15th day, the time spent by the animal in the each quadrant of the Morris water maze was also observed. When the time spent by the animal in the target quadrant was assessed in Morris water maze on last day (15th day), the naïve group animal spent the maximum time in the target quadrant whereas 3-NP treated rat decreased in the time in the target quadrant and these changes were significantly reversed by the WS root extract treatment (Figure 2).

### *Effect of WS root extract on memory performance in elevated plus maze paradigm in 3-NP treated rats*

In the present experiment, mean ITL on day 14 for each rat was relatively stable and showed no significant variation in elevated plus maze paradigms after 3-NP treatment. All the rats entered the closed arm within 60 sec. Following training, control (vehicle treated) and WS root extract treated (*per se*; 200 mg/kg, p.o.) rats entered closed arm quickly and mean retention transfer latency was shorter as compared to ITL. In contrast, 3-NP treated rats performed poorly throughout the experiment and increased in the mean retention transfer latency on day 15, demonstrating 3-NP-induced cognitive

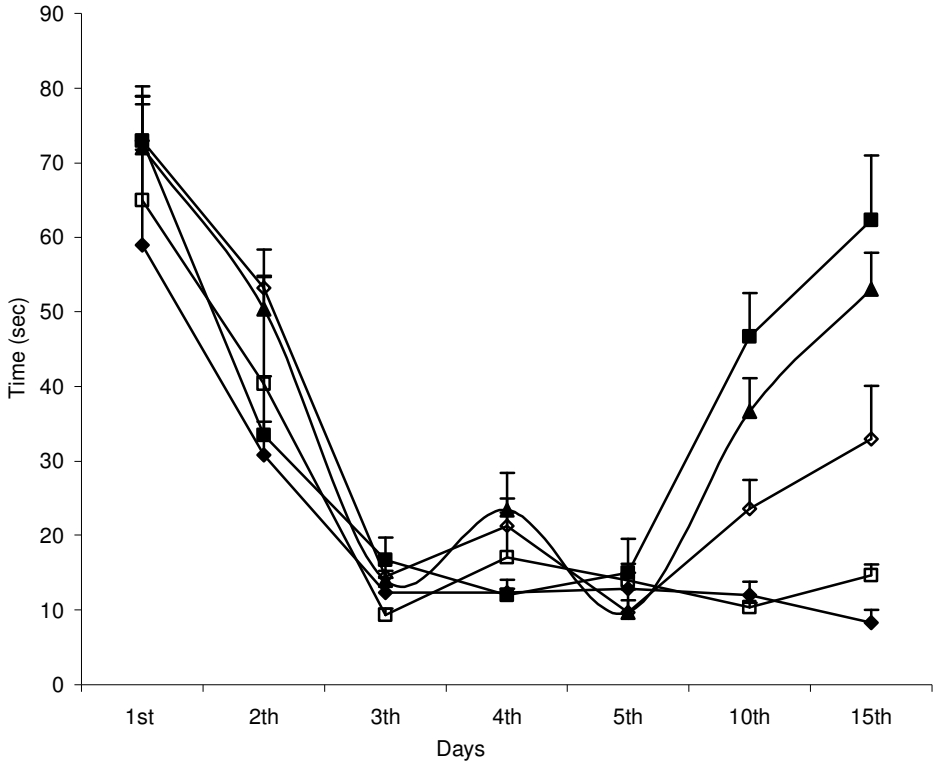


**Figure 1:** Effect of *Withania somnifera* (WS) root extract on Morris water maze in 3-nitropropionic (3-NP) acid treated rats. Values are mean ± S.E.M. [a]*p* < 0.05 as compared to vehicle treated control group; [b]p < 0.05 as compared to 3-NP injected group and WS root extract (200mg/kg) *per se* group. [c]p < 0.05 as compared to 3-NP + *WS* 100 mg/kg treated group. (♦, control; ▲, WS 100mg + 3-NP*;* □, WS 200 mg + 3-NP; ■, 3-NP; ◊, WS 200 mg + 3-NP)

dysfunction. In contrast, chronic administrations of WS root extract (100 and 200 mg/kg, p.o.) prior to 3-NP treatment significantly decreased mean retention latencies, indicating improvement of memory functions (Figure 3).

### *Effect of WS root extract on acetylcholinesterase and glutathione levels in 3-NP treated rats*

Further chronic 3-NP treatment significantly increased acetylcholinesterase enzyme level, reduced glutathione, total glutathione and oxidized glutathione and glutathione-S-transferase activity in striatum, cortex and hippocampus region of the rat brain when compared to the control group and WS root extract (200 mg/kg) *per se* (p < 0.05). In addition, WS root extract (100 and 200 mg/kg) treatment significantly restore glutathione (Table) and acetylcholinesterase activity in striatum, cortex and hippocampus of 3-NP treated rats brain (Figure 4).

## Discussion

Results of the present study indicate that WS root extract pretreatment significantly improved cognitive function, restored acetyl cholinesterase enzyme activity and glutathione enzyme level system in 3-NP treated animals. Striatal lesions-induced by systemic administrations of 3-NP caused an impairment in the memory tasks observed in Morris water maze and elevated plus maze paradigms. 3-NP has been reported to cause

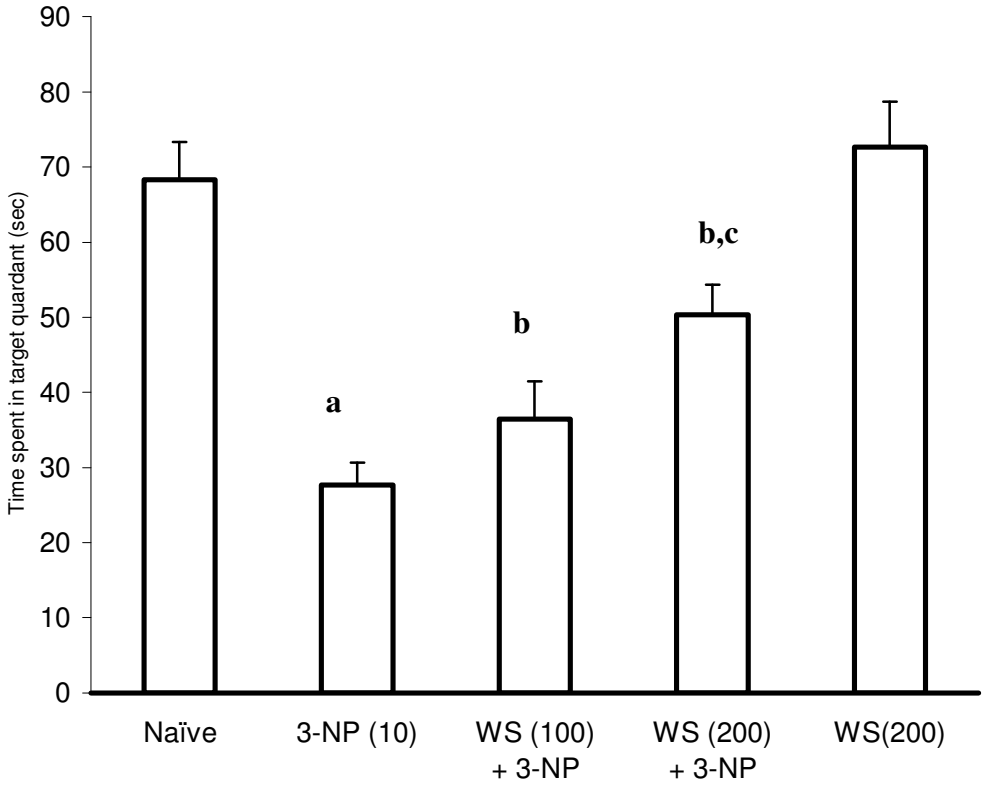


**Figure 2:** Effect of *Withania somnifera* (WS) root extract 100 and 200mg/kg, on time spent in target quadrant in Morris water maze in 3-nitropropionic acid treated rats. Values are mean ± S.E.M. [a]$p < 0.05$ as compared to vehicle treated control group; [b]$p < 0.05$ as compared to 3-NP injected group and WS root extract (200mg/kg) *per se* group. [c]$p < 0.05$ as compared to (3-NP + *WS* 100 mg/kg)treated group.

lesions in hippocampal CA1 and CA3 pyramidal neurons – the area of brain that is associated with cognitive performance[24]. Animal quickly learns during training period up to 5th day of 3-NP treatment in Morris water maze paradigm. However, significant difference was not observed in initial transfer latency between the treatment groups. Further, 3-NP treated group showed an increase in mean retention latency as compared to naïve group on day 10th and 15th. The time spent in the target quadrant was also decreased in the 3-NP treated rats in Morris water maze. When the animals were exposed to the plus maze, animal showed poor memory retention in 3-NP treated group. The observations indicate that chronic 3-NP systemic administration caused memory dysfunction as observed in the HD patients. Cognitive dysfunction is one of the characteristic features of HD patients [4, 25]. Alteration in cognition might be due to disruption of striatal –frontal circuits. Several investigators have shown that memory recall is severely affected in HD patients [3, 4]. In present study increased level of acetylcholinesterase enzyme activity particularly in the striatum, cortex and hippocampus of 3-NP treated rat was observed. Further, WS root extract treatment for two weeks significantly improved cognitive task and decrease

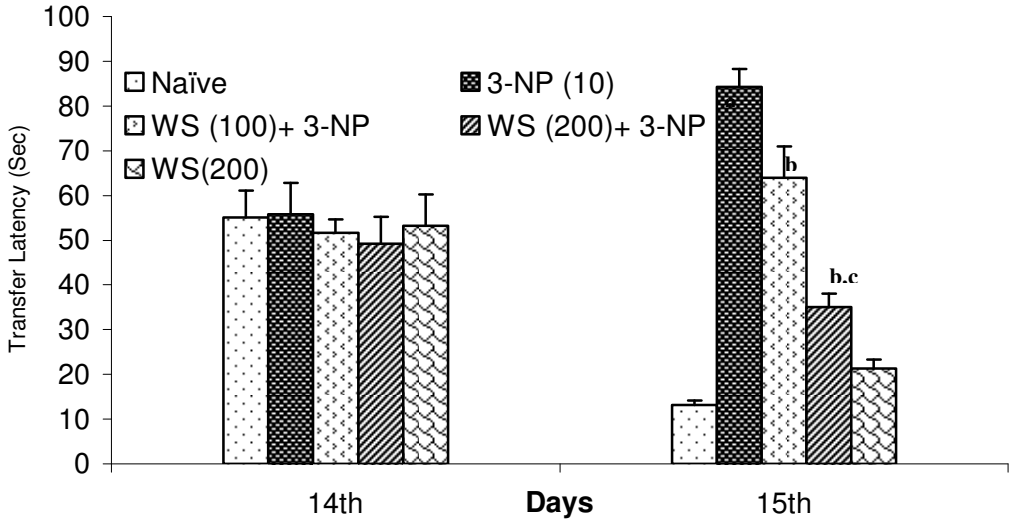


**Figure 3:** Effect of *Withania somnifera* (WS) root extract 100 and 200mg/kg, on transfer latency in plus maze in 3-nitropropionic acid treated rats. Values are mean ± S.E.M. [a]$p < 0.05$ as compared to vehicle treated control group; [b]$p < 0.05$ as compared to 3-NP injected group and WS root extract (200mg/kg) *per se* group. [c]$p < 0.05$ as compared to (3-NP + *WS* 100 mg/kg) treated group

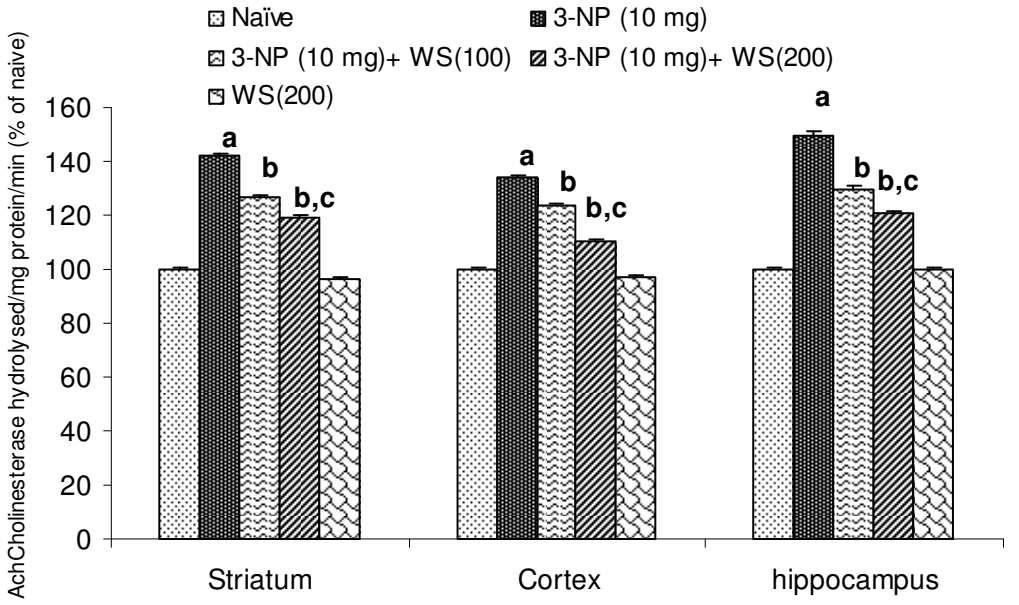


**Figure 4:** Effect of *Withania somnifera* (WS) root extract 100 and 200mg/kg on acetylcholinesterase enzyme in 3-nitropropionic acid treated rats. Values are mean ± S.E.M. [a]$p < 0.05$ as compared to vehicle treated control group; [b]$p < 0.05$ as compared to 3-NP injected group and WS root extract (200mg/kg) *per se* group. [c]$p < 0.05$ as compared to (3-NP + *WS* 100 mg/kg) treated group.

acetylcholinesterase enzyme activity in the 3-NP treated group, suggesting modulation of cholinergic neurotransmission. Systemic application of WS also affects AChE activity in basal forebrain nuclei [26]. These changes could be due to enhanced M1-muscarinic cholinergic receptor binding in lateral and medial septum as well as in frontal cortices, the drug-induced increase in cortical muscarinic acetylcholine receptor capacity partly explain the memory-improving effects of WS root extract extracts observed in animals [26]. Further, studies have shown the potential role of WS root extract in other animal models of tardive dyskinesia, Parkinson disease [16, 17, 27]. Withanoside IV has been reported to stimulate neurite outgrowth in cultured rat cortical neurons, improve memory deficits in Aβ (25-35)-injected mice and prevented loss of axons, dendrites, and synapses [28]. Sominone, an aglycone of withanoside IV causes axonal and dendritic regeneration and synaptic reconstruction [28]. These observations hypothesize that WS treatment may ameliorate neuronal dysfunction and improve memory.

Oxidative stress is a ubiquitously observed hallmark of neurodegenerative disorders. The chronic administration of the 3-NP raised lipid peroxidation and nitrite level in

**Table:** 3-Nitropropionic acid (3-NP) and *Withania somnifera (WS)* root extract treatment-induced glutathione changes in striatum, cortex and hippocampus of rats brain (percentage of control in parentheses)

| Treatment | | Total Glutathione (µmole of GSH/ mg protein) | Reduced Glutathione (µmole of GSH/ mg protein) | Oxidized Glutathione (µmole of GSH/ mg protein) | Glutathione-S-Transferase (µmole of CDNB formed/ min/mg protein) |
|---|---|---|---|---|---|
| Naïve | Striatum | 100± 0.05 | 100± 0.07 | 100± 0.07 | 100 ±0.07 |
| | Cortex | 100±0.07 | 100± .009 | 100±0.05 | 100 ±0.08 |
| | Hippocampus | 100±.007 | 100±0.04 | 100±0.07 | 100±0.08 |
| 3-NP (10 mg/kg) | Striatum | 56 ± 0.09[a] | 51± 0.07[a] | 72± 1.6 [a] | 29 ± 03 [a] |
| | Cortex | 71 ± 0.02[a] | 51± 0.07[a] | 83± 2.3 [a] | 49 ± 2.0 [a] |
| | Hippocampus | 74± 0.05[a] | 63±0.08 [a] | 94±.008 [a] | 75±0.09 [a] |
| *WS* root extract (100 mg/kg) + 3-NP (10 mg/kg) | Striatum | 64± 0.08 [b] | 74± 0.1[b] | 74± 0.12 [b] | 51 ± 0.13 [b] |
| | Cortex | 67± 0.14[b] | 60± 0.12[b] | 80± 0.13 [b] | 55 ± 0.12 [b] |
| | Hippocampus | 75±0.08 [b] | 60±0.05 [b] | 85±0.08 [b] | 79±0.09 [b] |
| *WS* root extract (200 mg/kg) + 3-NP (10 mg/kg) | Striatum | 81± 0.11[b,c] | 82± 0.11 [b,c] | 68± 0.06 [b,c] | 69± .05 [b,c] |
| | Cortex | 84± 0.07 [b,c] | 78± 0.09 [b,c] | 76± 0.5 [b,c] | 72± 0.07 [b,c] |
| | Hippocampus | 82±0.09 [b,c] | 73±0.08 [b,c] | 109±0.08 [b,c] | 83±0.07 [b,c] |
| *WS* root extract (200 mg/kg) | Striatum | 100± 0.09 | 104± 0.08 | 98± 0.08 | 99± 0.04 |
| | Cortex | 100± 0.09 | 97± 0.08 | 100± 0.03 | 101± 0.06 |
| | Hippocampus | 95±0.09 | 99±0.09 | 95±0.08 | 99±0.09 |

Values expressed as % of vehicle treated group. [a]$p<0.05$ versus naïve, [b]$p<0.05$ versus 3-NP, [c]$p<0.05$ versus 3-NP and *WS* root extract (100mg/kg), n=10 rats per group

the rat brain suggesting that oxidative the rat brain suggesting that oxidative damage damage play important role in 3-NP induced neurotoxicity [29]. In the present study, 3-NP significantly decreased total glutathione, reduced glutathione as well as oxidized glutathione levels in the striatum, cortex and hippocampus region of the brain, indicating weak antioxidant defense. 3-NP exposure significantly decreased the level of the glutathione–S-transferase in the same area of the brain. It seems that 3-NP induced neurotoxicity and cognitive dysfunction could be related to imbalance between reactive oxygen species and antioxidant defenses resulting in marked oxidative stress. 3-NP neurotoxicity triggers peroxidation processes, free radical generation and may weaken detoxifying endogenous antioxidant system such as glutathione. The antioxidant glutathione (GSH) is essential tripeptide for the cellular detoxification of reactive oxygen species in brain cells constitute more than 90% of the intracellular non-protein thiol pool. A compromised GSH system in the brain has been connected with the oxidative stress occurring in neurological diseases [30]. It regulates the endogenous protective mechanism, the glutathione redox cycle against chemical-induced toxicity[31]. It is specific substrate for the enzyme such as glutathione peroxidase and glutathione–S-transferase[32]. It plays a critical role in detoxification reactions by acting both as a nucleophilic scavenger of undesired compounds, toxic metabolites and a specific substrate for the enzymes including glutathione peroxidase and glutathione–S-transferase[33, 34]. GSH is known to react readily with a wide variety of free radical species leading to the formation of superoxide anions. The normal glutathione cycle involves oxidation of the GSH to GSSG. Further, GSH is regenerated back from GSSG within the cells in a reaction catalyzed by the flavoenzyme glutathione reductase. Therefore, decreased level leads to the imbalance of the redox status in the cell leading to oxidative stress. In the present study, a decrease in GSH in turn leads to decreased formation of GSSG and disturbs glutathione homeostasis in these brain areas. Further, WS treatment significantly reversed the disturbance in glutathione homeostasis caused by the 3-NP treatment. WS root extract might have direct free radical scavenging activity and spare antioxidant defense such as glutathione. The sitoindosides VII-X and withaferin A (glycowithanolides) active principles of WS, have been demonstrated to have antioxidant activity by increasing free-radical scavenging enzymes levels in the frontal cortex, striatum and hippocampus of rat brain[27,37]. In previous studies, administration of 3-NP increased the production of hydroxyl free radicals in the rat striatum suggesting that free radicals play a substantial role in 3-NP neurotoxicity [35,36]. The increase in glutamate levels may leads to increase in superoxide production and activation of calcium dependent nitric oxide synthase that cause oxidative stress and interactive stress [38,39].

## Conclusion

The root extract of *Withania Somnifera* has possible neuroprotective effect against a 3-NP-induced neurotoxicity in rats. This may have therapeutic benefit in the treatment of Huntington's disease.

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