Research article

Cytoprotective and anti-inflammatory effect of ethanolic extract of *Withania somnifera* root in combination with doxycycline in rotenone induced cytotoxicity in neuroblastoma cell lines

Vishala Epuri[1], Lavanya Prathap[2], Venkateshwar Reddy[1], B. Rajesh[3], B. Ramesh[4]

[1]Department of Anatomy, SVS Medical College, Mahaboobnagar, Telangana, India
[2]Department of Anatomy, Saveetha Dental College and Hospital, SIMATS, Chennai, India
[3]Department of Anatomy, [4]Department of Biochemistry, Arundathi Institute of Medical Sciences, Dundigal (V), Gandimaisamma (M), Medchal-Malkajgiri Dist., HMDA Hyderabad, 500043, Telangana, India

(Received: October 2022 Revised: December 2022 Accepted: February 2023)

Corresponding author: **B. Ramesh.** Email: rameshbiochem007@gmail.com

ABSTRACT

Introduction and Aim: Parkinson's disease (PD) is a neurodegenerative disorder that destroys dopaminergic neurons in the nigrostriatal area of the midbrain this results in a movement difficulty characterised by tremors, bradykinesia, and postural instability The present in vitro study is designed to assess the beneficial (cytoprotectant and anti-inflammatory) and cyto-toxicological effects of ethanolic *Withania somnifera* (Ashwagandha) root extract (EWSR) in presence of Rotenone (ROT) and other known neuroprotectants (Doxycycline and Ellagic acid).

Materials and Methods: The nine groups used in the in vitro studies were normal control, positive control (Rotenone or Rot), *W. somnifera* root extract (EWSR), Doxycycline (Doxy), Ellagic acid (EA), ROT with EWSR, ROT with doxy, ROT with EA, ROT with EWSR-doxy, and ROT with EWSR-doxy-EA. The EWSR, doxy and EA treatment was started on the 24th hour following the rotenone administration and continued for the next 36 hours. After the 36th hour of protective therapy, quantification of pro-inflammatory cytokines (ELISA), cell viability or analyses of neuronal cell loss, cell cycle analysis and molecular analysis (RTPCR) was performed as a part of assessment.

Results: Observations from the present in vitro studies clearly demonstrate the cytoprotective and anti-inflammatory properties of EWSR, and these beneficial or protective effects got enhanced when treated in combination with Doxy and EA indicating synergistic effect in the cells that were priorly treated with ROT, and later treated with EWSR, Doxy, and EA individually and in combination.

Conclusion: The findings imply that EWSR reduces the in vitro effects of ROT by acting as an anti-inflammatory and cytoprotective agent.

Keywords: Rotenone (ROT); *Withania somnifera* root extract (EWSR); Doxycycline; Anti-inflammation; Parkinson's disease.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder that selectively causes loss of dopaminergic neurons in the nigrostriatal area of the midbrain which results in a movement difficulty characterised by tremors, bradykinesia, and postural instability (1-6). Lewy bodies and Lewy neurites are present due to PD pathology, and the nigrostriatal dopaminergic pathway is disturbed (2). α-synuclein is the neural protein, it is over expressed in PD and inhibits neurotransmitter release in the CNS (4-6). However, there is evidence that α-synuclein is present in all CNS sites, as well as outside the brain. This extra CNS pile may be responsible for the disease process truly beginning (6). As a result, the disease is not pathologically limited to CNS sites. Supplementing or injecting dopamine into the striatum is one of the few known treatments for PD (6-10). However, it does not guarantee the recovery of motor and cognitive functions, slow the illness progression, or improve quality of life. Several theories and medical management have been proposed for the understanding of the pathogenesis of PD (9-12). This understanding has provided new approaches for novel treatments with few side effects for the underlying causes of motor dysfunction and cognitive impairments by slowing the disease's progression and neuronal loss (1-6). The use of phytochemicals or phytoextracts, regenerative therapies, gene therapies, cell-based medicines, and drug repurposing with various indications have all gained attention as therapy methods (12). Researchers have developed in vitro and in vivo models aiming to replicate as many PD characteristics as possible and understand the causes of PD (10-12). The most common neurotoxins used in animal models, Rotenone, 6-hydroxydopamine, Paraquat, and MPTP, are discussed here because one school of thinking holds that PD is the outcome of a toxic insult (11-14).

Rotenone is a well-known, highly-affinity inhibitor of complex I that is frequently used to pinpoint the complex's particular function (5-7). It is also a widely utilised organic pesticide that is naturally occurring and used in lakes and reservoirs to get rid of pesky fish. Due to its great lipophilicity, it easily and independently traverses cellular membranes (unlike MPP+), and it enters the brain very quickly (12-14). As a result, it works well for causing complex I to be systemically inhibited in test animals. Many significant consequences flow from this research. First, it offers strong experimental proof that parkinsonism can be brought on by a small, systemic flaw in complex I. Second, it suggests that complex I abnormalities are intrinsically sensitive to dopamine neurons in the substantia nigra. In other words, complex I was evenly blocked throughout the brain (and the rest of the body), but only the neurons in the substantia nigra aged. Thirdly, it lends "biological plausibility" to the PD "pesticide-environmental exposure hypothesis". Fourth, it implies that mechanisms other than a bioenergetic (ATP) deficit must be at work as degeneration occurred at levels of complex I inhibition that had little to no influence on respiration (3-5).

Withania somnifera (WS; Family: Solanaceae) is also known as Ashwagandha, Indian ginseng and termed as Horse smell in native language and has been used in indigenous medicine for over 3000 years (14-16). WS has been studied for anti-inflammatory (16,17), antitumor (17), antibacterial (13), antioxidant (13), anticonvulsant (18), and immunosuppressive properties (13-17). Many pharmacological studies on the properties of WS have been conducted to validate its use as a multi-purpose medicinal agent. The protective effect of WS has been reported due to presence of withanolides (18-20). The chemical composition of Withania species has been reported and purified the common constituents such as alkaloids, flavonoids, lactones, saponins, sitoindosides (i.e., Glucose attached to withanolide), steroidal lactones, withanolides and tannins from aerial parts, berries and roots of the WS plant (14-20). The withanolides are the primary and abundant bioactive constituent present in the roots used for therapeutic purposes (16).

Chronic neuroinflammation promotes neuronal cell death and significant brain atrophy by causing the ongoing release of pro-inflammatory cytokines. The antibiotic such as doxycycline (doxy) proved to be a potential multi-target therapy option due to its antibacterial activity but also pleiotropic action against amyloidosis, neuroinflammation, and oxidative stress (21-23). The second-generation tetracycline doxycycline (Doxy), which has a safer clinical profile and may pass the blood-brain barrier more easily, will receive special attention (22). Doxy showed convincing pre-clinical effects in mice models of AD against A oligomers and neuroinflammation, making it a promising preventive (21-23). Ellagic acid (EA), a naturally occurring polyphenolic molecule, is found in various fruits, nuts, and herbs as either free ellagitannins or coupled to polyols or sugars. Due to EA's remarkable neuroprotective properties, a sizable variety of botanicals and enhanced foods are now accessible commercially as nutraceuticals and used to prevent mild cognitive impairment (MCI) (24,25). The aim of the *in vitro* study is to evaluate the cytoprotective and anti-inflammatory role of EWSR alone and in combination with known cytoprotectants against rotenone-induced inflammation and cytopathic effects in neuronal cell lines.

MATERIALS AND METHODS

Materials

The cell lines Neuro A2 and HEK239 were obtained from a national cell line repository (National Centre for Cell Science, Pune, India). Hi-Gluta XLTM Dulbecco's Modified Eagle's Medium (High Glucose, Himedia) cell culture medium, L-Glutamine-Penicillin-Streptomycin solution, Dulbecco's Phosphate buffered saline (DPBS), 0.22 m sterile syringe driven filters, sterile cell scrapers, and 0.25 percent Trypsin-EDTA solution Gibco in South Africa provided the foetal bovine serum. Thermo Fisher, USA, provided sterile cell culture plasticware, as well as a flow-cytometer BD FACS calibre Apoptosis Kit - Annexin V Alexa Fluor 488 and propidium iodide (Thermo Fisher), and ultrapure water was generated using the Millipore RiOs-DI®3 system.

Preparation of stock solutions

50 percent ethanolic extract of *Withania somnifera* (EWS), doxycycline (Doxy), Ellagic acid (EA), and rotenone (ROT) solution were prepared. Rotenone stock solution was prepared in chloroform at 50 mg/mL, Doxycycline stock solution was prepared in 5 ml double distilled water, and Ellagic acid stock solution was prepared in 5 ml DMSO at 1.4 mg. After that, the solution was filter-sterilized with a 0.22 mm syringe filter. Diluting this stock solution yielded the required dosages.

Cell culture

Neuro A2 cells is a neuroblastoma cell line were cultured aseptically in 6 well culture plates in DMEM with glucose medium supplemented with 10% FBS (Gibco), 20 mM L-glutamine along with antibiotics such as 1X Pen-strep solution (100 units of penicillin-streptomycin/mL). Before treating the cells (70-80 percent confluency) with the toxins or phytochemicals or cytoprotectants and post

treatments the serum-free media is replaced with appropriate media. Different concentrations of cytoprotectants i.e., Doxycycline (Doxy 20 µg/mL), Ellagic acid (EA 20 µg/mL) and Phytoextract (5, 10, 20, and 30 µg/mL) were used added to the cells in varying amounts and final volume in each well is made up to 2 mL using corresponding media. Control cells were treated with vehicle solutions.

MTT assay

As previously described (30,31), the MTT assay was used to assess toxicity. Cells were seeded overnight in 96-well plates. Following 42 hours of incubation with crude extracts, the cells were rinsed with 1X PBS and incubated at 37 °C with 100 µL of 0.5 mg/mL MTT. After 30 minutes, the dark blue formazan crystals (MTT metabolites) were dissolved in 100 µL of DMSO and incubated at 37 °C for 30 minutes. Using a microplate reader, the level of reduced MTT was determined by measuring the difference in absorbance at 570 and 650 nm (SpectraMax M5, Molecular Devices). According to the NCI plant screening programme in the United States, a crude extract is generally thought to have in vitro cytotoxic activity with an IC_{50} of 20 mg/mL.

Cell cycle analysis

For the analysis the cultured cells were starved for 24 hours before treatment with phytoextracts (varied concentrations) and post treatment the cells were cultured for next 42 hours, cells were trypsinized, and fixed in 70% ethanol overnight. Post fixation cells were treated with Propidium Iodide, RNAseA, and Triton-X-100 before the DNA content analysis at various stages of the cell cycle by flowcytometry (Thermo Fisher).

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The expression of interested genes were analysed by using a semiquantitative RT-PCR method. Post treatment with respective drugs or phytoextracts the cells were fixed in Trizol reagent (cat no: 15596026; Thermo fishers) for controlling the RNase activity for purification of total RNA content from immortal cell lines (Neuro A2 and HEK239) as per the manufacturer's instructions. From the purified total RNA, only 2 µg of total RNA was then converted to cDNA using the commercial Verso cDNA synthesis kit (#AB-1453/A, Thermo Scientific). PCR amplification of cDNA solution was performed at a final volume of 20 µL using commercial Emerald GT PCR Master Mix (Cat# RR310A, Takara). The primer sequences used in the amplification of the genes is given in Table 1. The PCR cycling conditions for gene of interests were similar expect the annealing temperatures differed (*i.e.,* initial denaturation step 95°C for 5 min, extended denaturation step 95 for 30 s, elongation step 72°C for 30s for 40 cycles and final extension step at 72°C for 7 min. The RT-PCR products were separated on 1.5% agarose gel (Lonza) and observed under the gel documentation unit.

Table 1: Primer sequences of genes and annealing temperatures

Gene	Forward primer	Reverse primer	Annealing temperatures
TNF-α	GCAGGTCTACTTTGGAGTCATTG	CAGGTCACTGTCCCAGCATCT	58°C
MMP2	GAATGCCATCCCTGATAACCT	GCTTCCAAACTTCACGCTCTT	60°C
GAPDH	TGAGGTGACCGCATCTTCTTG	TGGTAACCAGGCGTCCGATA	58°C

RESULTS

ELISA analysis for the pro-inflammatory cytokines

The protein expression pro-inflammatory cytokines (TNF-alpha, IL6 and IFN-gamma) in cell culture isolates was estimated by ELISA and it is observed that their expression is highly significant in rotenone treated cells and its expression is less or reduced in the experimental drugs (Ashwagandha, Ellagic acid and doxycycline individually and in combination) used against rotenone (Fig.1).

MTT Assay

The survival of cell culture was observed in rotenone
treated cells and its cell survival was less or reduced in both HEK-239 and Neuro2A cell lines, but the experimental drugs Ashwagandha, Ellagic acid and doxycycline individually and in combination showed low toxicity in HEK-239 cell line and caused cytotoxic effects in the Neuro 2A cell line, indicating that these drugs exert anti-cancer and anti-inflammatory effects.

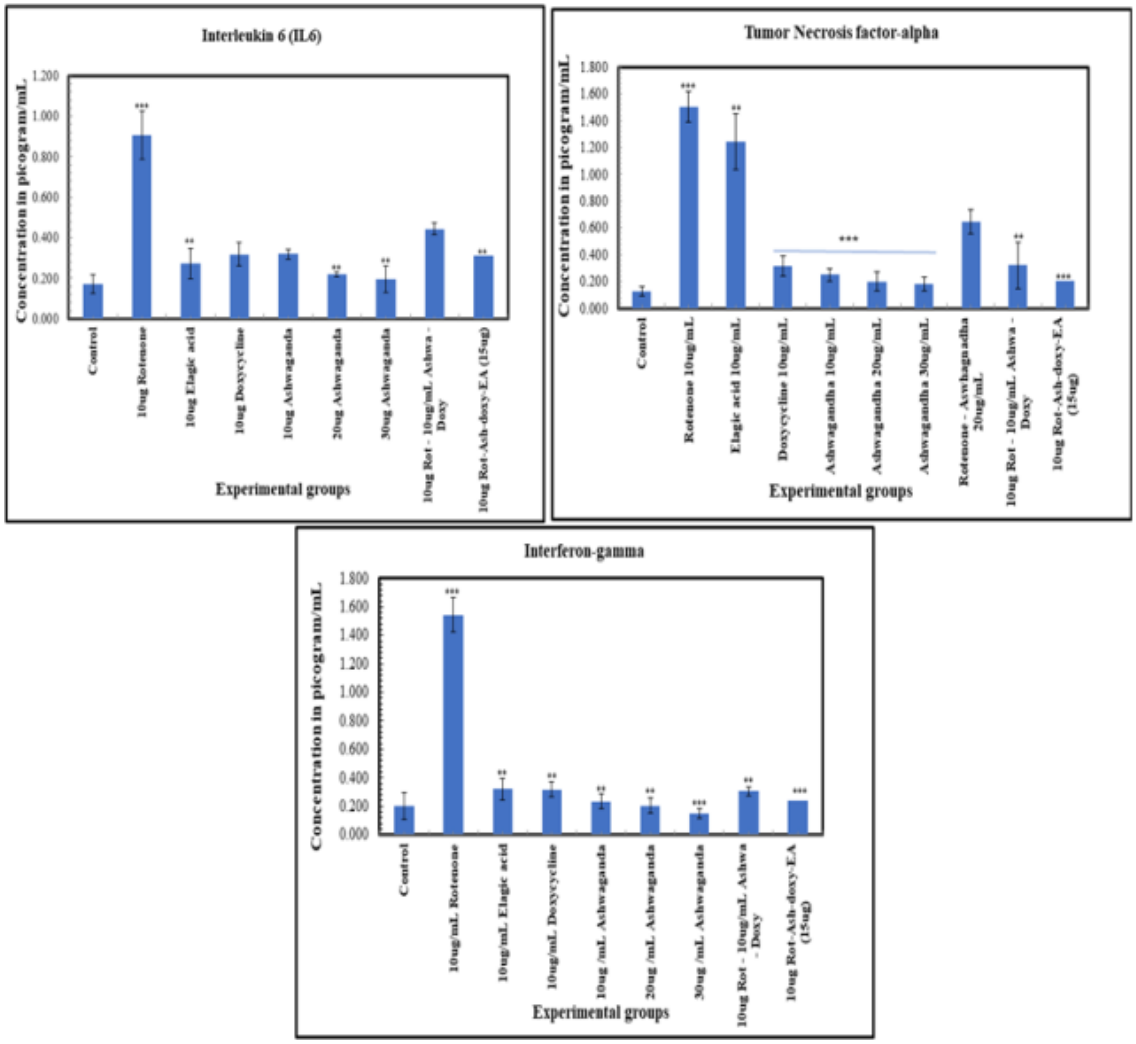

Fig. 1: ELISA analysis to check protein expression of individual pro-inflammatory cytokines (Interferon, Interleukin 6 and Tumor Necrosis Factor) in the cells treated with Rotenone (cytotoxin), Ashwagandha, Ellagic acid and doxycycline individually and in combination. p-value- <0.001** indicates significant, whereas p-value- <0.0001*** indicates highly significant.

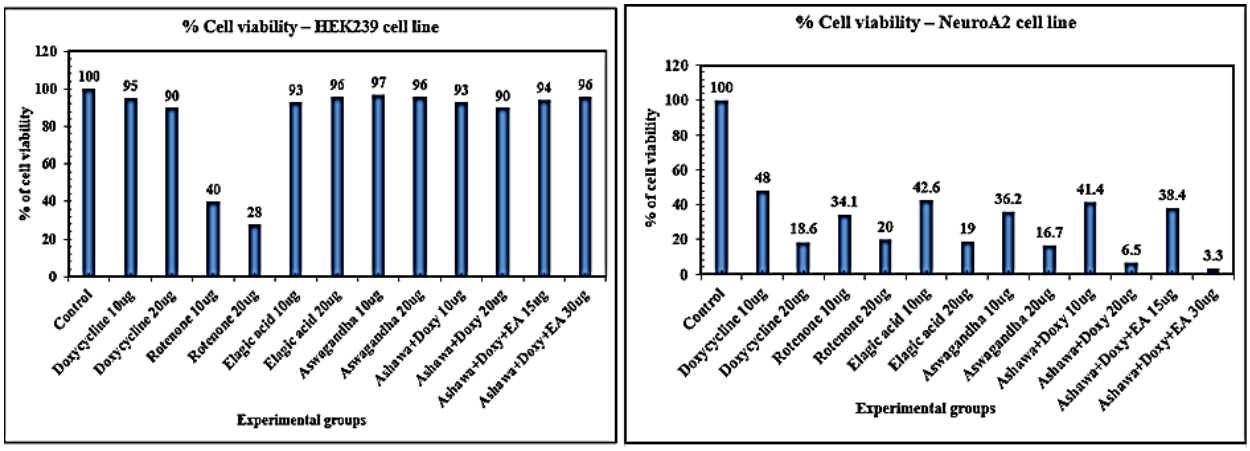

Fig. 2: MTT Assay (Cytotoxicity and cell viability) was performed in NeuroA2 and HEK293 cell lines by treating with Rotenone (cytotoxin), Ashwagandha, Ellagic acid and doxycycline individually (10.0 and 20.0 µg/mL) and in combination (10.0, 15.0, 20.0 and 30.0 µg/mL) for 36 h.

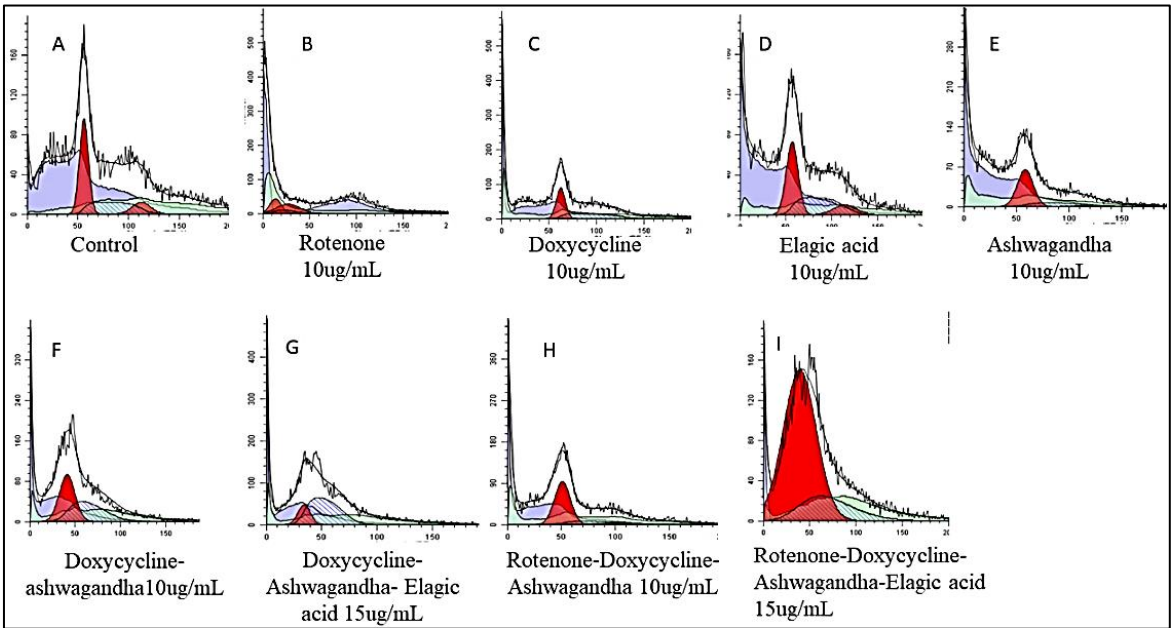

Fig.3. Cell cycle analysis in NeuroA2 cell line when treated with Rotenone, Ashwagandha, Ellagic acid and doxycycline individually and in combination

Table 2: Neuro2A cell cycle assay values when treated with Rotenone, Ashwagandha, Ellagic acid and doxycycline individually and in combination

Sample ID	**Cell cycle phases**		
	G0-G1	**S**	**G2**
Control	53.53	32.77	13.70
Rotenone 10 µg/mL	10.63	0.00	89.32
Doxycycline 10 µg/mL	58.42	41.13	0.45
Ellagic acid 10 µg/mL	49.05	41.26	9.69
Ashwagandha 10 µg/mL	71.92	23.39	4.69
Doxycycline-Ashwagandha 10 µg/mL	71.21	27.86	0.92
Doxycycline-Ashwagandha –Ellagic acid 15 µg/mL	79.36	20.16	0.49
Rotenone-Doxycycline- Ashwagandha 10 µg/mL	75.91	16.03	8.06
Rotenone-Doxycycline- Ashwagandha –Ellagic acid 15 µg/mL	22.40	77.60	0.00

G0-G1 and G2: Cell cycle growth stages; S: Synthetic phase

The cell cycle assay is performed to analyze the effect of the experimental drugs on the cell cycle stages. Neuro2A cells were treated with Rotenone (cytotoxin), Ashwagandha, Ellagic acid and doxycycline individually (10 µg/mL) and in combination (10 and 15 µg/mL) for 36 h and observed of the cells in different cell cycle stages (G0-G1 and G2 – preparatory phases; S- synthetic phase). Rotenone-treated cells were arrested mostly in G0-G1 and G2 phases, but the cells treated with experimental drugs showed presence of cells more in G0-G1 and synthetic phases indicating that cells are undergoing cell multiplication/ duplication (Table 2).

RT PCR studies

The gene expression profile of pro-inflammatory cytokine (TNF-α) and matrix metalloproteinase (MMP2) showed upregulation in rotenone treated groups. The GAPDH gene expression was included in every experiment as an indicator of the template concentration and qPCR success.

Fig. 4: Reverse transcriptase PCR analysis for the expression of inflammatory cytokine and matrix metalloproteinases 2 in Neuro2A cells treated with rotenone and experimental drugs (Positive control (PC/Rotenone/Rot); Negative control (NC); Ashwagandha extract (Ashwa) Ellagic acid (EA), Rotenone – Ashwagandha-doxycycline (Doxy), Ellagic acid (Rot-ADEA).

Expression of TNF-alpha and MMP2 is more significant in PC, EA, Rot-EA, whereas the

expression of TNF-alpha is lesser in the Ashwagandha alone, doxycycline, ROT-Ashwa, rot-doxy and ROT-ADEA.

DISCUSSION

Parkinson's disease (PD) is one the neurodegenerative disease with no proper aetiology for its incidence but, it is characterized as a progressive disease with selective loss nigrostriatal dopaminergic neurons, accumulation of fibrillar cytoplasmic inclusions (i.e., Lewies bodies, α-synuclein and ubiquitin) in the midbrain. According the epidemiologist exposure to environmental agents, such as pesticides (i.e., Rotenone, Paraquat, Dichlorodiphenyltrichloroethane (DDT) Dieldrin, etc) may increase PD risk. PD has also been connected to mitochondrial dysfunction. In PD brain, muscle, and platelets, there are systemic decreases in the activity of complex I of the mitochondrial electron transfer chain (ETC) (1-5). The discovery that MPP+ (1-methyl-4-phenyl-2,3-dihydropyridine), the active metabolite of the parkinsonism toxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), serves as a complex I inhibitor provides additional evidence for mitochondrial dysfunction in PD (3-5,14).

Rotenone (ROT) is a neurotoxicant naturally present in tropical plants traditionally used as an herbicide and pesticide (8). Due to the lipophilic nature of ROT, it can cross the blood brain barrier (BBB) and cause neurological disturbances in small animals and humans when they come into contact. Among the rodents or small animals, and as per the published report the Lewis rats show significant sensitivity towards ROT (9). ROT selectively destroys dopaminergic neurons, increased aggregation of α-synuclein and Lewy body inclusion in the midbrain when introduced intravenously (10-12). ROT administration is inducing pathologies very similar to that Paraquat, and ROT introduced neurological deficits are not restricted to the dopaminergic neurons but also disrupts other systems like serotonergic, noradrenergic and cholinergic systems as a result there is motor and cognitive disturbances being reported in the animal models (7).

The existing literature on *Withania somnifera* (WS) has provided information on the beneficial properties of so many medicinally important plants and their material in ameliorating the diseases or conditioning any ailments such as chronic inflammation or chronic and stubborn wounds (14-17). Ayurveda and Indian classical treatment regimens have also given importance to the medicinal plants with multiple health and beneficial properties, among them WS is one such important plant whose fruits, leaves and roots paste is believed to improve physical strength, longer energy levels, improved vigour, anti-stress, enhanced or conditioned immunity, gastrointestinal ulcers, reducing dermal complications such as leukoderma sores, swelling and scabies in the individual or animal upon administration (16,18-20). According to the latest published information the topical use of organic and aqueous extracts of WS have chemo preventive effects against skin cancer (15) and improved melanin production in patients (20). The WS extract contains withaferin A, which is insoluble in water but exerts significant anti-cancer effects by restricting the metastasis, angiogenesis, inflammation and drug induced stress. The use of animals in experimental pharmacological research has some drawbacks, including ethical concerns and the lack of justification for their usage when other adequate methods exist (15-18).

According to Vito *et al.,* neurodegeneration and neurological impairments are caused due to multi-factorial mechanisms such as chronic neuroinflammation, activation of brain immune cells i.e., microglia, altercating astroglia functioning in the brain (3-7,13,14). Aqueous leaf extract of WS is reported to exert anti-neuroinflammatory and neuroprotective effects by restricting the activation of pro-inflammatory cytokines by blocking the NF-B pathway and increased expression of MAP2 gene that helps in conditioning of activated microglia (dark coloured ameboid cells) to inactivated or normal microglia (light-coloured ramified cells) was demonstrated in a rat model (20). As a result, this extract may be a strong contender to stop neuroinflammation. Its capacity to preserve neuronal network and cognitive function is highlighted by the fact that withanolide A greatly reduced cerebral ischemia-induced apoptosis and necrotic cell death (12,13,20). Additionally, withanolide A has been reported to stop hypoxia-induced neurotoxicity through controlling glutathione biosynthesis (18). Additionally, it has been claimed that WS hydroalcoholic extract reduces stress in an animal model of depression (17). The withanolides possess unique ability to cross the BBB, as a result encouraging their use in the development of both therapeutic and preventative medications for neurological illnesses (20). According to a recent study, paste of ashwagandha (hot water mixed ashwagandha (ASH-WEX)) has shown different beneficial properties in comparison to the Withaferin-A which was supported by Bhat *et al.,* studies consumption of Ashwagandha tea has increased the natural killer-cell activity, and reduced the cytokine secretion or activation (12,13).

The current *in vitro* study was designed to evaluate the cytoprotective and anti-inflammatory property of ethanolic *Withania somnifera* root extract (EWSR) against ROT induced neuron loss and inflammatory response in the neuronal (NeuroA2) and normal (HEK239) cell line. The immunoassay (ELISA) was performed to quantify the pro-inflammatory markers

(Tumor necrosis factor alpha (TNF-α), Interleukin 6 (IL6) and Interferon gamma (IFN-g) expressed post rotenone induction with and with neuroprotectants to the cultured cells.The concentration of each cytokine was quantified in all the groups (control, 10ug ROT, 10ug Doxy, 10ug EA, Ashwa or WRSE (10,20, and 30ug/mL), 10ug ROT-5ug Ashw-5ug Doxy and 10ug ROT-5ug Ashw-5ug Doxy – 5ug EA). It was observed that in rotenone alone treated cells showed significant rise in the expression of pro-inflammatory cytokine in comparison to the controls and other neuroprotectant treated cells. A dose dependent response was observed in the groups when treated with Ashwa alone and in combination with known neuroprotectants (Fig. 1).

Cytotoxicity assay was performed taking two immortal cell lines and treated with different concentrations of ROT, Doxy, EA and Ashwa or EWSR (10 and 20µg/mL) alone, and in combination 10µg Ashwa-Doxy and 15µg Ashwa-Doxy– EA. ROT treated cells showed significant mortality in both cell lines. Whereas, the treatment of neuroprotectants alone and in combination on HEK239 cell lines showed no cytotoxicity. But similar treatment on the NeuroA2 cell line showed significant cytotoxicity in a dose dependent manner (Fig. 2).

Cell cycle analysis was performed after treating the Neuro A2 cell line with ROT, Doxy, EA, and Ashwa or EWSR alone and in combination to assess their individual effects on the cells when presented alone and in combination. From Fig. 3 and Table 1, one could assess their cytotoxic or cyto-proliferative effects of individual drugs or agents used in the study. ROT treated arrested the cells mostly in G2 phase, G0-G1 phases showed less cells, but no cells were observed in synthetic or S phase indicating severe cell loss and no signs of cell duplication. Similarly, other treatment (neuroprotectants (alone and in combination); and ROT along with neuroprotectants) were also assessed and it was observed that good number of cells were in G0-G1 and synthetic phases indicating that cells are healthy, undergoing cell multiplication/ duplication, which is comparable to that of control group. Based on the observation, these neuroprotectants or experimental agents are safe to use and increase the cell vitality and vigor even in the presence of ROT (Table 1).

Qualitative RT PCR analysis was done to assess the effects of ROT, and other neuroprotectants on the gene expression of TNF-a (tumor necrosis factor alpha) and MMP2 (matrix metalloproteinases 2). According to the findings from RT PCR studies, TNF-α and MMP2 gene expression was significant in comparison to the control and neuroprotectant groups and it can be inferred that ROT is a good pro-inflammatory. Gene expression of TNF-α and MMP2 in the cells treated with neuroprotectant alone and combination with ROT also showed reduced expression indicating the EWSR exerts anti-inflammatory properties. The observations made in the present study, clearly indicates EWSR to be a good natural source with neuroprotective and anti-neuroinflammatory efficacy against ROT (Fig. 4).

CONCLUSION

The present study is an attempt to assess the neuroprotective and cytoprotective efficiency of *Withania somnifera* root extract (EWSR) or Ashwagandha against rotenone induced cytotoxicity and neuroinflammation in the neuronal cell line. The results of the *in vitro* study suggest that EWSR or Ashwagandha alone and in combination with doxycycline and Ellagic acid, has a synergistic effect in controlling the Rotenone-induced cytopathic or cytotoxic and inflammatory responses in NeuroA2 cells. As a result, the combination therapy could be a potential treatment regimen in controlling neurodegenerative diseases. In order to validate and support the study findings, *in vivo* should be performed before taking into clinics.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Kouli, A., Torsney, K.M., Kuan, W.L. Parkinson's disease: Etiology, neuropathology, and pathogenesis. In: Stoker TB, Greenland JC, editors. Parkinson's Disease: Pathogenesis and Clinical Aspects (Internet). Brisbane (AU): Codon Publications; 2018. Chapter1. doi: 10.15586/ codonpublicationsparkinsonsdisease.2018.ch1
2. Salari, S., Bagheri, M. *In vivo, in vitro* and pharmacologic models of Parkinson's disease. Physiol Res. 2019; 68(1): 17-24.
3. Moradi, V.S., Nasrolahi, A., Ghaderi, S., Belali, R., Rashno, M., Farzaneh, M., *et al.,* Mitochondrial dysfunction and Parkinson's disease: Pathogenesis and therapeutic strategies. Neurochem Res. 2023. doi: 10.1007/s11064-023-03904-0.
4. Lee, Y., Lee, S., Chang, S.C., Lee, J. Significant roles of neuroinflammation in Parkinson's disease: therapeutic targets for PD prevention. Arch Pharm Res. 2019; 42(5): 416-425.
5. Ibarra-Gutiérrez, M.T., Serrano-García, N.,Orozco-Ibarra, M. Rotenone-induced model of Parkinson's Disease: Beyond mitochondrial complex I inhibition. Mol Neurobiol 2023; 60: 1929-1948.
6. Radad, K., Al-Shraim, M., Al-Emam, A., Wang, F., Kranner, B., Rausch, W.D., *et al.,* Rotenone: from modelling to implication in Parkinson's disease. Folia Neuropathol. 2019;57(4):317-326.
7. Raza, C., Anjum, R., Shakeel, N.U.A. Parkinson's disease: Mechanisms, translational models and management strategies. Life Sci. 2019 June 1;226:77-90.
8. Betarbet, R., Sherer, T.B., MacKenzie, G., Garcia-Osuna, M., Panov, A.V., Greenamyre, J.T. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 2000; 3: 1301-1306.
9. Zhang, M., He, Q., Chen, G., Li, PA. Suppression of NLRP3 Inflammasome, pyroptosis, and cell death by NIM811 in rotenone-exposed cells as an *in vitro* model of Parkinson's disease. Neurodegener Dis. 2020; 20(2-3): 73-83.
10. Marogianni, C., Sokratous, M., Dardiotis, E., Hadjigeorgiou, G.M., Bogdanos, D., Xiromerisiou, G. Neurodegeneration

and inflammation-An interesting interplay in Parkinson's disease. Int J Mol Sci. 2020;21(22):8421.

11. Bhat, J., Damle, A., Vaishnav, P.P., Albers, R., Joshi, M., Banerjee, G.*In vivo* enhancement of natural killer cell activity through tea fortified with ayurvedic herbs. Phytother Res. 2010; 24:129-135.
12. Dutta, R., Khalil, R., Green, R., Mohapatra, S.S., Mohapatra, S. *Withania somnifera* (Ashwagandha) and Withaferin A: Potential in Integrative Oncology. Int J Mol Sci. 2019 Oct 25;20(21):5310.
13. Bhatnagar, M., Goel, I., Roy, T., Shukla, S.D., Khurana, S. Complete Comparison Display (CCD) evaluation of ethanol extracts of *Centella asiatica* and *Withania somnifera* shows that they can non-synergistically ameliorate biochemical and behavioural damages in MPTP induced Parkinson's model of mice. PLoS One. 2017; 12(5): e0177254.
14. Limanaqi, F., Biagioni, F., Busceti, C.L., Ryskalin, L., Polzella, M., Frati, A., *et al.*, Phytochemicals bridging autophagy induction and alpha-synuclein degradation in parkinsonism. International Journal of Molecular Sciences. 2019; 20(13):3274.
15. Sikandan, A., Shinomiya, T., Nagahara, Y. Ashwagandha root extract exerts anti-inflammatory effects in HaCaT cells by inhibiting the MAPK/NF-κB pathways and by regulating cytokines. Int J Mol Med. 2018;42: 425-434.
16. Sangita, C., Chatterjee, P., Dey, P., Bhattacharya, S. Evaluation of Anti-inflammatory Effect of Ashwagandha: A preliminary study in vitro. Pharmacognosy Journal. 2012; 4(29): 47-49.
17. Gupta, M., Kaur, G. *Withania somnifera* as a potential anxiolytic and anti-inflammatory candidate against systemic lipopolysaccharide-induced neuroinflammation. Neuromol Med. 2018; 20 (3): 343-362.
18. Gupta, M., Kaur, G. *Withania somnifera* (L.) dunal ameliorates neurodegeneration and cognitive impairments associated with systemic inflammation. BMC Complement. Altern Med. 2019;19 (1): 217.
19. Ahmad, M., Saleem, S., Ahmad, A.S., Ansari, M.A., Yousuf, S., Hoda, M.N., *et al.*, Neuroprotective effects of *Withania somnifera* on 6-hydroxydopamine induced Parkinsonism in rats. Hum Exp Toxicol. 2005; 24:137-147.
20. Vareed, S.K., Bauer, A.K., Nair, K.M., Li, Y., Jayaprakasam, B., Nair, M.G., et al., Blood-brain barrier permeability of bioactive withanamides present in Withania somnifera fruit extract. Phytother. Res. 2014; 28 (8): 1260-1264.
21. Singh, S., Khanna, D., Kalra, S. Minocycline and Doxycycline: More Than Antibiotics. Curr Mol. Pharmacol. 2021;14(6):1046-1065.
22. Balducci, C., Santamaria, G., La Vitola, P., Brandi, E., Grandi, F., Viscomi, A.R., *et al.*, Doxycycline counteracts neuroinflammation restoring memory in Alzheimer's disease mouse models. Neurobiol Aging. 2018;70:128-139.
23. Santa-Cecília, F.V., Leite, C.A., Del-Bel, E., Raisman-Vozari, R. The Neuroprotective Effect of Doxycycline on Neurodegenerative Diseases. Neurotox Res. 2019;35(4):981-986.
24. Dornelles, G.L., de Oliveira, J.S., de Almeida, E J. R., Mello, C.B.E. Rodrigues, B.R., da Silva, C. B, *et al.*, Ellagic acid inhibits neuroinflammation and cognitive impairment induced by lipopolysaccharides. Neurochem Res. 2020;45(10):2456-2473.
25. Ardah, M.T., Bharathan, G., Kitada, T., Haque, M.E. Ellagic Acid prevents dopamine neuron degeneration from oxidative stress and neuroinflammation in MPTP model of Parkinson's disease. Biomolecules. 2020; 10(11):1519.