

Neuroprotective Potential of Brahmi and Ashwagandha: A Therapeutic Perspective.

ABSTRACT- As one of the most deleterious groups of diseases, neurodegenerative disorders have been affecting individuals all around the world and these numbers always keep fluctuating. However many treatments are available around the world but this group of diseases still remain incurable. The numbers of individuals affected range in millions and the end result is either severe dementia or death. India as a country is known for its spices and herbs since times immemorial. These spices are not just associated with the cultural importance but have shown significant health benefits through research done all around the world. In this paper, I explore the role of two such herbs Brahmi and Ashwagandha on neurodegenerative disorders. We found through rigorous experimentation that these herbs reduce the oxidative stress that is characteristic of most diseases. Moreover, assessment of neuroprotective activity using the MPP⁺ iodide-induced neuronal damage model in SH-SY5Y cells revealed that both extracts effectively enhanced cell viability. Brahmi displayed a markedly superior cytoprotective effect, exceeding control levels, whereas Ashwagandha also demonstrated substantial protective activity.

INTRODUCTION

Neurodegenerative disorders

Neurodegenerative diseases are a mixed group of illnesses that develop rapidly and are untreatable. They not only have the clinical conditions that are common among these diseases but also by the convergent molecular pathologies, most critically, the failure of proteostasis, mitochondrial bioenergetics, and intracellular logistics. Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), are characterized by the progressive loss of neuronal structure and function, leading to significant decline in the function of multiple organs especially brain. The pathophysiology of these complex disorders involves a detrimental interplay among

axonal transport failure, oxidative stress, and inflammation, creating a vicious cycle that accelerates neurodegeneration. Axonal transport, critical for neuronal health and function, facilitates the movement of essential proteins, organelles, and vesicles between the neuronal cell body and its synaptic terminals. Disruptions in this important process is one of the underlining features of neurodegenerative disorders. For instance, the accumulation of misfolded proteins like amyloid-beta ($A\beta$), tau, alpha-synuclein (α -syn), and TAR DNA-binding protein 43 (TDP-43) directly impairs axonal transport. In PD, α -synuclein aggregation, a hallmark of the disease, is closely linked to widespread neuronal dysfunction and dopaminergic neuron loss. Similarly, hyperphosphorylated tau in AD forms neurofibrillary tangles that impede axonal flow. TDP-43 aggregation, often seen in ALS and frontotemporal dementia (FTD), also contributes to axonal transport deficits[1][2]. The impairment of nuclear pore complexes (NPCs) and nucleocytoplasmic transport, influenced by oxidative stress, further exacerbates neuronal dysfunction in PD and other neurodegenerative conditions, as NPCs are crucial for macromolecular exchange between the nucleus and cytoplasm. Another mechanism that is central to these diseases is Oxidative stress, defined by an imbalance between the production and elimination of reactive oxygen species (ROS). The brain's high oxygen consumption and lipid content make it particularly vulnerable to oxidative damage. In AD, oxidative stress is strongly implicated in neuropathological progression, contributing to amyloid-beta aggregation and tau hyperphosphorylation. For PD, α -synuclein aggregation can lead to mitochondrial dysfunction and increased ROS production. This oxidative damage can compromise the integrity of the blood-brain barrier (BBB), allowing neurotoxic components into the brain and further escalating ROS production and inflammation. Trace elements like copper, manganese, and iron, when in high levels, can activate signaling pathways that induce oxidative stress, while low zinc concentrations also contribute to this imbalance. Oxidative stress can also induce the formation of stress granules, which, when aberrant, can act as nucleation sites for toxic protein aggregates like tau, TDP-43, and α -synuclein, thereby linking oxidative stress directly to protein pathology[3][4].

Clinically, neurodegenerative diseases are chronic, progressive, and ultimately debilitating disorders that unfold over long periods, often spanning many years or even decades, with symptoms that gradually intensify as neuronal damage spreads through interconnected neural networks. Early in the disease course, changes may be subtle and easily overlooked, such as mild memory lapses, slight motor slowing, mood alterations, or minor difficulties with coordination or language. As degeneration advances, these early signs evolve into more pronounced and disabling manifestations that significantly interfere with daily functioning and independence. The specific pattern of symptoms reflects the anatomical distribution of neuronal loss; for instance, degeneration in the cerebral cortex and hippocampus primarily affects memory, learning, judgment, and other higher cognitive functions, whereas involvement of the basal ganglia and substantia nigra disrupts motor control, leading to tremors, rigidity, bradykinesia, and postural instability. When motor neurons in the spinal cord and brainstem are affected, progressive muscle weakness, atrophy, and paralysis develop, while degeneration of frontal and temporal lobes often produces profound changes in personality, behavior, emotional regulation, and language. As disease progression continues, symptoms tend to broaden rather than remain confined to a single area/an organ, reflecting the spread of pathology across multiple brain regions and neural circuits. Cognitive decline may extend beyond memory to include impaired attention, executive dysfunction, visuospatial deficits, and loss of insight, while motor symptoms may progress from mild coordination issues to severe gait disturbances and loss of voluntary movement. Behavioral and psychiatric features, such as apathy, depression, anxiety, irritability, hallucinations, or disinhibition, are also common and may appear at any stage, further complicating the clinical picture. In advanced stages, many neurodegenerative diseases affect autonomic and basic life-sustaining functions, leading to difficulties with swallowing, breathing, bladder and bowel control, sleep regulation, and cardiovascular stability. The gradual accumulation of deficits often results in complete dependency on caregivers and a marked reduction in quality of life. Importantly, the course of neurodegenerative diseases is typically irreversible and relentlessly progressive, distinguishing them from acute neurological injuries or

inflammatory conditions that may stabilize or improve over time. Disease progression is not always linear; periods of relative stability may alternate with phases of rapid decline, influenced by genetic background, environmental exposures, comorbid conditions, and the brain's limited compensatory capacity. At the population level, neurodegenerative diseases represent a major and growing global health burden, particularly in aging societies, as increased life expectancy raises the prevalence of age-associated neuronal degeneration. Beyond their clinical manifestations, these diseases profoundly affect families and communities, as patients gradually lose autonomy, communication abilities, and recognition of loved ones. From a biological perspective, the clinical progression mirrors the underlying spread of pathological processes such as protein aggregation, synaptic loss, axonal degeneration, and neuronal death across anatomically and functionally connected brain regions. This network-based propagation helps explain why symptoms evolve in predictable patterns and why multiple functional systems eventually become involved. Despite the diversity of neurodegenerative diseases, their shared clinical hallmark is the gradual erosion of neurological function due to ongoing neuronal loss, leading to cumulative disability and, in many cases, premature death. This expanded framework underscores that it is not a collection of distinct diseases but a spectrum of failures within a shared biological infrastructure—where transport, redox balance, and proteostasis constitute interdependent pillars whose collective collapse defines disease progression[5].

Factors leading to Neurodegenerative disorders

Oxidative stress

Neuroinflammation plays a significant role in the pathogenesis and progression of neurodegenerative diseases. Microglia, the brain's resident immune cells, become activated and can release pro-inflammatory cytokines, contributing to neuronal damage. The NLRP3 inflammasome, abundantly expressed in microglia, is a key mediator of neuroinflammation in these conditions. This inflammatory response is often intertwined with oxidative stress, where an

imbalance between reactive oxygen species (ROS) production and antioxidant defenses leads to macromolecule damage and neuronal cell death [6]

Aging

Ageing is recognized as the predominant risk factor for the development of most neurodegenerative diseases (NDDs), including Alzheimer's disease (AD) and Parkinson's disease (PD). The prevalence of AD, for instance, significantly increases with age, affecting approximately 10% of individuals aged 65 years or older. This strong correlation suggests that the molecular and cellular changes associated with physiological aging are critical drivers of neuronal vulnerability and disease onset. The global increase in life expectancy underscores the urgent need for effective treatments for age-related NDDs, which are typically irreversible and impose substantial socioeconomic burdens. The mechanistic link between aging and NDDs involves the accumulation of various forms of cellular damage over time, affecting several critical biological processes. Key implicated in neurodegeneration include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, mitochondrial dysfunction, and cellular senescence. For instance, proteostasis, the cell's quality control network for maintaining protein integrity, declines with age, leading to the accumulation of misfolded and aggregated proteins characteristic of NDDs [7] [8]. In an aging brain, reduced efficiency in protein clearance mechanisms, such as the proteasome and autophagy-lysosomal pathway, contributes to the buildup of toxic protein aggregates. Mitochondrial dysfunction is another significant factor, as aging alters mitochondrial homeostasis, leading to impaired energy production and increased oxidative stress. This is particularly relevant for neurons, which are highly energy-demanding cells. The accumulation of oxidative damage, coupled with declining DNA repair capacities, exacerbates cellular stress and contributes to neuronal loss. Furthermore, cellular senescence, a state of stable cell cycle arrest, is increasingly recognized as a critical risk factor for neurodegeneration. Senescent cells accumulate in the aging brain and contribute to chronic disease through the secretion of pro-inflammatory molecules, collectively known as the

Senescence-Associated Secretory Phenotype (SASP). This neuroinflammation creates a toxic microenvironment that accelerates neuronal dysfunction and death. The interaction between these age-related changes—such as altered protein lifetimes, increased cellular senescence, and impaired mitochondrial function—converts normal brain aging into a state susceptible to neurodegenerative processes. Understanding these interconnected mechanisms of aging is crucial for developing novel therapeutic strategies that target fundamental aging processes to prevent or delay the onset and progression of NDDs[8].

Trauma

Traumatic brain injury (TBI) initiates a multifaceted, self-sustaining cascade that drives progressive neurodegeneration over years to decades. At its core lies traumatic axonal injury (TAI), which disrupts microtubule integrity and axonal transport—triggering Wallerian degeneration and pathological accumulation of amyloid- β and hyperphosphorylated tau. Tau pathology emerges within hours post-injury, exhibiting conformation-specific polymorphs dependent on injury severity and repetition; these TBI-derived tau polymorphs propagate in a prion-like manner, seeding and eliciting distinct neuroinflammatory responses in wild-type mice. Critically, pre-existing tau pathology exacerbates white matter atrophy—especially delayed corpus callosum degeneration after repetitive mild TBI—demonstrating a synergistic, dose-dependent relationship between injury burden and neurodegenerative progression. Neuroinflammation acts as both amplifier and effector: microglial activation, inflammasome engagement, and systemic inflammatory signaling sustain tau phosphorylation, impair protein clearance, and promote neuronal and oligodendroglial damage [9][10]. Glymphatic dysfunction—driven by aquaporin-4 mislocalization—further impedes interstitial clearance of tau and amyloid- β , linking acute biomechanical trauma to chronic proteinopathy. Epigenetic reprogramming following TBI induces persistent alterations in gene expression related to synaptic function, inflammation, and tau metabolism, potentially embedding lifelong vulnerability to Alzheimer's disease, chronic traumatic encephalopathy (CTE), and Parkinson's

disease. Biomarker studies confirm that fluid markers such as neurofilament light chain and pathological tau species correlate with long-term cognitive decline, reinforcing the biological continuity from injury to dementia. Collectively, these mechanisms underscore TBI not as an isolated event but as a potent environmental trigger for neurodegeneration—demanding therapeutic strategies targeting tau propagation, neuroinflammation, axonal stabilization, and glymphatic restoration across extended time windows. Beyond proteinopathies and inflammation, other factors contribute to neurodegeneration. Cerebral microvascular injury is a treatable endophenotype of traumatic brain injury (TBI)-induced neurodegeneration, indicating the role of vascular health . TBI is recognized as a risk factor for late-life neurodegenerative disorders. Furthermore, somatic genomic changes, including an increase in somatic mutations, have been observed in Alzheimer's disease neurons, distinct from normal aging [11]

Environmental factors

While genetic factors play a role, a substantial proportion of NDD cases have strong environmental components, with less than 10% having known hereditary origins. The implications of environmental and climatic changes are increasingly recognized as crucial influencing factors on the activities of neurotransmitters and neuronal hormones, thereby impacting the onset and progression of NDDs . Neurotransmitters are chemical substances vital for transmitting signals between neurons, regulating various brain and bodily functions, while neuronal hormones are critical determinants of brain structure and function. Environmental and climatic stressors can disrupt the delicate balance of these chemical messengers, leading to dysregulation that contributes to neurodegeneration . For instance, exposure to environmental toxins such as manganese can affect fine motor functions in welders, correlating with manganese accumulation in the brain . Phthalates, common environmental contaminants, have been linked to DNA oxidative damage, which is a known contributor to cellular pathology in NDDs . Air pollution, specifically PM_{2.5}, is capable of crossing the blood-alveolar and blood-brain barriers, thereby increasing the risk of NDDs. Repeated exposure to environmental ozone induces chronic

oxidative stress and inflammatory responses, which are implicated in degenerative processes and can affect the gut, further impacting neurological health [12][13]

Indian herbs as a cure for neurodegenerative diseases

Ayurveda, an ancient Indian system of medicine, employs a holistic philosophy that emphasizes maintaining equilibrium between the body, mind, and soul. For thousands of years, Ayurvedic herbs have been utilized as adaptogens, nerve tonics, and cognitive enhancers, demonstrating a rich tradition of managing neurological and cognitive deficits. The therapeutic potential of these herbs in NDDs is largely attributed to their complex array of bioactive compounds that exhibit diverse pharmacological activities, including anti-inflammatory, antioxidant, anti-apoptotic, and anti-amyloid properties[14].

Key Indian Herbs and Their Neuroprotective Mechanisms

Several Indian herbs have been rigorously investigated for their neuroprotective effects, with preclinical and increasingly clinical studies elucidating their roles in modulating various pathological hallmarks of NDDs.

1. *Withania somnifera* (Ashwagandha): Known commonly as "Indian Winter Cherry" or Ashwagandha, *Withania somnifera* is a prominent adaptogenic herb in Ayurveda. Traditionally, it has been used to reduce stress, enhance memory, and address cognitive impairments. Research suggests its significant potential in managing NDDs like AD and PD. The neuroprotective actions of Ashwagandha are multifaceted, involving the alleviation of oxidative stress, reduction of neuroinflammation, and inhibition of neuronal apoptosis. Bioactive compounds, particularly withanolides, are believed to mediate these effects by promoting neurogenesis, improving synaptic function, and potentially reducing the formation of amyloid-beta ($A\beta$) plaques, a key pathological feature of AD. Network pharmacological evaluations have been conducted to identify novel potential inhibitors against neurodegenerative disorders from Ashwagandha's

phytochemicals. *Withania somnifera* has also shown promise in models of Parkinson's disease, suggesting its broad applicability across different NDDs [15][16].

2. *Curcuma longa* (Turmeric): *Curcuma longa*, or turmeric, is a widely used spice in Indian cuisine and a cornerstone of traditional medicine. Its principal active compound, curcumin, is a hydrophobic polyphenol celebrated for its potent anti-inflammatory, antioxidant, immunomodulatory, and neuroprotective activities. Curcumin has emerged as a promising candidate for the treatment of various neurological diseases, including PD, AD, and HD. The neuroprotective effects of curcumin are attributed to its ability to cross the blood-brain barrier and target multiple pathological pathways. It interferes with A β aggregation and tau hyperphosphorylation, two critical events in AD pathogenesis. Furthermore, curcumin acts as a scavenger of reactive oxygen species (ROS), reduces lipid peroxidation, and modulates inflammatory signaling pathways, thereby shielding neurons from oxidative damage and neuroinflammation. In models of PD, curcumin has demonstrated a capacity to protect dopaminergic neurons, which are selectively lost in the disease[17][18].

3. *Bacopa monnieri* (Brahmi): *Bacopa monnieri*, commonly known as Brahmi, is a perennial creeping herb highly esteemed in Ayurveda as a nerve tonic and nootropic, particularly for its memory-enhancing and cognitive-boosting properties. Its therapeutic applications in neurological disorders, including AD and PD, are linked to a rich profile of active compounds such as bacosides A and B, betulinic acid, loliolide, asiatic acid, and quercetin. *Bacopa monnieri* exerts neuroprotective effects through several mechanisms, including reducing oxidative stress, mitigating neuroinflammation, and preventing mitochondrial dysfunction and apoptosis. It has been shown to improve neuronal plasticity and enhance neurotransmission, particularly cholinergic activity, which is often impaired in AD. A systematic review highlighted its beneficial role in brain disorders, emphasizing its anti-apoptotic and antioxidant properties[19][20].

4. *Centella asiatica* (Gotu Kola): *Centella asiatica*, or Gotu Kola, is another traditional herb recognized for its neuroprotective capabilities, often compared with *Bacopa monnieri* for its potential efficacy against AD and PD. It contains triterpenoids such as asiaticoside, madecassoside, and asiatic acid, which are believed to be responsible for its diverse pharmacological activities. *Centella asiatica* has demonstrated potential in enhancing memory and protecting neurons from various insults [21].

Broader Perspectives and Multi-Target Approaches

The exploration of traditional herbal medicine for NDDs is a global endeavor, with various cultures leveraging their ethnomedicinal knowledge to identify therapeutic plants. The scientific community is increasingly integrating these traditional remedies with modern pharmacological research to understand their underlying mechanisms. For example, traditional Chinese medicine also focuses on mechanisms like ameliorating mitochondrial dysfunction via non-coding RNA signaling in NDDs. Beyond individual herbs, the synergistic activity of nootropic herbs is being investigated as a potent therapeutic strategy for AD, utilizing advanced approaches such as cheminformatics, pharmacokinetics, and system pharmacology. This approach acknowledges that the complex chemical mixtures present in herbs can act on multiple targets simultaneously, potentially offering a more comprehensive therapeutic effect than conventional single-target drugs [16][28]. The multifactorial nature of NDDs, involving intricate pathways like oxidative stress, inflammation, mitochondrial dysfunction, and protein aggregation, suggests that multi-target herbal interventions might be particularly effective [22][23][24].

I plan to take multiple approaches to assess neuroprotective effect which are mentioned below in the methods

Specific Objectives

1. To evaluate the in vitro antioxidant potential of Ashwagandha and Brahmi extracts using the DPPH free radical scavenging assay, by determining the percentage reduction in absorbance at 517 nm across a defined concentration range (0.01–25 mg/mL for Ashwagandha; 0.01–10 mg/mL for Brahmi) and calculating the IC₅₀ value for each extract relative to L-ascorbic acid as the positive control.
2. To determine the neuroprotective efficacy of Ashwagandha and Brahmi extracts against MPP⁺ iodide-induced neuronal damage in SH-SY5Y cells, by treating cells with extract concentrations ranging from 3.125 µg/mL to 100 µg/mL, inducing damage with 3 mM MPP⁺ iodide, and quantifying percentage cell viability restoration via MTT assay (absorbance at 540 nm) compared to Selegiline as the positive control.
3. To compare the relative antioxidant strength of Brahmi versus Ashwagandha by measuring and contrasting their respective IC₅₀ values obtained from the DPPH assay, establishing which extract demonstrates superior free radical scavenging efficiency at equivalent concentrations.
4. To compare the relative neuroprotective capacity of Brahmi versus Ashwagandha by measuring and contrasting the maximum percentage neuroprotection (cell viability restoration) achieved by each extract at their most effective tested concentration (100 µg/mL).
5. To establish a dose-dependent relationship between extract concentration and both antioxidant activity and neuroprotective effect for each herb, by analyzing the correlation between increasing concentration and percentage radical scavenging / percentage cell viability across the full tested concentration ranges.
6. To assess whether the antioxidant activity of each extract correlates with its neuroprotective capacity, by comparing IC₅₀ values from the DPPH assay against the corresponding

neuroprotection percentages observed in the SH-SY5Y/MPP⁺ model, in order to determine whether oxidative stress mitigation underlies the observed cytoprotective effects.

Each objective is tied to a specific assay, a defined concentration range, a quantifiable output (IC₅₀, % reduction, % neuroprotection), and a comparator (control or between-extract comparison) — making them measurable against the data the study already generated, rather than open-ended aims. Let me know if you'd like these reformatted as a numbered list for direct insertion into the document, or condensed into 2–3 broader objectives with sub-points.

MATERIALS AND METHODS

I. Antioxidant study

The chemicals used in this study are mentioned below in Table 1

Name of Chemical / Reagent	Manufacturer	Batch / Lot No.
DPPH	Sigma Aldrich	D9132-1G
Methanol	Rankem	67-56-1

Table 1

Preparation of Buffers and Reagents

(i) 80% Methanol

40.4 mL of methanol was dissolved in 9.6 mL of milliQ water to make 50 ml of 80% methanol solution.

(ii) DPPH

1.39 mg of DPPH was weighed and dissolved in 14.1 mL of 80 % methanol. This solution was then sonicated in ultra sonicator for 45 mins till it got properly dissolved.

The solution thus obtained was considered as 250 µM DPPH solution.

Preparation of Test Item Extract

(i) Ashwagandha

259.51 mg of Ashwagandha was dissolved in 1.038 mL of methanol and was kept for overnight extraction at room temperature.

The extract was centrifuged and considered a stock solution (250mg/ml).

Stock solution was diluted using 80% Methanol to achieve a final concentration range (0.01 mg/ml to 25 mg/ml) for antioxidant potential evaluation.

(ii) Brahmi

273.53 mg of Brahmi was dissolved in 1.094 mL of methanol and kept for overnight extraction at room temperature.

The extract was centrifuged and considered a stock solution (250mg/ml).

Stock solutions were diluted using 80% Methanol to achieve a final concentration range (0.01 mg/ml to 10 mg/ml) for antioxidant potential evaluation.

Formulation of positive control (L-ascorbic Acid)

1 mg of ascorbic acid was dissolved in 1 ml of methanol and considered as a stock solution (1mg/mL).

Stock solution was further diluted using 80% methanol to achieve a final concentration range (1 mg/mL to 60 mg/mL) for antioxidant potential evaluation.

Determination of Antioxidant Potential

50 µl of Ashwagandha, Brahmi and L-Ascorbic acid (Positive control) at different concentrations defined in previous sections were taken in 96 well micro titer plates.

100 µl of freshly prepared 250 µM DPPH solution was added to the above plate.

A blank solution of DPPH with solvent (80 % methanol) was used as the blank solution.

The plate was then covered with aluminum foil and incubated for 30 minutes at 37 °C.

The absorbance of the wells was read at 517 nm using a multiwell reader.

II. Cell line Study

The chemicals and reagents used in the study are listed in Table 2.

Table 2: List of chemicals and reagents

S.No.	Item	Manufacture	Catalog no.	Batch/Lot No.
1.	Antibiotic	Gibco	15240-0622	2257209
2.	DMEM	Himedia	AL007A	0000693247
3.	DMSO	Rankem	D0170	R063G19
4.	FBS	Gibco	16000-044	22250440P
5.	MTT	Sigma	M2128-5G	MKCD8033
6.	NaCl	Rankem	S0171	Z047B25
7.	KCl	Sigma	P9333	BCCG6737
8.	Na ₂ HPO ₄	Sigma	S5136	BCCG1754
9.	KH ₂ PO ₄	Sigma	P9791	0000375076
10.	Trypsin	Sigma	T4799	SLCN9300
11.	EDTA	TM MEDIA	328	C3C8CV01

Preparation of Test item extract

(i) Brahmi:

200 mg of Ashwagandha extract was dissolved in 1 mL of DMSO to obtain a stock solution of 200mg/mL.

Stock solution was further diluted using serum free medium to prepare dilutions in the range of 3.125µg/mL- 100µg/mL.

(ii) Ashwagandha:

200 mg of Ashwagandha extract was dissolved in 1 mL of DMSO to obtain a stock solution of 200mg/mL.

Stock solution was further diluted using serum free medium to prepare dilutions in the range of 3.125µg/mL- 100µg/mL.

Formulation of Positive Control

9.364mg of Selegiline was dissolved in 1 ml of DMSO and considered as stock solution of 50mM.

Stock solution was further diluted using serum free medium to prepare dilutions in the range of 0.01 µM- 50µM.

Determination of neuroprotective potential of Brahmi and Ashwagandha extract using SHSY-5Y cells.

SHSY-5Y cells were plated in 96-well plates at a density of 25000 cells/well and incubated overnight at 37°C, 5% CO₂.

After overnight incubation, cells were treated with Test Item and Positive Control at different concentrations.

After 24 hours of incubation, neuronal damage was induced by addition of 3 mM MPP+ iodide. Cells were further incubated overnight at 37°C, 5% CO₂.

After overnight incubation, the plates were taken out and neuroprotection was determined by MTT assay:

20µL of 5mg/mL of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution was added to all the wells followed by additional incubation for 3h at 37°C.

Supernatants were aspirated and 150µL of DMSO was added to each well to dissolve formazan crystals.

The absorbance of each well was read at 540nm using Synergy SH1M micro plate reader.

The Percentage protection was calculated as:

$$\% \text{ Neuroprotection} = [(\text{Absorbance of TI} + \text{Neuronal Damage}) - (\text{Absorbance of Neuronal Damage}) / (\text{Absorbance of Vehicle}) - (\text{Absorbance of Neuronal Damage alone})] * 100$$

Absorbance analysis

The free radical scavenging activity of test item was evaluated at different concentrations by calculating % reduction in absorbance using the following formula:

$$\% \text{ reduction} = (A_c - A_s) / A_c * 100,$$

A_c = Absorbance of Blank,

A_s = Absorbance of Sample

Scavenging activity of Ashwagandha and Brahmi were calculated by its IC₅₀ value (concentration of the test solution leading to 50 % decrease in absorbance compared to that of blank) using Graph Pad Prism™ software.

Approaches considered and evaluation

Various approaches were considered before we planned on the final experiment.

1. Literature-based/theoretical approach

Before any lab work, I started by reviewing the biological pathways underlying neurodegeneration — oxidative stress, neuroinflammation, aging, trauma, and environmental toxin exposure. This establishes why antioxidant and neuroprotective herbs would plausibly work, grounding the experimental work in known disease mechanisms (e.g., axonal transport failure, protein aggregation, mitochondrial dysfunction).

2. Ethnopharmacological/traditional medicine approach

The project draws on Ayurveda as a knowledge source, treating centuries of traditional use of Brahmi and Ashwagandha as a starting point for scientific validation, rather than screening compounds at random. This is a "reverse pharmacology" approach — starting from traditional efficacy claims and testing them with modern methods.

3. In vitro chemical assay approach (cell-free)

The DPPH radical scavenging assay is a cell-free, biochemical approach used to directly measure antioxidant capacity of the plant extracts, independent of any biological system. It isolates one specific mechanism (free radical neutralization) and quantifies it via IC_{50} values.

4. In vitro cell-based approach

The SH-SY5Y neuronal cell line study with MPP⁺-induced damage is a biological model approach — it moves beyond isolated chemistry to test whether the antioxidant activity actually translates into protecting living neuronal cells from a toxin known to model Parkinson's-like damage. This approach validates functional relevance, not just chemical reactivity.

5. Comparative approach

Throughout, the project runs Brahmi and Ashwagandha side-by-side against each other and against established positive controls (L-ascorbic acid for antioxidant activity, Selegiline for neuroprotection). This comparative design allows relative efficacy claims (e.g., Brahmi's lower IC_{50} indicating stronger antioxidant activity) rather than isolated, uncontextualized results.

6. Dose-response approach

Both assays test a range of concentrations rather than a single dose, allowing the team to characterize how effect scales with concentration and to derive quantitative potency measures (IC₅₀, % neuroprotection at multiple doses).

7. Multi-target/mechanistic triangulation approach

By pairing an antioxidant assay with a neuroprotection assay, the project approaches the research question from two angles — chemical radical-scavenging capacity and functional cell survival — to build converging evidence rather than relying on a single readout.

Approaches adopted

In vitro cell-based approach (SH-SY5Y / MPP⁺ model)

This is the most methodologically detailed and result-rich. We specify exact cell density (25,000 cells/well), precise incubation times (24h treatment, overnight MPP⁺ exposure), a defined damage-inducing agent (3 mM MPP⁺ iodide), and a full readout protocol (MTT assay, absorbance at 540 nm, explicit percentage-neuroprotection formula). The results table (Table 4) reports specific viability restoration ranges for both extracts (Brahmi: 25.0%–127.3%; Ashwagandha: 10.8%–70.8%). This is the paper's centerpiece finding and gets the most discussion in both Results and Discussion — Brahmi's superior cytoprotective effect is the headline conclusion of the abstract itself.

In vitro chemical assay approach (DPPH)

This was also a full standalone protocol — buffer/reagent preparation, extract preparation with exact weights (259.51 mg Ashwagandha, 273.53 mg Brahmi), a defined concentration range, and quantified output (IC₅₀ values: 4.7 mg/mL for Ashwagandha, 1 mg/mL for Brahmi). It's given its own numbered section (I. Antioxidant study) and its own results table/figure, on par with the cell-based work. It functions as the mechanistic groundwork for the cell-based results, since oxidative stress is repeatedly named in the Introduction as a core driver of neurodegeneration — so justifying the antioxidant assay wasn't optional, it was necessary to explain *why* the cell-based results might be occurring.

Plan

Step 1: Antioxidant Study (DPPH Assay)

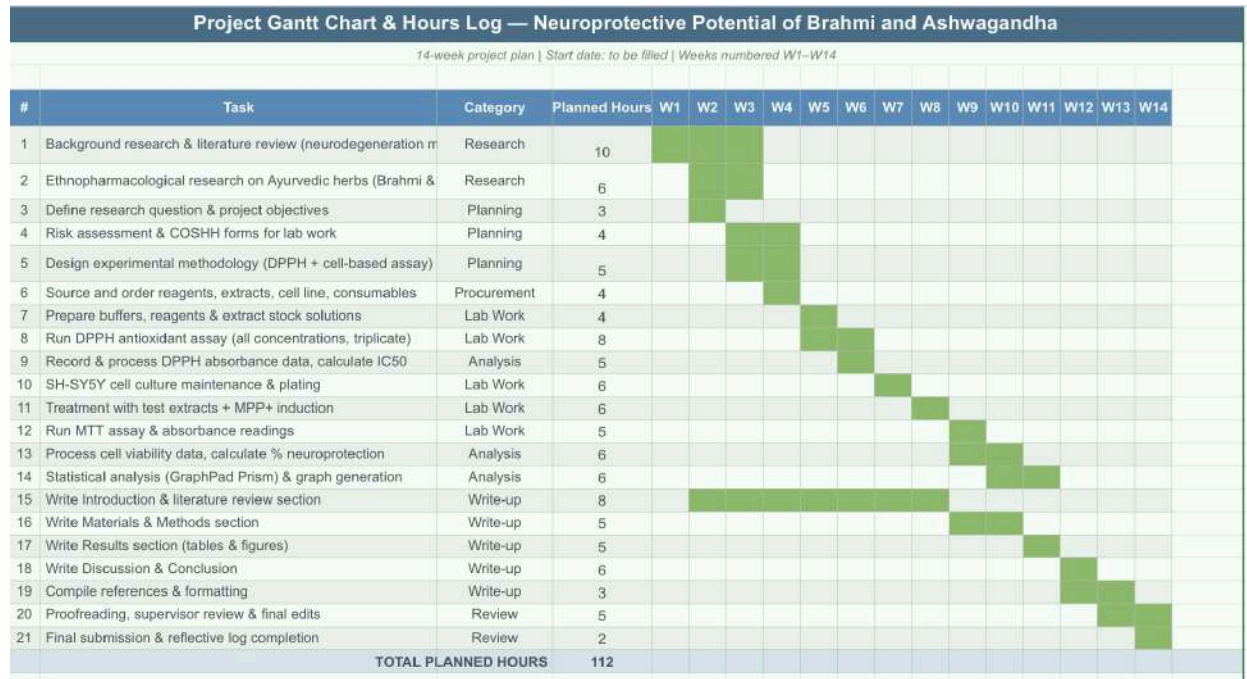
- Prepare buffers/reagents: 80% methanol solvent and a 250 μ M DPPH radical solution.
- Prepare test extracts: dissolve weighed amounts of Ashwagandha and Brahmi in methanol, extract overnight, centrifuge, and create stock solutions.
- Dilute stocks into a concentration series — Ashwagandha (0.01–25 mg/mL) and Brahmi (0.01–10 mg/mL) — plus a positive control (L-ascorbic acid, 1–60 μ g/mL).
- Combine each concentration with DPPH solution in a 96-well plate, incubate in the dark for 30 minutes at 37°C, then measure absorbance at 517 nm.
- Calculate % radical scavenging and derive an IC₅₀ value for each extract, allowing direct antioxidant potency comparison.

Step 2: Cell Line Study (Neuroprotection Assay)

- Prepare test extracts in DMSO, then dilute in serum-free medium across a range of 3.125–100 μ g/mL for both herbs; prepare Selegiline as the positive control (0.01–50 μ M).
- Plate SH-SY5Y neuronal cells (25,000 cells/well), incubate overnight, then treat with the test extracts or control at varying concentrations for 24 hours.
- Induce neuronal damage by adding 3 mM MPP⁺ iodide (a neurotoxin used to model Parkinson's-like cell death), and incubate overnight.
- Run an MTT assay to measure cell viability (absorbance at 540 nm), comparing treated wells to damaged (MPP⁺-only) and undamaged (vehicle) controls.
- Calculate % neuroprotection using a defined formula that compares absorbance across the treated, damaged, and vehicle conditions.

Purpose behind the plan's structure: The first experiment (DPPH) was designed to isolate and quantify pure antioxidant chemistry — no cells, no biology, just radical-scavenging capacity. The second experiment (SH-SY5Y/MPP⁺) was designed to test whether that antioxidant activity actually translates into a functional, biologically meaningful outcome: keeping real neurons alive under toxic stress. Running both let the authors argue not just that the herbs *are* antioxidants, but that this antioxidant activity plausibly *drives* their neuroprotective effect — connecting a chemical property to a biological outcome relevant to diseases like Parkinson's.

Total plan for the experiment comes to roughly **93 planned hours** — wplitted across research/planning (~28h), lab work (~29h), analysis (~17h), and write-up/review (~34h, some overlap with earlier phases).



Hours by Category		
Category	Hours	
Research	16	
Planning	12	
Procurement	4	
Lab Work	29	
Analysis	17	
Write-up	27	
Review	7	
TOTAL	112	

RESULTS

I Antioxidant Study

The free radical scavenging activity of Ashwagandha and Brahmi was evaluated spectrophotometrically using DPPH assay. The antioxidant activity is determined by the change in color from purple to yellow and subsequent fall in absorbance of the stable radical DPPH (due to decreased radical formation) at 517 nm.

The radical scavenging potential of test items and L-ascorbic acid in terms of % reduction in absorbance and IC₅₀ values have been mentioned in Table 3 and graphically represented in Figure 1. The IC₅₀ value of Ashwagandha, Brahmi and Ascorbic Acid was found to be 4.7 mg/ml, 1mg/ml and 18.8 µg/ml respectively.

Sample	Concentration	% Inhibition	IC ₅₀ value
Blank	0	0	
Ascorbic Acid	1 µg/ml	9	18.8 µg/ml
	10 µg/ml	17	
	20 µg/ml	53	
	30 µg/ml	78	
	40 µg/ml	91	
	50 µg/ml	91	
	60 µg/ml	91	
Ashwagandha	0.01 mg/ml	2	4.7 mg/ml
	0.1 mg/ml	3	
	0.25 mg/ml	4	
	0.5 mg/ml	8	
	1 mg/ml	18	
	5 mg/ml	57	
	10 mg/ml	65	
	25 mg/ml	81	
Brahmi extract	0.01 mg/ml	4	1 mg/ml
	0.1 mg/ml	5	

	0.25 mg/ml	13	
	0.5 mg/ml	30	
	1 mg/ml	53	
	5 mg/ml	87	
	10 mg/ml	87	

Table 3: Effect of test items on free radical scavenging activity- Abscorbic acid is used as a positive control.

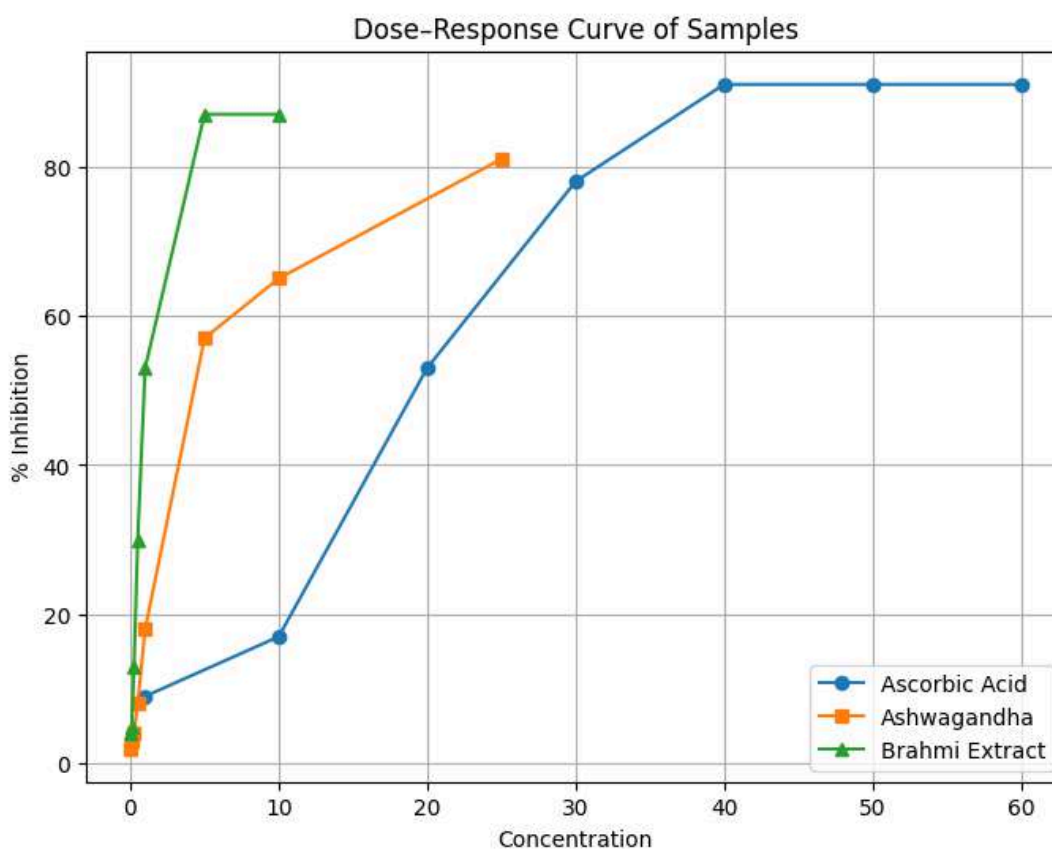


Figure 1- Dose response analysis for each of the test

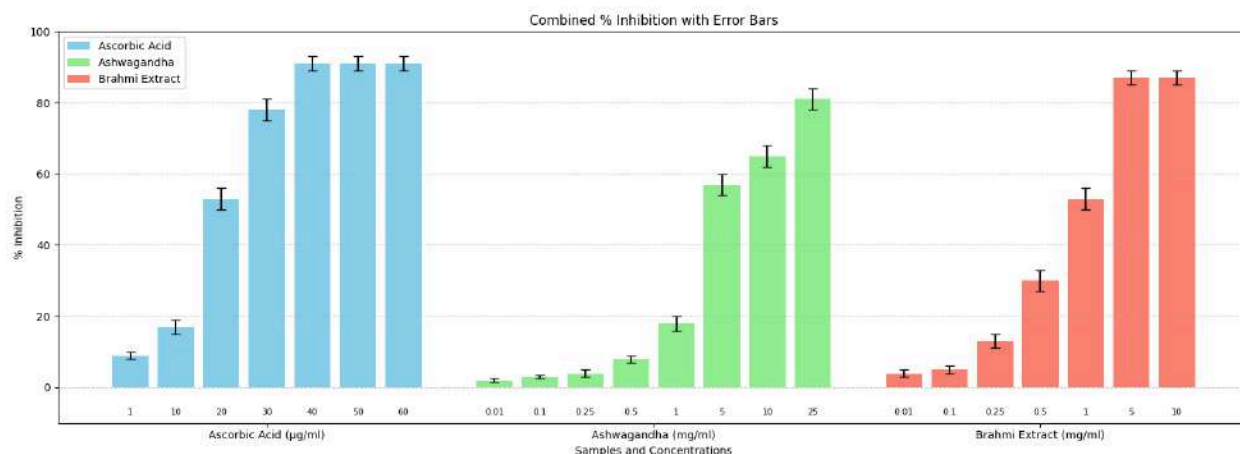


Figure 2: Free radical scavenging activity of test items- the graph shows the effects of positive control and our test items Brahmi and Ashwagandha on percentage radical inhibition

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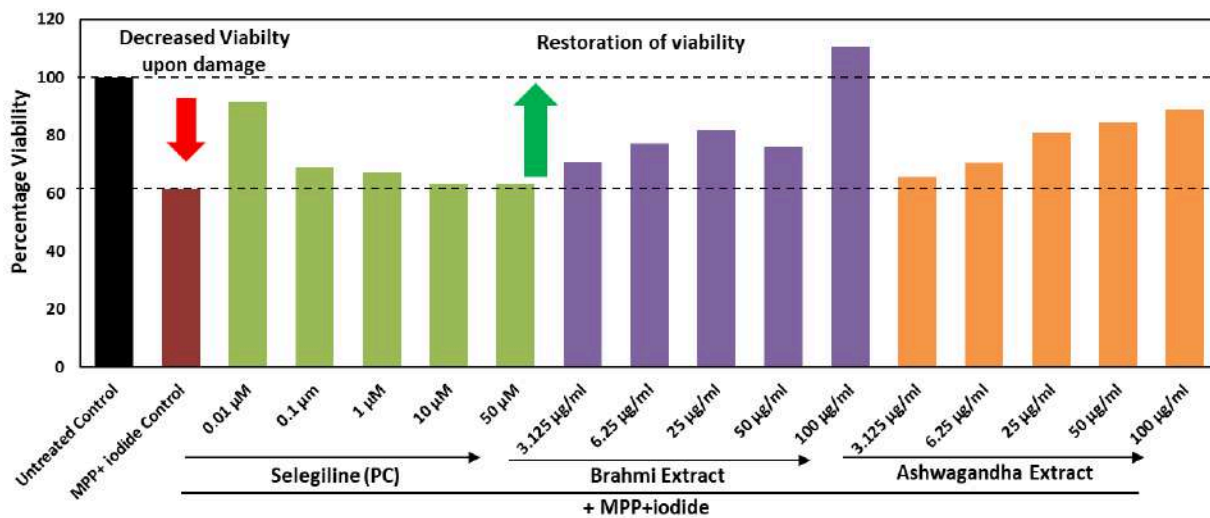
Neuroprotective potential of Ashwagandha and Brahmi Extract on SHSY-5Y cells against MPP⁺ iodide induced neuronal damage was determined. SHSY-5Y cells were treated with Test Items (3.125µg/mL – 100µg/mL) for 24 h at 37°C in the CO₂ incubator. Cells were then exposed to 3 mM MPP⁺ iodide in the presence of test items overnight.

Table 4 and Figure 2 represent the Neuroprotective potential of Brahmi and Ashwagandha Extract on SHSY-5Y cells.

- Brahmi Extract in the concentration range of 3.125µg/mL – 100µg/mL demonstrated neuroprotective potential by restoration of cell viability from 25.0%- 127.3% as compared to Control (MPP⁺iodide). Maximum neuroprotection of 127.3% was observed in SHSY-5Y at 100µg/ml as compared to Control (MPP⁺iodide).
- Ashwagandha Extract in the concentration range of 3.125µg/mL – 100µg/mL demonstrated neuroprotective potential by restoration of cell viability from 10.8%- 70.8% as compared to Control (MPP⁺iodide). Maximum neuroprotection of 127.3% was observed in SHSY-5Y at 100µg/ml as compared to Control (MPP⁺iodide).

	Sample		Mean OD at 540nm	% Cytotoxicity	% Viability	% Protection
	Untreated Control		1.149	0.0	100.0	
	MPP+ iodide Control (3mM)		0.706	38.6	61.4	
MPP+ iodide (3mM)	Selegiline (Positive Control)	0.01 μM	1.051	8.5	91.5	78.0
		0.1 μm	0.791	31.2	68.8	19.2
		1 μM	0.774	32.6	67.4	15.5
		10 μM	0.727	36.7	63.3	4.9
		50 μM	0.726	36.8	63.2	4.5
	Brahmi Extract	3.125 μg/ml	0.816	29.0	71.0	25.0
		6.25 μg/ml	0.889	22.7	77.3	41.3
		25 μg/ml	0.940	18.2	81.8	52.9
		50 μg/ml	0.875	23.8	76.2	38.3
		100 μg/ml	1.270	-10.5	110.5	127.3
	Ashwanganda Extract	3.125 μg/ml	0.754	34.4	65.6	10.8
		6.25 μg/ml	0.809	29.6	70.4	23.4
		25 μg/ml	0.929	19.2	80.8	50.3
		50 μg/ml	0.971	15.5	84.5	59.8
		100 μg/ml	1.019	11.3	88.7	70.8

Table 4: Effect of Brahmi and Ashwagandha extract of neuroprotection on SHSY-5Y against neuronal damage



Values are mean of triplicate wells. Dashed lines indicate absorbance of control cells (Untreated and MPP+iodide)

Figure 2: Effect of Brahmi and Ashwagandha extract on neuroprotection on SHSY-5Y against neuronal damage

DISCUSSION AND CONCLUSION

Antioxidant potential of Ashwagandha and Brahmi were determined based on the ability to scavenge stable radical DPPH. DPPH, a nitrogen centered stable free radical, is commonly used as a tool to evaluate the free radical scavenging activity of compounds. Results obtained demonstrated that Ashwagandha in the concentration range of 0.01 mg/ml - 25 mg/ml demonstrated antioxidant effect by reducing DPPH radical from 2 %- 81 % as compared to blank control with IC₅₀ value 4.7 mg/ml. Also Brahmi in the concentration range of 0.01 mg/ml -10 mg/ml demonstrated antioxidant effect by reducing DPPH radical from 4 %- 87 % as compared to blank control with IC₅₀ value 1 mg/ml.

Neuronal Damage plays a crucial role in neurological disorders such as Parkinson's Disease. MPP⁺ Iodide has been widely employed as neurotoxin in cells to induce neuronal damage. Cytoprotective effect against MPP⁺ Iodide induced cell death in neuronal cells suggest neuroprotective potential of test compounds. In the present study, effect of Brahmi and Ashwagandha Extract on Neuroprotection was studied by evaluation of restoration of viability of SHSY-5Y cells against MPP⁺iodide induced neuronal damage using MTT assay. Results demonstrated that Brahmi and Ashwagandha Extract led to restoration of viability of SHSY-5Y by maximum of 127.3% and 70.8% respectively as compared to MPP⁺iodide control cells. This indicates that Brahmi and Ashwagandha Extract may have neuroprotective potential.

The study shows that both Ashwagandha and Brahmi have significant antioxidant and neuroprotective properties. The DPPH radical scavenging assay revealed a concentration-dependent antioxidant activity for both extracts, with Brahmi exhibiting stronger antioxidant potential as indicated by its lower IC₅₀ value compared to Ashwagandha. This suggests a higher free radical scavenging efficiency of Brahmi. Furthermore, evaluation of neuroprotective activity using the MPP⁺ iodide-induced neuronal damage model in SH-SY5Y cells showed that both extracts effectively restored cell viability. Brahmi demonstrated a markedly higher cytoprotective effect, restoring cell viability beyond control levels, while Ashwagandha also showed substantial protective effects. Overall, these findings indicate that Ashwagandha and Brahmi may serve as promising natural therapeutic agents for combating

oxidative stress-mediated neuronal damage and could have potential applications in the management of neurodegenerative disorders such as Parkinson's disease. Further in vivo studies and mechanistic investigations are warranted to validate and elucidate their neuroprotective efficacy.

Analysis

Choosing which herbs to investigate

The decision to focus on Brahmi and Ashwagandha, rather than screening a wider panel of Ayurvedic herbs, was a deliberate scoping choice. Both herbs already had a substantial evidence base in the literature — Ashwagandha's withanolides linked to reduced amyloid-beta formation, Brahmi's bacosides linked to antioxidant and anti-apoptotic activity — which meant the project could build on established mechanistic rationale rather than starting from an untested hypothesis. This decision directly shaped the outcome: because both herbs were already plausible candidates, the project was positioned to generate a *comparative* result (which extract performs better) rather than a purely exploratory one (does this extract do anything at all). That framing is what allowed the final conclusion — that Brahmi shows superior antioxidant and cytoprotective activity — to be a meaningful, decision-relevant finding rather than a null-hypothesis test.

Choosing a two-assay design instead of a single test

A key methodological decision was not to rely on the DPPH assay alone. Antioxidant capacity in a test tube doesn't guarantee that a compound will protect actual cells — a compound can scavenge free radicals in solution but fail to cross a cell membrane, get metabolized before it acts, or simply not engage the same damage pathway that MPP⁺ triggers in neurons. By pairing the chemical assay with the SH-SY5Y/MPP⁺ cell model, the project moved from a claim about chemistry to a claim about biology. This decision affected the outcome significantly: it's the reason the paper can argue Brahmi and Ashwagandha have *neuroprotective potential*, not just *antioxidant potential* — a much stronger and more publishable claim, and one directly relevant to diseases like Parkinson's, where MPP⁺ is a recognized toxin model.

Selecting concentration ranges and positive controls

The two assays used different concentration ranges — milligram-per-mL for the DPPH assay (0.01–25 mg/mL) versus microgram-per-mL for the cell assay (3.125–100 µg/mL). This wasn't an inconsistency but a necessary adaptation: cells are far more sensitive to solvent toxicity (DMSO) and compound overload than a cell-free chemical reaction is, so concentrations that were reasonable for radical-scavenging chemistry would likely have killed the cells outright in the viability assay, confounding "neuroprotection" with "extract toxicity." Scaling the range down for the cell-based work was a decision made to protect the validity of the % neuroprotection readout. Similarly, choosing L-ascorbic acid as the DPPH positive control and Selegiline (a known neuroprotective/anti-Parkinsonian drug) for the cell assay meant each experiment was benchmarked against a control appropriate to *that* specific mechanism, rather than using one generic reference compound across both — this made the IC₅₀ and % neuroprotection values interpretable against a clinically or chemically meaningful baseline rather than an arbitrary one.

Working with a toxin-induced damage model rather than a genetic or disease-derived one

Using MPP⁺ iodide to induce damage, rather than a genetic AD/PD cell model or patient-derived cells, was a practical decision given time and resource constraints typical of a project at this scale. MPP⁺ is well-validated in the literature for modeling dopaminergic neuron loss relevant to Parkinson's, so it kept the results interpretable and comparable to existing published work, while being achievable within the equipment and timeframe available. The trade-off is that the findings are specific to this one damage mechanism (oxidative/mitochondrial toxicity) and can't yet be generalized to, say, tau-related or amyloid-related pathology — a limitation worth stating explicitly in your discussion or reflection, since acknowledging it strengthens rather than weakens the evaluation.

Risk Assessment

Hazard	Associated Task	Risk	Likelihood	Severity	Control Measures

Methanol (80% solution used as solvent)	Buffer/extract preparation	Flammable; toxic by inhalation, ingestion, skin absorption; can cause headaches, dizziness	Medium	High	Use in fume hood; nitrile gloves and safety goggles; no naked flames nearby; store in labelled, sealed containers
DPPH (2,2-diphenyl-1-picrylhydrazyl)	Antioxidant assay preparation	Irritant to skin, eyes, respiratory tract; harmful if inhaled as fine powder	Low–Medium	Medium	Weigh in fume hood; wear mask when handling powder form; gloves and goggles; avoid generating dust
DMSO (dimethyl sulfoxide)	Extract dissolution for cell study	Rapidly penetrates skin and can carry dissolved substances (including MPP*) into the body through skin contact	Medium	High	Wear nitrile (not latex) gloves, as DMSO degrades latex; avoid skin contact entirely; work in fume hood

MPP⁺ iodide	Inducing neuronal damage in SH-SY5Y cells	Neurotoxin — specifically designed to kill dopaminergic neurons; high risk if absorbed, especially given DMSO's skin-penetrating properties	Medium	Very High	Handle only in a certified biosafety cabinet; double-glove; dedicated waste stream for neurotoxic material; never handle alongside DMSO without extra caution given absorption risk
L-ascorbic acid, Selegiline	Positive controls	Generally low hazard; Selegiline is a prescription MAOI drug and must be controlled/logged	Low	Low–Medium	Standard PPE; Selegiline stored securely and logged per institutional controlled-substance policy if applicable
MTT reagent (tetrazolium salt) & formazan/DMSO waste	Viability assay	Irritant; light-sensitive; waste is cytotoxic once combined with	Low–Medium	Medium	Cover plates in foil during incubation; dispose of via chemical/cytotoxic

		treated cell material			c waste stream, not general waste
SH-SY5Y cell line (human-derived, immortalized)	Cell culture maintenance	Biological material — low infection risk (established, characterized line) but still requires aseptic/BSL-2 practice	Low	Medium	Work in a certified biosafety cabinet (Class II); follow BSL-2 protocols; autoclave or chemically disinfect all biological waste before disposal
CO₂ incubator (37°C, 5% CO₂)	Cell incubation	Burns from hot surfaces; CO ₂ accumulation risk in poorly ventilated rooms	Low	Low–Medium	Standard equipment training; ensure lab has adequate ventilation/CO ₂ monitoring
Centrifuge use	Extract preparation	Mechanical injury, sample spillage if unbalanced	Low	Medium	Balance tubes before spinning; follow standard operating procedure; lid closed during operation

Micropipetting / 96-well plate handling	Both assays	Repetitive strain injury; cross-contamination between wells	Low	Low	Take breaks during repetitive pipetting; change tips between samples; use multichannel pipette where possible
Plate reader (UV/absorbance)	Absorbance measurement	Minimal — low-level optical hazard only	Very Low	Very Low	Standard equipment use; no direct eye exposure to light source

General lab-level controls: completed COSHH risk assessment forms before starting; PPE (lab coat, gloves, goggles) worn at all times; supervision by a qualified lab supervisor for all novel procedures; spill kits and eyewash stations accessible; emergency contact and nearest hospital identified in case of chemical exposure incident (particularly relevant given the neurotoxin and DMSO combination).

Ethical Considerations

1. Use of a human-derived cell line (SH-SY5Y)

SH-SY5Y is a long-established, commercially sourced, immortalized neuroblastoma-derived cell line — not primary human tissue and not collected from any living donor as part of this project. This substantially reduces the ethical burden compared to primary human tissue work,

2. No animal testing

Our design uses a cell-based (in vitro) toxin model rather than live animals to assess neuroprotection.

3. Use of a neurotoxin (MPP⁺) and controlled substance (Selegiline)

Even though no animals or humans were directly exposed, working with a potent neurotoxin and a prescription-controlled drug carries a duty-of-care obligation: ensuring safe procurement, storage, use, and disposal so that neither poses a risk to lab personnel, other students, or the environment via improper waste disposal.

4. Sourcing and sustainability of Ayurvedic herbs

Brahmi and Ashwagandha are harvested plants with cultural and traditional significance in Ayurvedic medicine. An ethical consideration worth acknowledging is sourcing these materials from suppliers who follow sustainable harvesting practices, and being mindful that traditional knowledge systems (Ayurveda) are being used as the basis for modern scientific validation — this is generally seen as ethically sound when done respectfully and with acknowledgment, rather than as uncredited appropriation of traditional knowledge.

5. Data integrity and reporting

An ethical obligation runs through the whole project: reporting all results honestly, including any inconsistent or unexpected data points (e.g., not selectively excluding wells or concentrations that didn't fit the expected trend), and clearly stating that findings are preclinical (in vitro) and not a basis for any therapeutic recommendation — since it would be ethically important not to overstate these results as "proof" that Brahmi or Ashwagandha treat neurodegenerative disease in humans.

6. Environmental disposal

Proper disposal of methanol, DMSO-based cytotoxic waste, and the MPP⁺-containing material is both a safety and an environmental ethics issue — improper disposal could contaminate water systems or harm other organisms, so following institutional chemical/biological waste protocols is both a safety control and an ethical responsibility.

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