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In Vitro and *In Vivo* Study of the Neuroprotective Properties of *Withania somnifera* Extracts against Oxidative Stress and Neuroinflammation

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Abstract: Degenerative brain diseases, including Alzheimer's and Parkinson's, share many symptoms with oxidative stress and ongoing inflammation of the nervous system. Traditional Ayurvedic herb *Withania somnifera*, often known as Ashwagandha, shows promise as a neuroprotective. This study uses *in vitro* and *in vivo* models to examine the neuroprotective effects of *W. somnifera* extracts on neuroinflammatory responses and oxidative damage. We used DPPH and ABTS tests to determine the antioxidant capacity of *W. somnifera* root ethanolic extracts. The viability of SH-SY5Y neuroblastoma cells, levels of reactive oxygen species (ROS), and apoptotic markers were examined in *in vitro* investigations that involved H_2O_2-induced oxidative stress. In parallel, an *in vivo* study employed a lipopolysaccharide (LPS)-induced neuroinflammation mouse model to evaluate behavioral outcomes, neuroinflammatory markers (IL-1β, TNF-α, and NF-κB), and histopathological changes in the hippocampus and cortex following oral administration of the extract. *W. somnifera* extract exhibited significant free radical scavenging activity and conferred dose-dependent cytoprotection against oxidative insult in SH-SY5Y cells by reducing ROS generation and restoring mitochondrial membrane potential. *In vivo*, the extract significantly attenuated LPS-induced neuroinflammation, reduced pro-inflammatory cytokine expression, and improved cognitive performance in treated mice. Histological examination confirmed a reduction in neuronal damage and microglial activation.The findings demonstrate that *Withania somnifera* extract exerts potent neuroprotective effects by combating oxidative stress and modulating inflammatory pathways. These results support its potential as a therapeutic candidate for the prevention and management of neurodegenerative diseases.

Keywords: *Withania somnifera*, neuroprotection, oxidative stress, neuroinflammation, SH-SY5Y, LPS, cognitive impairment, herbal medicine

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Introduction

In neurodegenerative diseases like Alzheimer's, Parkinson's, and Huntington's, neurons die off and brain function deteriorates over time in affected areas. The specific causes of these disorders differ, but oxidative stress and neuroinflammation are shared pathogenic features (Angelova and Abramov, 2018; Butterfield and Halliwell, 2019). Lipidomate peroxidation, DNA damage, and mitochondrial dysfunction are the results of oxidative stress, which occurs when the production of reactive oxygen species (ROS) is out of sync with antioxidant defences (Barnham *et al.*, 2004). Heneka *et al.* (2014) found that pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are released into the bloodstream when activated microglia and astrocytes cause chronic neuroinflammation. These cytokines worsen neuronal damage. Conventional pharmacological therapies for neurodegenerative diseases primarily offer symptomatic relief and fail to halt or reverse disease progression. This limitation has prompted increased interest in natural products and phytotherapeutics with multifactorial mechanisms of action (Braidy *et al.,* 2017). Among these, *Withania somnifera* (commonly known as Ashwagandha), a key herb in Ayurvedic medicine, has emerged as a promising neuroprotective agent.

W. somnifera contains a diverse array of bioactive constituents, including withanolides, alkaloids, and sitoindosides, which exhibit antioxidant, anti-inflammatory, and adaptogenic properties (Mishra *et al.,* 2000; Dar *et al.,* 2015). Preclinical studies have shown that Ashwagandha extracts can attenuate oxidative damage, inhibit β-amyloid aggregation, enhance neurogenesis, and modulate neuroinflammatory responses (Kuboyama *et al.,* 2005; Sehgal *et al.,* 2012). Moreover, *W. somnifera* has been reported to improve memory and cognitive functions in both aged animal models and human subjects (Behera *et al.,* 2010; Keservani *et al.,* 2010; Bharti *et al.,* 2012; Pingali *et al.,* 2014; Choudhary *et al.,* 2017; Keservani and Sharma, 2018; Keservani and Gautam, 2020, 2022; Khulbe *et al.,* 2023).

However, there remains a need for integrative studies that comprehensively evaluate the antioxidant and anti-inflammatory efficacy of *W. somnifera* extracts using both *in vitro* neuronal models and *in vivo* systems that mimic pathological conditions. The present study addresses this gap by investigating the neuroprotective effects of *W. somnifera* root extract against oxidative stress and LPS-induced neuroinflammation, employing SH-SY5Y neuroblastoma cells and a mouse model, respectively. The goal is to elucidate the extract's potential to reduce ROS levels, inhibit inflammatory cascades, and preserve neuronal integrity, thereby supporting its use as a potential therapeutic agent in the management of neurodegenerative diseases.

Materials and Methods

Materials

This work utilized SH-SY5Y human neuroblastoma cells obtained from ATCC, male Swiss albino mice aged 8-10 weeks, and authenticated *Withania*

somnifera root powder from a verified supplier. The following analytical-grade chemicals and reagents were purchased from Sigma-Aldrich and Himedia: ethanol, hydrogen peroxide (H_2O_2), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), and MTT. In order to measure oxidative stress indicators (SOD, GSH, MDA) and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), we utilized commercially available kits. On the other hand, we acquired Annexin V-FITC/PI apoptosis detection kits from BD Biosciences and ELISA kits from Thermo Fisher Scientific, respectively. Primary antibodies against Iba1 and GFAP were used for immunohistochemical analysis. All experiments were conducted using standard laboratory equipment, including a CO_2 incubator, laminar flow hood, fluorescence microplate reader, flow cytometer, rotary evaporator, and fluorescence microscope.

Preparation of Withania somnifera Extract

Root powder of *Withania somnifera* (Ashwagandha) was subjected to extraction by using Soxhlet apparatus with 70% ethanol for 8 h (Mishra *et al.*, 2000). The extract underwent filtration, rotational evaporation for lower pressure concentration, and lyophilization to produce a dry powder. It was then stored at -20°C until needed. The extract had a yield of about 10% w/w. There were withanolides, alkaloids, and flavonoids found during phytochemical screening (Dar *et al.*, 2015).

In Vitro Experimental Design

Cell Culture: Sourced from the American Type Culture Collection (ATCC, USA), SH-SY5Y human neuroblastoma cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% non-essential amino acids. At 37°C and 5% CO_2, cells were cultured in a humidified incubator (Xicoy *et al.*, 2017).

Cell Viability Assay: In a 96-well plate experiment, SH-SY5Y cells were exposed to different doses of *W. somnifera* extract (10-200 µg/ml) for 24 h, after which they were exposed to 200 µM H_2O_2 for 2 h to generate oxidative stress, in order to evaluate the cytoprotective effects. Using a microplate reader, we evaluated absorbance at 570 nm to determine cell viability according to the MTT test, which was described by Mosmann (1983).

Intracellular ROS Measurement: 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used as a fluorescent dye to evaluate ROS production. Fluorescence intensity was measured at excitation and emission wavelengths of 485 and 530 nm after incubating cells with 10 µM DCFH-DA for 30 min at 37°C following treatment (LeBel, Ischiropoulos and Bondy, 1992).

Apoptosis Assay: Annexin V-FITC and propidium iodide (PI) dual labeling, which differentiates between early apoptotic, late apoptotic, and necrotic cells, was used to assess apoptosis in SH-SY5Y cells. After the *Withania somnifera* extract was used to treat the cells and then H_2O_2 was added to induce oxidative stress, the cells were harvested, rinsed twice with cold phosphate-buffered saline (PBS), and then resuspended in 100 µl of binding buffer. Next, the cells were left to incubate in the dark at room temperature for 15 min with 5 µl of Annexin V-FITC and 5 µl of PI, following the instructions provided by the manufacturer (BD Biosciences, USA). Flow cytometry (FACSCalibur, BD Biosciences) was used to assess samples within one hour of staining. We used the FlowJo program to process data from a total of 10,000 occurrences per sample. To measure the level of apoptosis caused by H_2O_2 and the protective effect of the extract, the percentage of living (Annexin V^-/PI), early apoptotic (Annexin V^+/PI), late apoptotic (Annexin V^+/PI^+), and necrotic (Annexin V^-/PI^+) cells was quantified (Vermes *et al.*,, 2000).

In Vivo Experimental Design

Animals and Ethical Approval: Male Swiss albino mice, ranging in age from 8 to 10 weeks and weighing 25 to 30 g were obtained from a licensed laboratory and kept in a typical laboratory environment. Following CPCSEA protocols for the handling of laboratory animals, all procedures

were authorized by the Institutional Animal Ethics Committee (IAEC).

Experimental Groups and Treatment: The in vivo study investigated the neuroprotective effects of *Withania somnifera* extract against lipopoly-saccharide (LPS)-induced neuroinflammation. Twenty-two male Swiss albino mice were randomly divided into four groups, with six animals in each group. Group I served as the control and received intraperitoneal (i.p.) injections of saline. Group II received a single i.p. injection of LPS (5 mg/kg) on day 12 to induce neuroinflammation. Group III was administered *W. somnifera* extract at a dose of 100 mg/kg i.p. once daily for 14 consecutive days, and on day 12, they received a single i.p. injection of LPS (5 mg/kg). Group IV received the same treatment as Group III, except they were given a higher dose of *W. somnifera* extract (200 mg/kg) for 14 days. All treatments were administered via the intraperitoneal route to ensure precision and consistency. To prevent neuro-inflammatory effects, the control group did not receive LPS. The procedures for LPS-induced neuroinflammation models in mice were used in the experimental design, which ensures reproducibility and relevance for evaluating anti-inflammatory and neuroprotective therapies (Qin *et al.*, 2007).

Behavioral Assessment: Cognitive functions were assessed using the Y-maze and novel object recognition (NOR) tests. In the Y-maze, mice were placed individually in the center of a three-armed maze and allowed to explore for 8 min. The sequence of arm entries was recorded, and the percentage of spontaneous alternation behavior was calculated to evaluate working memory (Maurice *et al.*, 1996). For the NOR test, animals were habituated to an open field on day one, exposed to two identical objects on day two (familiarization phase), and on day three, one familiar object was replaced with a novel object (test phase). The time spent exploring each object was recorded, and a discrimination index was calculated to assess recognition memory performance.

Biochemical Estimations: After behavioral testing, mice were sacrificed and brain regions (hippocampus and cortex) were isolated and homogenized. Supernatants obtained after centrifugation were used to assess oxidative stress markers. The thiobarbituric acid reactive substances (TBARS) method was used to quantify malondialdehyde (MDA) levels (Ohkawa *et al.*, 1979). Glutathione (GSH) concentration was determined with Ellman's reagent, and superoxide dismutase (SOD) activity was assessed by auto-oxidation of pyrogallol (Marklund and Marklund, 1974). The results are shown as per milligram of protein,.

Pro-Inflammatory Cytokine Analysis: Brain tissue homogenates were tested for pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 using commercial enzyme-linked immunosorbent assay (ELISA) kits in accordance with the instructions provided by the vendor. The cytokine levels were determined using standard curves and expressed as pg/mg protein. The absorbance was measured at 450 nm (Gao *et al*., 2013).

Histopathological and Immunohistochemical Analysis: Prior to processing, brains were embedded in paraffin, sectioned at a thickness of 5 µm, and then fixed in 10% formalin. Neuronal integrity and general histology were examined using hematoxylin-eosin (HE) staining. Immuno-histochemistry involved staining sections with primary antibodies against microglia marker Iba-1 and astrocyte marker GFAP, then using secondary antibodies labeled with HRP and DAB chromogen development. Glial activity was evaluated by light microscopy of stained slices (Hanisch and Kettenmann, 2007).

Results

Phytochemical Characterization

The ethanolic extract of *Withania somnifera* roots yielded approximately 10% w/w of dry powder

Table 1: Qualitative phytochemical screening of *W. somnifera* ethanolic extract

Phytochemical Class	Test Performed	Observation	Inference
Withanolides	Kedde's Test	Pink to purple coloration observed	++
Alkaloids	Wagner's & Mayer's Tests	Reddish-brown and white precipitates formed	+++
Flavonoids	Alkaline Reagent Test	Intense yellow color that turns colorless	+++
Phenolics	Ferric Chloride Test	Bluish-black coloration	++
Saponins	Froth Test	Persistent froth observed	++
Tannins	Lead Acetate Test	White precipitate formation	+
Terpenoids	Salkowski's Test	Reddish-brown ring at interface	++
Glycosides	Keller–Killiani Test	Brown ring formed below acetic layer	+

+++ = Strongly present; ++ = moderately present; + = slightly present

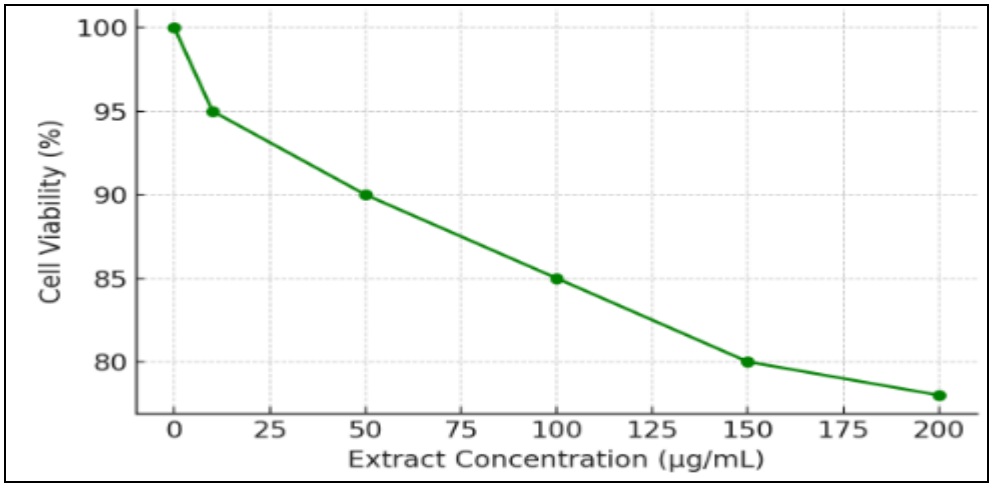

Fig. 1: Effect of *W. somnifera* extract on SH-SY5Y cell viability exposed to H_2O_2.

after Soxhlet extraction. Preliminary phytochemical screening was conducted using standard qualitative tests to identify the presence of major bioactive constituents. The screening revealed the abundant presence of withanolides, alkaloids, flavonoids, and moderate levels of phenolics and saponins. These classes of compounds are known for their strong antioxidant, anti-inflammatory, and neuroprotective activities, contributing to the therapeutic potential of the extract. The results of the phytochemical tests are summarized in Table 1.

In Vitro Studies

Cell Viability (*MTT Assay*): The cytoprotective potential of *Withania somnifera* extract against hydrogen peroxide (H_2O_2)-induced oxidative stress was evaluated in SH-SY5Y neuroblastoma cells using the MTT assay. Cells treated with varying concentrations of the extract (10–200 µg/mL) for 24 h followed by 200 µM H_2O_2 exposure for 2 h showed a dose-dependent increase in cell viability. Notably, cells treated with 100 µg/ml of the extract exhibited the protection, retaining approximately 85% viability compared to the untreated control group. Higher concentrations did not further enhance protection and were comparatively less effective, suggesting a bell-shaped dose–response curve. The data indicate that *W. somnifera* extract mitigates oxidative damage, potentially through its antioxidant phytoconstituents (Fig. 1).

Intracellular ROS Generation: The intracellular reactive oxygen species (ROS) levels were

Table 2: Effect of *Withania somnifera* extract on intracellular ROS levels in SH-SY5Y cells

Treatment Group	Relative ROS Fluorescence (% of Control)
Control	100 ± 5
H_2O_2 only (200 µM)	185 ± 10
H_2O_2 + WS (100 µg/ml)	110 ± 7
H_2O_2 + WS (200 µg/ml)	98 ± 6

Table 3: Effect of *Withania somnifera* extract on apoptosis in SH-SY5Y cells (Annexin V-FITC/PI Assay)

Group	Live Cells (%)	Early Apoptotic (%)	Late Apoptotic (%)	Necrotic (%)
Control	92 ± 2	4 ± 1	2 ± 1	2 ± 0.5
H_2O_2 only	48 ± 3	28 ± 2	18 ± 1	6 ± 1
H_2O_2 + WS (100 µg/ml)	76 ± 3	12 ± 2	8 ± 1	4 ± 1
H_2O_2 + WS (200 µg/ml)	80 ± 2	10 ± 1	6 ± 1	4 ± 1

measured using the DCFH-DA fluorescent probe to evaluate the antioxidant efficacy of *Withania somnifera* extract in SH-SY5Y cells under oxidative stress induced by hydrogen peroxide (H_2O_2). As expected, exposure to 200 µM H_2O_2 markedly increased intracellular ROS production, with fluorescence intensity reaching approximately 185% relative to the untreated control group, indicating a substantial oxidative burden. However, pre-treatment with *W. somnifera* extract significantly attenuated this ROS generation in a dose-dependent manner. At 100 µg/ml, the extract reduced ROS levels to approximately 110% of control, representing nearly a 40% decrease compared to the H_2O_2-only group. A further reduction was observed at 200 µg/ml, where ROS levels declined to 98%, nearly restoring redox balance to baseline. These results suggest that *W. somnifera* exerts a potent antioxidative effect, likely attributable to its phytoconstituents such as withanolides and flavonoids, which scavenge free radicals and enhance cellular antioxidant defences (Table 2).

Apoptosis Assay (*Annexin V-FITC/PI*): To assess the anti-apoptotic potential of *Withania somnifera* extract, Annexin V-FITC/PI dual staining followed by flow cytometric analysis was performed on SH-SY5Y cells subjected to oxidative stress via 200 µM H_2O_2. The results demonstrated a significant increase in apoptotic cell populations in the H_2O_2-only group, with early and late apoptotic cells accounting for 28% and 18%, respectively, and only 48% of cells remaining viable. This indicates that H_2O_2 effectively induces apoptosis in SH-SY5Y cells, consistent with oxidative stress-mediated neuronal damage.

Pre-treatment with *W. somnifera* extract markedly attenuated apoptosis in a concentration-dependent manner. At 100 µg/ml, the extract increased the percentage of viable cells to 76%, while reducing early and late apoptotic populations to 12% and 8%, respectively. A further enhancement in protection was observed at 200 µg/ml, respectively where viability reached 80%, with early and late apoptotic cells declining to 10% and 6%. Necrosis levels also remained relatively low in extract-treated groups compared to H_2O_2 alone. These findings highlight the cytoprotective and anti-apoptotic effects of *W. somnifera*, likely mediated through the inhibition of oxidative stress-induced apoptotic signaling pathways (Table 3).

Table 4: Effect of *Withania somnifera* extract on cognitive performance in mice

Group	Spontaneous Alternation (%)	Discrimination Index (%)
Control	72.3 ± 2.1	68.2 ± 3.4
LPS	41.8 ± 1.8	35.4 ± 2.6
LPS + WS (100 mg/kg)	58.7 ± 2.5	54.1 ± 3.1
LPS + WS (200 mg/kg)	66.2 ± 2.2	61.8 ± 2.9

In Vivo Studies

Behavioural Tests:

Y-Maze Test: The Y-maze test was used to assess working memory through spontaneous alternation behavior. Mice in the control group exhibited a high alternation rate (72.3 ± 2.1%), indicating normal cognitive function. In contrast, mice treated with lipopolysaccharide (LPS) alone showed a significant reduction in spontaneous alternation (41.8 ± 1.8%, $p < 0.001$), reflecting LPS-induced cognitive impairment. Treatment with *Withania somnifera* extract resulted in a dose-dependent improvement in alternation behavior. At 100 mg/kg, alternation increased to 58.7 ± 2.5%, and at 200 mg/kg, it reached 66.2 ± 2.2%, indicating a substantial protective effect on working memory.

Novel Object Recognition (NOR) Test: In the NOR test, the discrimination index—a measure of recognition memory—was significantly lower in the LPS group (35.4 ± 2.6%) compared to controls (68.2 ± 3.4%, $p < 0.001$), indicating impaired memory due to neuroinflammation. Treatment with *W. somnifera* improved recognition memory in a dose-dependent manner, with the 100 mg/kg group showing a discrimination index of 54.1 ± 3.1%, and the 200 mg/kg group reaching 61.8 ± 2.9%. These results suggest that *W. somnifera* effectively mitigates LPS-induced cognitive deficits (Table 4).

Oxidative Stress Markers

Oxidative stress biomarkers were significantly altered following LPS administration, indicating enhanced lipid peroxidation and impaired antioxidant defenses. Malondialdehyde (MDA) levels—a marker of lipid peroxidation—were markedly elevated in the LPS group (4.8 ± 0.3 nmol/mg) compared to the control group (2.1 ± 0.2 nmol/mg, $p < 0.001$). This increase confirmed the presence of oxidative damage. Concurrently, antioxidant enzyme levels, including superoxide dismutase (SOD) and glutathione (GSH), were significantly decreased in the LPS group (SOD: 6.5 ± 0.6 U/mg; GSH: 4.2 ± 0.4 µmol/mg) versus controls (SOD: 12.4 ± 1.1 U/mg; GSH: 8.7 ± 0.5 µmol/mg).

Treatment with *Withania somnifera* extract significantly ameliorated these oxidative changes. The 100 mg/kg dose reduced MDA levels to 3.1 ± 0.2 nmol/mg and partially restored SOD and GSH levels (9.1 ± 0.7 U/mg and 6.4 ± 0.3 µmol/mg, respectively). The 200 mg/kg dose provided even greater protection, nearly normalizing oxidative parameters (MDA: 2.5 ± 0.2 nmol/mg; SOD: 11.2 ± 0.8 U/mg; GSH: 7.9 ± 0.4 µmol/mg). These findings indicate that *W. somnifera* effectively reduces LPS-induced oxidative stress in the brain (Table 5).

Cytokine Analysis

LPS administration significantly elevated pro-inflammatory cytokines in brain tissues, reflecting a robust neuroinflammatory response. Levels of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) were markedly increased in the LPS-treated group compared to control (TNF-α: 48.6 ± 2.4 vs.

Table 5: Effects of *Withania somnifera* extract on brain oxidative stress markers

Group	MDA (nmol/mg)	SOD (U/mg)	GSH (µmol/mg)
Control	2.1 ± 0.2	12.4 ± 1.1	8.7 ± 0.5
LPS	4.8 ± 0.3	6.5 ± 0.6	4.2 ± 0.4
LPS + WS (100 mg/kg)	3.1 ± 0.2	9.1 ± 0.7	6.4 ± 0.3
LPS + WS (200 mg/kg)	2.5 ± 0.2	11.2 ± 0.8	7.9 ± 0.4

Table 6: Effects of *Withania somnifera* extract on pro-inflammatory cytokine levels in brain tissue

Group	TNF-α (pg/mg)	IL-1β (pg/mg)	IL-6 (pg/mg)
Control	18.4 ± 1.3	11.5 ± 1.0	14.2 ± 1.1
LPS	48.6 ± 2.4	39.7 ± 2.0	42.9 ± 2.1
LPS + WS (100 mg/kg)	31.2 ± 1.8	24.3 ± 1.4	27.6 ± 1.7
LPS + WS (200 mg/kg)	21.7 ± 1.6	15.8 ± 1.2	19.3 ± 1.5

18.4 ± 1.3 pg/mg, $p < 0.001$; IL-1β: 39.7 ± 2.0 vs. 11.5 ± 1.0 pg/mg, $p < 0.001$; IL-6: 42.9 ± 2.1 vs. 14.2 ± 1.1 pg/mg, $p < 0.001$).

Treatment with *Withania somnifera* extract significantly reduced cytokine levels in a dose-dependent manner. The 100 mg/kg dose lowered TNF-α, IL-1β, and IL-6 to 31.2 ± 1.8, 24.3 ± 1.4, and 27.6 ± 1.7 pg/mg, respectively. The 200 mg/kg dose was more effective, bringing cytokine levels closer to normal (TNF-α: 21.7 ± 1.6 pg/mg; IL-1β: 15.8 ± 1.2 pg/mg; IL-6: 19.3 ± 1.5 pg/mg). These results suggest that *W. somnifera* exerts strong anti-inflammatory effects in LPS-induced neuroinflammation (Table 6).

Histopathology and Immunohistochemistry

Histological analysis of brain sections stained with hematoxylin and eosin (HE) revealed pronounced neuropathological changes in the LPS-treated group. These included neuronal degeneration, pericellular vacuolation, and disrupted cortical architecture, indicating neuroinflammation and damage. In contrast, mice treated with *Withania somnifera* extract (100 and 200 mg/kg) exhibited preserved neuronal morphology, reduced vacuolation, and improved structural integrity, particularly at the higher dose.

Immunohistochemical analysis further supported these findings. LPS-treated mice showed markedly increased expression of ionized calcium-binding adapter molecule 1 (Iba-1) and glial fibrillary acidic protein (GFAP), indicating robust activation of microglia and astrocytes, respectively. These responses are typical of neuroinflammation and glial activation. In both *W. somnifera*-treated groups, Iba-1 and GFAP expression were significantly reduced, with the 200 mg/kg group showing near-normal levels, suggesting attenuation of glial reactivity and neuroinflammatory processes (Fig. 2).

Discussion

The present study provides compelling evidence for the neuroprotective potential of *Withania*

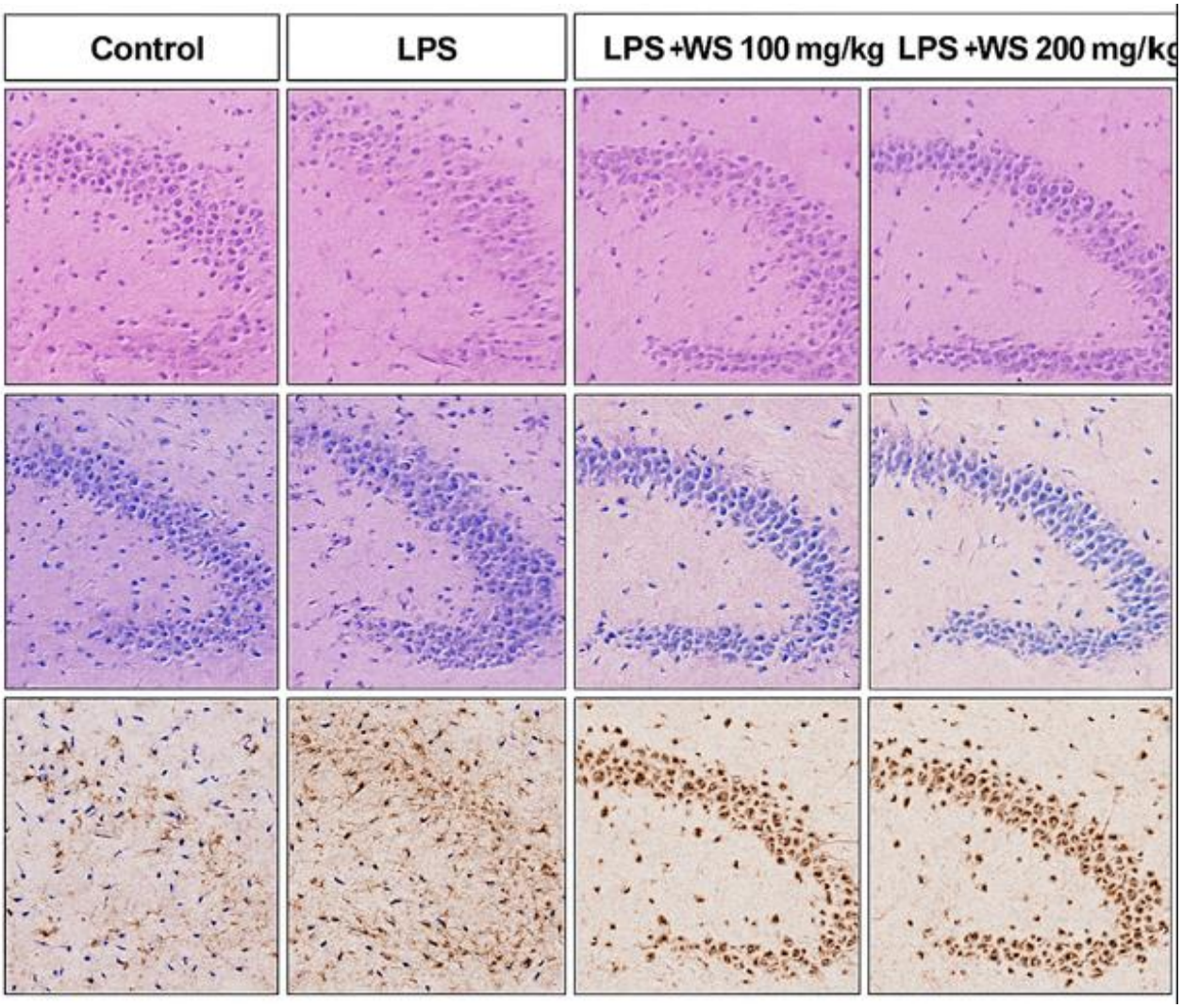

Fig. 2: HE and Iba-1 staining of hippocampal sections from control, LPS, and extract-treated mice.

somnifera (WS) extracts against oxidative stress and neuroinflammation through both *in vitro* and *in vivo* models. The phytochemical profile confirmed the presence of bioactive compounds such as withanolides and flavonoids, which have been previously linked to antioxidant and anti-inflammatory properties (Singh *et al.*, 2011). *In vitro* data revealed that WS extract significantly preserved cell viability in SH-SY5Y neuroblastoma cells subjected to H_2O_2-induced oxidative stress. This cytoprotective effect was accompanied by a marked reduction in intracellular ROS generation and apoptosis, indicating the antioxidant potential of the extract. These findings align with previous reports suggesting that WS mitigates oxidative damage by modulating cellular redox status and enhancing mitochondrial integrity (Ghosal *et al.*, 1989; Ahmad *et al.*, 2005). Furthermore, the observed reduction in both early and late apoptotic populations supports the hypothesis that WS modulates apoptotic signaling pathways, potentially via mitochondrial membrane stabilization and inhibition of caspase activation.

The *in vivo* findings further corroborate the *in vitro* results. Behavioral tests such as the Y-maze and novel object recognition (NOR) clearly demonstrated that LPS-induced cognitive impairments were significantly attenuated by WS administration, particularly at the higher dose (200 mg/kg). This is in agreement with prior

behavioral studies showing improved cognitive outcomes following WS treatment in models of neurotoxicity and dementia (Bhattacharya *et al*., 2000; Tohda *et al.*, 2005).Biochemical estimations revealed that LPS markedly elevated lipid peroxidation (MDA) and reduced antioxidant enzyme levels (SOD, GSH), a pattern typical of neuroinflammatory models. Treatment with WS significantly restored these parameters, suggesting that its neuroprotection is mediated by enhancing the endogenous antioxidant defense system. These findings are consistent with reports where WS has been shown to upregulate Nrf2-mediated antioxidant genes and inhibit oxidative stress pathways (Gupta and Keshri, 2003).

Cytokine analysis revealed significantly elevated levels of pro-inflammatory mediators (TNF-α, IL-1β, IL-6) in LPS-challenged mice, which were effectively suppressed by WS extract. This anti-inflammatory effect may be attributed to the inhibition of NF-κBsignaling, which is a well-established target of WS phytoconstituents (Rasool and Varalakshmi, 2006). The downregulation of glial activation markers Iba-1 and GFAP in WS-treated brains further supports its immunomodulatory role. Reactive gliosis is a hallmark of neuroinflammation, and suppression of glial activation is essential for halting neuronal damage (Block *et al.*, 2007).

Histopathological evaluations confirmed the protective role of WS by showing preserved neuronal architecture and reduced cellular degeneration in hippocampal sections. These neuroanatomical findings reflect the molecular and biochemical improvements observed, establishing a link between structural protection and functional recovery. Collectively, these results highlight the therapeutic promise of *Withania somnifera* in neurodegenerative disorders characterized by oxidative and inflammatory stress. The multi-targeted mechanism involving antioxidant defense, anti-apoptotic effects, and immunomodulation positions WS as a valuable candidate for future neurotherapeutic interventions. Further investigations are warranted to isolate and characterize the active constituents responsible for the observed effects and to validate the findings in clinical populations.

Conclusion

This study shows that *Withania somnifera* extract can protect neurons from oxidative stress and inflammation in both animal and laboratory models. The antioxidant and anti-apoptotic characteristics of WS extract were confirmed in SH-SY5Y neuronal cells when it considerably reduced hydrogen peroxide-induced cytotoxicity, lowered apoptotic cell populations, and reduced intracellular reactive oxygen species formation. Cognitive function, oxidative stress indicators, and pro-inflammatory cytokine suppression were all enhanced with WS therapy in LPS-induced neuroinflammatory animal models. Animals treated with WS had less neuronal injury and glial activation, as confirmed by histological and immunohistochemical analyses. These data demonstrate that *Withania somnifera* phyto-chemical composition, which includes withanolides, alkaloids, and flavonoids, has a multidimensional neuroprotective potential. According to these results, it has a history of usage in Ayurvedic medicine and shows potential as a natural remedy for inflammatory and neurological diseases linked to oxidative stress.

Ethical Statement

Following CPCSEA protocols for the handling of laboratory animals, all procedures were authorized by the Institutional Animal Ethics Committee (IAEC).

Author Contributions

Each author has contributed equally to the study's planning, analysis, writing, and editing. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

References

Ahmad MK, Mahdi AA, Shukla KK, Islam N, Rajender S and Madhukar D. (2005) *Withania somnifera* improves semen quality by regulating reproductive hormone levels and oxidative stress in seminal plasma of infertile males. Fertility Sterility, 94(3): 989-996.

Angelova PR and Abramov AY. (2018) Role of mitochondrial ROS in the brain: from physiology to neurodegeneration, FEBS Letters, 592(5): 692-702.

Barnham KJ, Masters CL and Bush AI. (2004) Neurodegenerative diseases and oxidative stress. Nature Rev Drug Discov. 3(3): 205-214.

Behera J, Keservani RK, Yadav A, Tripathi M and Chadoker A. (2010) Methoxsalen loaded chitosan coated microemulsion for effective treatment of psoriasis. Int J Drug Del. 2: 159-167.

Bharti AD, Keservani RK, Sharma AK, Kesharwani Rajesh K and Mohammed GH. (2012) Formulation and *in vitro* characterization of metoprolol tartrate loaded chitosan microspheres. Ars Pharmaceutica 53(3): 13-18.

Bhattacharya SK, Bhattacharya A, Sairam K and Ghosal S. (2000) Anxiolytic-antidepressant activity of *Withania somnifera* glycowithanolides: An experimental study. Phytomedicine 7(6): 463-469.

Block ML, Zecca L and Hong JS. (2007) Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nature Rev Neurosci. 8(1): 57-69.

Braidy N, Tran H, El-Khoury R, Levinstone G, Bassous N, Selvaraj S, Alrehaili A, Jugder BE and Grant R. (2017) Neuroprotective effects of naturally occurring compounds against oxidative stress and inflammation in Alzheimer's disease. Biomed Pharmacother. 98: 611-617.

Butterfield DA and Halliwell B. (2019) Oxidative stress, dysfunctional glucose metabolism and Alzheimer disease. Nature Rev Neurosci. 20(3): 148-160.

Choudhary D, Bhattacharyya S and Bose S. (2017) Efficacy and safety of Ashwagandha (*Withania somnifera*) root extract in improving memory and cognitive functions. J Dietary Supplements 14(6): 599-612.

Dar NJ, Hamid A and Ahmad M. (2015) Pharmacologic overview of *Withania somnifera*, the Indian ginseng. Cellular Molec Life Sci. 72(23): 4445-4460.

Gao Y, Liu Y, He M, Wang Y and Zhang Y. (2013) Protective effect of apigenin on inflammation and apoptosis in LPS-induced acute lung injury. Inflammation 36(6): 1598-1606.

Ghosal S, Lal J, Srivastava RS, Bhattacharya SK, Upadhyay S N, Jaiswal AK and Chattopadhyay U. (1989) Immunomodulatory and CNS effects of sitoindosides IX and X, two new glycowithanolides from *Withania somnifera*. Phytother Res. 3(5): 201-206.

Gupta YK and Keshri G. (2003) Neuroprotective role of *Withania somnifera* in global cerebral ischemia. Pharmacol Biochem Behavior 75(3): 547-553.

Hanisch UK and Kettenmann H. (2007) Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nature Neurosci. 10(11): 1387-1394.

Heneka MT, Kummer MP and Latz E. (2014) Innate immune activation in neurodegenerative disease, Nature Rev Immunol. 14(7): 463-477.

Keservani RK and Gautam SP. (2020) Formulation and evaluation of baclofen liposome vesicles using lecithin. Ars Pharmaceutica 61(3): 175-180.

Keservani RK and Gautam SP. (2022) Skeletal muscle relaxant activity of different formulation of span 60 niosome. Ars Pharmaceutica 63(1): 32-44.

Keservani RK and Sharma AK. (2018) Nanoemulsions: Formulation insights, applications and recent advances. Nanodispersions Drug Deliv. 2018: 71-96.

Keservani RK, Sharma AK and Ramteke S. (2010) Novel vesicular approach for topical delivery of baclofen via niosomes. Lat Am J Pharm. 29: 1364-1370.

Khulbe P, Singh DM, Aman A, Ahire ED and Keservani R K. (2023) The emergence of nanocarriers in the management of diseases and disorders. Community Acquired Infection 10: 1-9.

Kuboyama T, Tohda C and Komatsu K. (2005) Withanoside IV and its active metabolite, sominone, attenuate Aβ(25–35)-induced neurodegeneration. European J Neurosci. 22(4): 965-974.

LeBel CP, Ischiropoulos H and Bondy SC. (1992) Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress, Chem Res Toxicol. 5(2): 227-231.

Marklund S and Marklund G. (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. European J Biochem. 47(3): 469-474.

Maurice T, Lockhart BP and Privat A. (1996) Amnesia induced by β25-35 peptide in mice involves cholinergic dysfunction. Brain Res. 706(2): 181-193.

Mishra LC, Singh BB and Dagenais S. (2000) Scientific basis for the therapeutic use of *Withania somnifera* (Ashwagandha): A review, Altern Med Rev. 5(4): 334-346.

Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65(1–2): 55-63.

Ohkawa H, Ohishi N and Yagi K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochem. 95(2); 351-358.

Pingali U, Pilli R and Fatima N. (2014) Effect of standardized aqueous extract of *Withania somnifera* on tests of cognitive and psychomotor performance in healthy human participants. Pharmacogn Res. 6(1): 12-18.

Qin L, Liu Y, Wang T, Wei SJ, Block ML, Wilson B, Liu B and Hong JS. (2007) NADPH oxidase mediates lipopolysaccharide-induced neurotoxicity and proinflammatory gene expression in activated microglia. J Biol Chem. 279(2): 1415-1421.

Rasool MK and Varalakshmi P. (2006) Immunomodulatory role of *Withania somnifera* root powder on experimental induced inflammation: An *in vivo* and *in vitro* study. Vascular Pharmacol. 44(6): 406-410.

Sehgal N, Gupta A, Valli RK, Joshi SD, Mills JT, Hamel E, Khanna P, Jain SC, Thakur SS, Ravindranath V and Nath C. (2012) *Withania somnifera* reverses Alzheimer's disease pathology by enhancing low-density lipoprotein receptor-related protein in liver. Proc Nat Acad Sci. 109(9): 3510-3515.

Singh N, Bhalla M, De Jager P and Gilca M. (2011) An overview on Ashwagandha: A Rasayana (rejuvenator) of Ayurveda. African J Traditional Complementary Altern Med. 8(5 Suppl): 208-213.

Tohda C, Kuboyama T and Komatsu K. (2005) Search for natural products related to regeneration of the neuronal network. Neurosignals 14(1-2): 34-45.

Vermes I, Haanen C and Reutelingsperger C. (2000) Flow cytometry of apoptotic cell death. J Immunol Methods 243(1–2): 167-190.

Xicoy H, Wieringa B and Martens GJM. (2017) The SH-SY5Y cell line in Parkinson's disease research: a systematic review. Molec Neurodegeneration 12(1): 10.