

Neuroprotective effects of *Geranium Robertianum* L. Aqueous extract on the cellular Parkinson's disease model

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Abstract. – OBJECTIVE: In recent years, botanical medicines alone or in conjunction with existing therapies have attracted considerable popularity as an alternative treatment for Parkinson's Disease (PD). For instance, *Geranium robertianum* L. (Geraniaceae family) has been used in folk medicine for its antioxidant and anti-inflammatory properties. However, its neuroprotective potential has not been well demonstrated.

MATERIALS AND METHODS: Herein and for the first time, we have investigated the *in vitro* neuroprotective effects of leaf extract of *G. Robertianum* over a wide dose range (0-200 µg/mL) on the PD model using retinoic acid (RA)-differentiated SHSY-5Y cells and 1-methyl-4-phenylpyridinium (MPP⁺)-induced neurotoxicity. The neuroprotective effects were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) release assays. The antioxidant activity of the extract was measured by total antioxidant status (TAS) and total oxidant status (TOS). The effect of leaf aqueous extracts on acetylcholinesterase (AChE) activity was also determined. Finally, cell death mechanisms were analyzed by flow cytometry.

RESULTS: Our results showed that *G. Robertianum* leaf extract ameliorated cytotoxicity and oxidative damage by MPP⁺. Moreover, *G. Robertianum* extract exhibited a protective activity against MPP⁺ induced apoptosis.

CONCLUSIONS: The current findings could lead to a promising new candidate for a possible cure of Parkinson's disease through neuroprotective mechanisms with respect to antioxidant and apoptosis inhibitory properties of *G. Robertianum* water extracts.

Key Words:

Antiapoptotic, *In vitro*, *Geranium Robertianum*, MPP⁺ toxicity, neurotoxicity, Parkinson's disease.

Introduction

Parkinson's disease (PD), which is one of the most common neurodegenerative disorders, was

first described by James Parkinson (nearly 200 years ago) as "shaky paralysis". James Parkinson defined the symptoms of the disease as involuntary tremors, decreased muscle strength, and poor balance without mental and sensory disorders^{1,2}. The disease was coined by Jean-Martin Charchot, who disagreed with the name "shaky paralysis". While studying the clinical spectrum of the disease with his students, Charcot discovered that tremors were not a necessary symptom for all individuals with PD³. The PD incidence around the world is approximately 10-50 people per 100,000 people, and the prevalence is estimated to be 250-570 people per 100,000 people. The incidence and prevalence trend of the disease increases with age. When the aging population is considered, it is expected that the number of individuals with PD will double in densely populated countries by 2030^{4,5}. Motor symptoms associated with PD include akinesia, bradykinesia, tremor, rigidity, and postural instability (primary) as well as gait and speech disorders (secondary)⁶. However, non-motor symptoms, such as anxiety and depression are common in PD as well. Anxiety disorders associated with PD may include generalized anxiety disorder, agoraphobia, panic disorder, obsessive-compulsive disorder, and social or specific phobia^{7,8}.

The discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin, which degenerates dopaminergic neurons in substantia nigra (SN) causing a Parkinson-equivalent syndrome⁹, accelerated research on PD pathogenesis related to environmental factors. MPTP is a small, lipophilic compound that can pass through the blood-brain barrier (BBB). This small compound is transformed into the toxic form called MPP⁺ in astrocytes by the glial monoamine oxidase B (MAO-B) enzyme and up-taken to dopaminergic neurons through the organic cation transporter (OCT-3) enzyme. Among dopaminergic neurons, MPP⁺ acts as a complex inhibitor and

prevents oxidative phosphorylation¹⁰. This inhibition decreases adenosine triphosphate (ATP) levels in the cell and increases ROS production, and dopaminergic neuronal death by causing lipid peroxidation¹¹. Thus, MPP⁺ is considered the most effective toxin for designing *in vitro* PD models¹². Since there is no known definitive cure, therapeutic approaches for PD have focused on symptomatic remedies. The medications used in the treatment of PD are aimed at increasing the dopamine level in the brain and simulating the effects of dopamine deficiency. Treatment of PD is based on the control of motor symptoms with levodopa supplementation. However, long-term exposure to a high dose of levodopa is associated with the development of motor complications, characterized by levodopa-induced dyskinesia¹³.

The Geraniaceae family includes 750 species that are cultivated for their economic importance in aromatherapy and cosmetics perfumery¹⁴. *Geranium Robertianum*, also known as “Red Robin” or “Herb-Robert”, has been used in several countries as an herbal medicine with therapeutic benefits for different types of lesions, such as ulcers, diarrhea, and wounds¹⁵. *G. Robertianum* is native to Europe, Asia, North America, and North Africa^{16,17} and exhibits good antioxidant and free radical scavenging properties¹⁸. Furthermore, multiple studies¹⁹⁻²¹ have reported the anticarcinogenic activity of *G. Robertianum*.

Today, herbal medicine therapy is particularly common in the treatment of PD to reduce the side effects of existing medicines as well as drug dependence. Various herbs have been investigated²²⁻²⁵ for the treatment of PD including the herbs traditionally used by local people for many years to treat different diseases. The strategy of such herbal approaches is to reduce oxidative stress, which plays a crucial role in the pathogenesis of PD. For this purpose, isolates obtained from natural variegated plants have been tested *in vivo* and *in vitro* PD models which also reported their neuroprotective actions²⁶. The current literature shows that the neuroprotective effects of *G. Robertianum* have not been fully identified in the cellular PD model. Collapsed oxidative stress machinery in the brain was claimed to be one of the most important reasons for the PD emergence and the potential antioxidant properties of *G. Robertianum* were suggested through several scientific investigations. Therefore, this study aims to examine the neuroprotective potentials of leaf aqueous extracts obtained from *G. Robertianum* *in vitro* PD model.

Materials and Methods

Plant Extract Preparation

G. Robertianum samples were collected from the Yedigöller region of Erzurum Province in Turkey and morphological identifications of the plant species were performed by Prof. Dr. Meryem Sengul Koseoglu. After plant identifications were completed, air-dried *G. Robertianum* leaves were grounded by using a tissue lyser (Qiagen®, Hilden, Germany) to obtain a powder after being frozen at -80°C and incubated in ultra-pure distilled water at room temperature overnight. The supernatant was obtained by centrifugation followed by lyophilization. The concentrations of the lyophilized aqueous extracts were determined with an analytical balance (Shimadzu®, Kyoto, Japan). The aqueous extracts were filtered with a 0.40 µm membrane filter (Merc Millipore™, Burlington, MA, USA) for application to SHSY-5Y cell cultures.

Cell Culture

SHSY-5Y cell line (ATCC, CRL-2266) was used to establish the Parkinson's disease toxicity model. The growth culture media consists of 1% penicillin/streptomycin, 5% fetal bovine serum and 94% DMEM/F12. The prepared medium was heated to 37°C temperature, 5 mL was collected and transferred into a T25 flask and SHSY-5Y cells were seeded. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ until achieving approximately 80% confluent. Every two days, the old medium in the flask was removed, cell debris washed with phosphate buffered saline (PBS) and incubation continued with a fresh medium²⁷.

Neuronal Differentiation of SHSY-5Y Cells

When the cells reached about 80% confluence, the old medium was removed, and the cell debris was washed three times with 2 ml of PBS. Afterward, 10 µM retinoic acid (RA) was added to the culture and the cells were incubated at 37°C and 5% CO₂. After 11 days of culture, neuronal cell differentiation can be achieved by exposing the cells to a medium containing RA one day and a medium containing no RA the other day, respectively. Cell culture analysis was examined under an inverted microscope after each medium change. Also, cellular differentiation was further confirmed by using flow cytometry cell cycle analysis²⁸.

In Vitro PD Model

At the end of the 11th day, differentiated SHSY-5Y cells were harvested with trypsin and seeded

into a 96-well plate with approximately 10^4 cells per well. MPP⁺ iodide was applied to the cells (in triplicate, $n=3$) at a concentration range of 12, 24, 47, 94, 188, 375, 750, and 1,500 $\mu\text{g/mL}$, with a total volume of 200 μL for each well. While negative control wells contained only medium and cells, 1% Triton X-100 was added to positive control wells in addition to cells and medium. After 24 hours of incubation period, the cells were tested for viability, and the IC_{50} concentration of MPP⁺ toxic substance in differentiated SHSY-5Y cells was determined²⁹.

Cytotoxicity Screening of Plant Extract

Differentiated SHSY-5Y cells were harvested with trypsin and inoculated into a 96-well plate with 104 cells per well. *G. Robertianum* extract was applied to the cells (in triplicates) at a concentration range of 3, 6, 12, 25, 50, 100, 200, and 400 $\mu\text{g/mL}$, with a total volume of 200 μL in each well. While negative control wells contained only medium and cells, 1% Triton X-100 was added to positive control wells in addition to cells and medium. After 24 hours of the incubation period, the cells were tested for viability and the cytotoxicity was measured.

Application of Aqueous Extracts to In Vitro PD Model

Differentiated SHSY-5Y cells were harvested with trypsin and inoculated into a 96-well plate with approximately 104 cells per well. MPP⁺ iodide was added to the cells at a predetermined inhibitory concentration (IC_{50}). Next, extract at a concentration range of 3, 6, 12, 25, 50, 100 and 200 $\mu\text{g/mL}$ were added (in triplicate), with a total volume of 200 μL in each well. While negative control wells contained only medium and cells, 1% Triton X-100 was employed as a positive control in addition to cells and medium. MPP⁺ control was constituted by the application of MPP⁺ iodide at IC_{50} concentration to the cell culture and cell viability tests were performed to determine the neuroprotective effects of *G. Robertianum* leaf extract following 24 hours of incubation.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

The MTT solution used in viability tests was prepared by dissolving the compounds in PBS to yield a 5 mg/mL concentration rate. After 24 hours of experimental incubation, prepared MTT solution was added to each sample with up to 10% of the total volume in the well. The cells were in-

cubated again at 37°C and 5% CO_2 for 3 hours. Afterward, the supernatant was removed, and the formazan crystals attached to the bottom of the well were dissolved by using dimethyl sulfoxide (DMSO). The wells were measured at 570 and 630 nm wavelengths with a microplate reader³⁰.

Lactate Dehydrogenase (LDH) Release Assay

The LDH solution used in viability tests was prepared according to the manufacturer's protocols (Thermo Fisher Scientific, Waltham, MA, USA). At the end of 24 hours of incubations, 50 μL supernatant was transferred from each well to a new plate and a 50 μL reaction mixture was added. The plates were incubated in the dark at room temperature for 30 min. Then, 50 μL of stop solution was added to each well, and measurements were recorded at 490 and 680 nm wavelengths with a microplate reader³¹.

Acetylcholinesterase (AChE) Activity Assay

AChE activity was determined by using an assay kit (Abcam, Cambridge, UK) and analyses were conducted based on the manufacturer's instructions. Briefly, cell lysates were prepared by incubating with lysis buffer at room temperature. Then, 50 μL acetylthiocholine reaction buffers were added to each sample to reach 100 μL /well volume and incubated for 30 min at room temperature. After the incubation period, samples were analyzed by a microplate reader at 410 nm. Experimental samples were compared with the kit's standards to assess the activity levels³².

Total Antioxidant Status (TAS) And Total Oxidative Status (TOS) Analysis

Total Antioxidant Status (TAS) and Total Oxidative Status (TOS) were investigated by using commercial kits (Rel Assay Diagnostics, Gaziantep, Turkey). For TAS analysis, cell culture supernatants were used and culture samples, kit standard (equivalent to 1 mmol/L of Trolox), and dH₂O were mixed with Reagent 1 in 96-well plates and incubated for 5 min according to the manufacturer's protocols. Then, Reagent 2 was added, and mixtures were incubated at 37°C for 5 min; absorbance was monitored by a microplate reader at 660 nm. Each sample data was calculated *via* the kit's standard (equivalent to 1 mmol/L of Trolox). For TOS analysis, Reagent 1 was added to each well, and absorbances were measured at 530 nm. Next, Reagent 2 was added, and sam-

ples were incubated for 5 min at 37°C. Finally, absorbance was monitored at 530 nm by using a microplate reader. Oxidative status values were calculated by using the kit's standard ($\mu\text{M H}_2\text{O}_2$ Equiv./L)^{33,34}.

Flow Cytometry Analysis

SHSY-5Y cells treated with *G. Robertianum* extract and MPP⁺ iodide for were harvested 24 hours with trypsin and precipitated by centrifugation. After the cells were suspended with 500 μL binding buffer, 5 μL Annexin V-FITC and propidium iodide were added. Samples were incubated at room temperature for 5 min and analyzed by flow cytometry³⁵.

Statistical Analysis

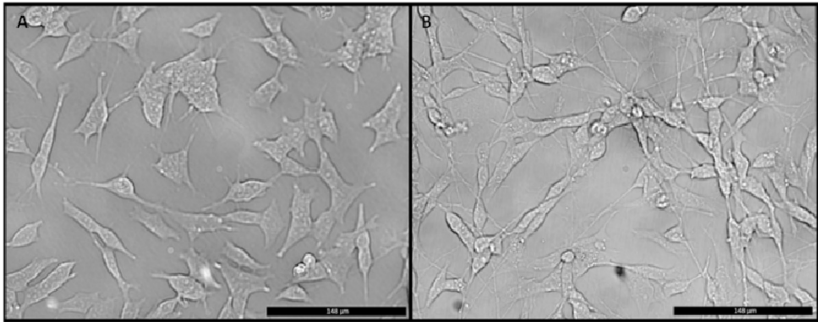
The obtained data were analyzed by the GraphPad Prism (GraphPad Software®, La Jolla, CA, USA) statistical program. Statistical evaluations were performed by applying one-way ANOVA followed by the Dunnett comparison test. The maximum statistical significance level was accepted as $p < 0.05$.

Results

SHSY-5Y cell cultures were incubated with all-trans RA for 11 days to induce cell differentiation into mature neuron-like cells. After the differentiation period, SHSY-5Y cells (initially deltoid-shaped) began to develop elongated body structures and avoid clustering behavior after 11 days of RA exposure. In addition, an increasing number of dendrite-like structures were observed between cells. Moreover, flow cytometry cell cycle analysis was utilized for further validation of differentiation. Prior to differentiation, SHSY-5Y cell culture was presented as S phase ($47.15 \pm 2.25\%$) and G2 phase ($11.88 \pm 1.05\%$). After differentiation, cell cultures were more likely to be in the G1 phase ($75.07 \pm 2.94\%$) as shown in Table I.

In order to determine the IC₅₀ concentration of MPP⁺ compound, spectrum wide range of concentrations (12, 25, 47, 94, 188, 375, 750, and 1,500 $\mu\text{g/mL}$) were applied to differentiated SHSY-5Y cell culture. After viability tests, a logarithmic plot of MPP⁺ concentrations were constructed and the IC₅₀ concentration value for MPP⁺ was

Table I. 40x image of normal and differentiated SHSY-5Y cells. **A**, Undifferentiated SHSY 5Y cells; **B**, differentiated SHSY-5Y cells, and **C**, Cell cycle analysis of differentiated SH-SY5Y cells after 11 days of retinoic acid application (RA: Retinoic acid).



Cell population (%)				
Group	G1 phase	G2 phase	S phase	G2/G1
Control	36.41 ± 1.77	11.88 ± 1.05	47.15 ± 2.25	1.74 ± 0.07
RA treated	$75.07 \pm 2.94^*$	$1.42 \pm 0.07^*$	$25.61 \pm 0.92^*$	1.92 ± 0.05

Values are expressed as the mean \pm standard deviation. Symbol (*) indicates statistically significant difference at $p < 0.01$ as compared with the negative control.

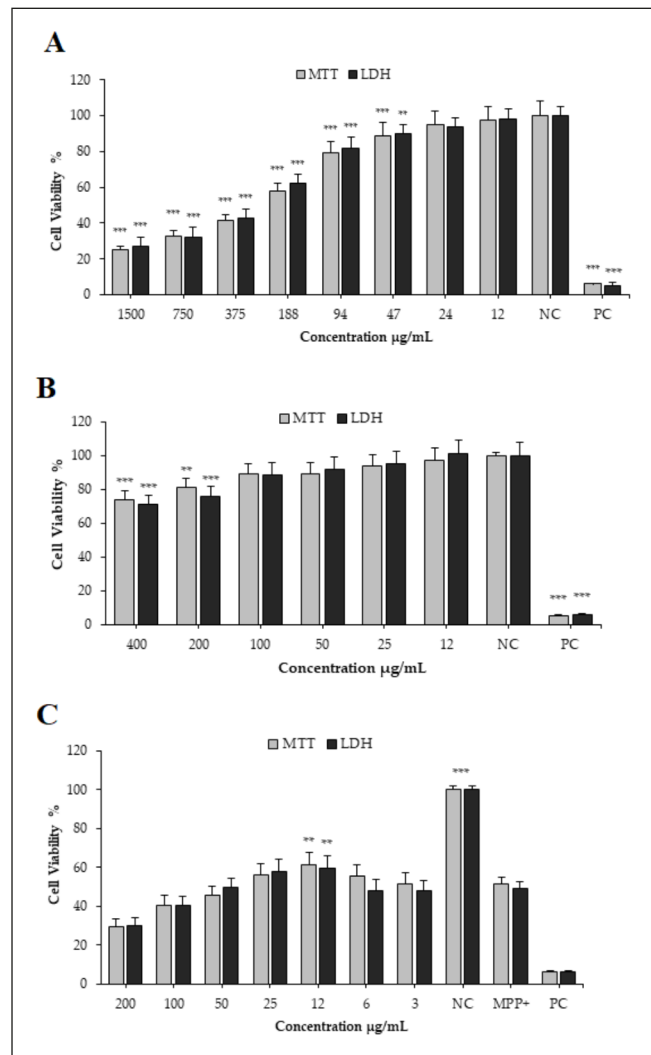


Figure 1. Cell viability analyses. **A**, 24 hours of MPP⁺ exposure to differentiated SHSY-5Y cells; **B**) 24 hours of *G. Robertianum* extract application to differentiated SHSY-5Y cells and **C**), *Geranium Robertianum* extract treatment on the experimental *in vitro* Parkinson model for 24 hours. Significant difference as compared with the negative control was shown as *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

found to be 313.9 µg/mL. After determination of IC₅₀ concentration of MPP⁺ compound on differentiated SHSY-5Y cell culture, *G. Robertianum* extract was applied at different concentration ranges of 6, 12, 25, 50, 100, 200, and 400 µg/mL to identify toxicological properties of isolates on the differentiated SHSY-5Y cell culture for determining suitable extract concentrations. We found that the highest concentrations (200 and 400 µg/mL) caused significant decreases in cell viabilities, whereas concentrations lower than 200 µg/mL did not induce any neurotoxicity (Figure 1). *G. Robertianum* extract was applied to differentiated SHSY-5Y cells that were treated with MPP⁺

compound at a predetermined IC₅₀ concentration (313.9 µg/mL) at a concentration range of 3, 6, 12, 25, 50, 100, and 200 µg/mL. We noticed that *G. Robertianum* leaf extract (12 µg/mL) caused a statistically significant increase in cell viability (approximately 10%) when treated with MPP⁺ (Figure 1).

Flow cytometry was used to assess cell death in the *in vitro* PD model. Herein, applications of MPP⁺ at IC₅₀ concentration for 24 hours mainly increased apoptotic cell deaths (early apoptosis = 32.98% and necrosis = 14.78%) in the differentiated SHSY-5Y cell culture. After the cells were incubated for 24 hours with MPP⁺ at IC₅₀ concen-

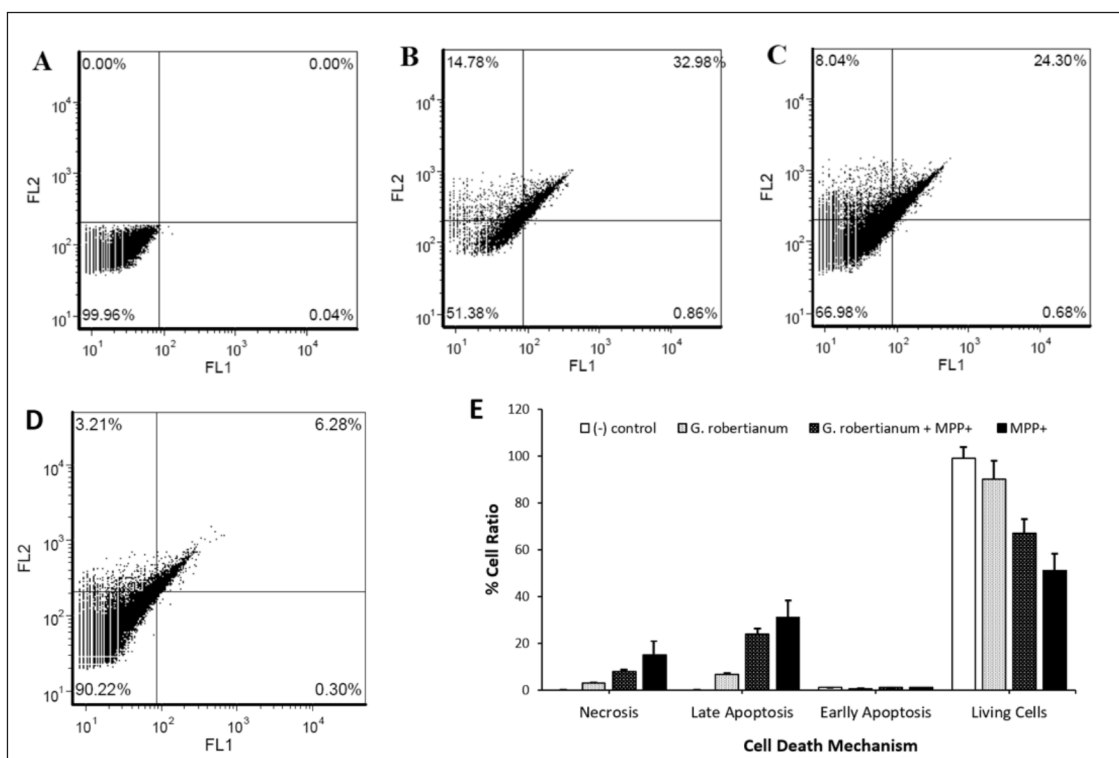


Figure 2. Flow cytometry analysis of *in vitro* Parkinson's Disease model. **A**, Negative control; **(B)**, MPP⁺ application; **(C)**, *G. Robertianum* + MPP⁺ application; **(D)**, only *G. Robertianum* application and **(E)**, summary of flow cytometry analysis

tration with *G. Robertianum* extract at 12 $\mu\text{g}/\text{mL}$, apoptosis-related death levels were reduced when compared to the MPP⁺ group (from 32.98% to 24.30%) as shown in Figure 2.

Decreased AChE activity, one of the most common causes of PD, was used to verify that the MPP⁺ applied *in vitro* PD model is a usable system for anti-PD compound administration. Our results revealed that AChE activity was significantly decreased after the application of MPP⁺ into differentiated SHSY-5Y cell culture. On the other hand, there were significant changes in AChE activity compared to the negative control when *G. Robertianum* extract was applied to cell culture, and a significant ameliorative effect of *G. Robertianum* extract when applied with MPP⁺ (Table II) was detected.

G. Robertianum extract was applied to differentiated SHSY-5Y cells with MPP⁺ (IC₅₀ concentration) to evaluate the oxidant-antioxidant effects. We have found that MPP⁺ significantly decreased antioxidant levels (from 1.62 to 1.05 mM) and increased oxidant levels compared to the negative control (from 5.61 to 8.28 μM H₂O₂

Equiv./L). Additionally, we have seen that the application of *G. Robertianum* extract with MPP⁺ compound significantly elevates the antioxidant level (from 1.05 to 1.54 mM) enough to reach the total antioxidant status of the negative control. Furthermore, the application of *G. Robertianum* extract with MPP⁺ compound was found to decrease oxidative status (from 8.28 to 5.76 μM H₂O₂ Equiv./L) and the regulated oxidative state had a similar level to the negative control (Table III)..

Table II. Acetylcholinesterase activity (1 $\mu\text{mol}/\text{min}$) of *in vitro* Parkinson's disease model in respect to the application of *G. Robertianum* + MPP⁺.

Groups	Acetylcholinesterase Activity ($\mu\text{u}/\text{mL}$)
Control (-)	445.75 \pm 5.67 ^a
MPP ⁺	346.85 \pm 7.44 ^b
<i>G. Robertianum</i>	435.27 \pm 8.36 ^a
<i>G. Robertianum</i> + MPP ⁺	358.32 \pm 9.12 ^b

Means with the same letter in the column are not significantly different at level of $p < 0.05$. Same superscripted letters (^{a,b}) define statistically similar results.

Table III. TAS and TOS levels in an experimental *in vitro* Parkinson model treated with *G. Robertianum* and MPP⁺ compound for 24 hours.

Groups	TAS (mM Trolox Equiv./L)	TOS ($\mu\text{M H}_2\text{O}_2$ Equiv./L)
Control (-)	1.62 \pm 0.11 ^b	5.61 \pm 0.39 ^a
MPP ⁺	1.05 \pm 0.07 ^a	8.28 \pm 0.58 ^b
<i>G. Robertianum</i>	2.45 \pm 0.25 ^c	5.32 \pm 0.45 ^a
<i>G. Robertianum</i> + MPP ⁺	1.54 \pm 0.27 ^b	5.76 \pm 0.40 ^a

TAS: Total Antioxidant Status, TOS: Total Oxidative Status, NC: Negative control, MPP⁺: MPP⁺ treated cells. Means with the same letter in the column are not significantly different at level of $p < 0.05$. Same superscripted letters (^{a,b,c}) define statistically similar results.

Discussion

Although the underlying pathological mechanism of PD has not been fully elucidated, previous studies^{36,37} have concluded that different mechanisms, such as mitochondrial disorders, oxidative stress, lipid peroxidation, and heavy metal accumulation could contribute to the disease by accelerating neuronal cell death. At this point, certain plant aqueous extracts exhibited crucial anti-apoptotic roles in Parkinsonism³⁸. In this study, we investigated the neuroprotective effects of aqueous extracts obtained from *G. Robertianum* leaves in an *in vitro* PD model induced by MPP⁺. Furthermore, AChE activity, TAS and TOS levels, and flow cytometry were performed to reveal the neuroprotective mechanism of *G. Robertianum* extract against MPP⁺ toxicity. The neurotoxicity induced by the MPP⁺ in SHSY-5Y cells has been of use to mimic the PD model³⁹⁻⁴¹. In this study, we aimed to differentiate SHSY-5Y cells into primary neuronal cells using retinoic acid, which is in line with other studies⁴²⁻⁴⁴ in the literature. After 11 days of all-trans RA application, the cells that initially had a deltoid body structure and tended to clump together started to move away from each other by moving into a thin-long body structure and also form axon-dendrite-like cellular structures between each other. Furthermore, flow cytometry analysis of the cell cycle showed that SHSY-5Y cells that were in the S phase progressed to the G1 intermediate phase. These results proposed that the RA application could differentiate cancerous cells into stable and undivided neuron-like cell cultures.

According to the MPP⁺ toxicity tests, the IC₅₀ concentration of the MPP⁺ was found to be approximately 300 $\mu\text{g}/\text{mL}$; the finding is in line with others^{45,46}. It has to be noted that *G. Robertianum* leaves aqueous extracts up to a concentration rate of 100 $\mu\text{g}/\text{mL}$ did not show any toxic effects on dif-

ferentiated SHSY-5Y cells as compared to the negative control. Moreover, *G. Robertianum* extract (12 $\mu\text{g}/\text{mL}$) resulted in elevations in cell viability in differentiated SHSY-5Y cell culture exposed to MPP⁺ compared to MPP⁺ treated group. Present results revealed that MPP⁺ would inhibit the activity of AChE in differentiated SHSY-5Y cell culture. This finding indicates that differentiated cell cultures after MPP⁺ could stimulate PD-like neurotoxicity and might be used as a disease model for drug discovery and toxicity studies^{30,47,48}. However, *G. Robertianum* extract has a significant effect on the AChE activity against MPP⁺ toxicity. It could be concluded that the underlying mechanism behind the neuroprotective effect of the plant extract was most probably due to the regulation of AChE activity.

In previous tissue culture studies^{11,49} and animal experiments, MPP⁺ has been reported to inhibit oxidative phosphorylation by acting as a mitochondrial *complex I* protein inhibitor. As a result, it activates the apoptotic pathway through ROS-induced lipid peroxidation. Herein, we have found that MPP⁺ could contribute to oxidative stress by decreasing the total antioxidant level and increasing the total oxidant level in differentiated SHSY-5Y cells, significantly. The application of *G. Robertianum* aqueous extracts with MPP⁺ tends to increase the antioxidant status and decrease oxidative stress in cell cultures. The survival-enhancing properties of *G. Robertianum* extract against MPP⁺ toxicity on differentiated SHSY-5Y culture could be attributed to oxidative stress ameliorative effects of aqueous extracts.

MPP⁺ application was found to induce apoptosis in different cell cultures and some of them were proven to have resulted from the increase of oxidative stress^{50,51}. Also, this study correlated with the literature by flow cytometry and antioxidant analyses that cell treated with MPP⁺ at IC₅₀ concentration would activate the apoptotic pa-

thway presumably as a result of oxidative stress. Moreover, *G. Robertianum* extract has shown antiapoptotic properties against MPP⁺ induced toxicity in the *in vitro* PD model. Antioxidants were reported^{52,53} to block oxidative stress-mediated cellular damages and suppress apoptosis. Besides, antioxidants executed remarkable cell protection and apoptosis inhibition. Again, some naturally occurring compounds, such as pioglitazone and melatonin, are being in consideration for clinical studies on PD therapy in humans⁵⁴. In this context, the observed antiapoptotic effect could be attributed to the most abundant components in *G. Robertianum*, including linalool, α -terpineol, geraniol, α -caryophyllene, germacrene D, phytol acetate as well as oxidized alkenes like 2-(E)-hexenal and 3-(Z)-hexen-1-ol that exhibited more or less antioxidative property^{16,55,56}.

Conclusions

In vitro PD model constituted of SHSY-5Y cell culture differentiation and MPP⁺ application could be a useful model to investigate PD-like conditions for drug discovery and toxicological analyses. Furthermore, *G. Robertianum* was shown to exhibit neuroprotective features against MPP⁺ induced toxicity through regulating oxidative stress machinery and modulating antiapoptotic systems. Additionally, it was observed that the application of *G. Robertianum* plant aqueous extracts alter AChE enzyme activity. The current findings suggest that *G. Robertianum* extracts could be a promising candidate for the treatment of PD pathology.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

Not applicable.

Informed Consent

Not applicable.

Availability of Data and Materials

All the data used to support the findings of this study are included within the article and are available from the corresponding author by a reasonable request.

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Authors' Contribution

Conceptualization: M.E.A.; methodology: A.Y. and M.E.A.; writing, original draft preparation: M.E.A. and A.Y.; writing, review and editing: M.E.A.; supervision: M.E.A. All authors have read and agreed to the published version of the manuscript.

References

- 1) Parkinson J. An Essay on the Shaking Palsy. *J Neuropsychiatry Clin Neurosci* 2002; 14: 223-236.
- 2) AlMahadin G, Lotfi A, Zysk E, Siena FL, Carthy MM, Breedon P. Parkinson's disease: current assessment methods and wearable devices for evaluation of movement disorder motor symptoms - a patient and healthcare professional perspective. *BMC Neurol* 2020; 20: 419.
- 3) Charcot JM. *Leçons sur les Maladies du Système Nerveux*. Cambridge University Press, 2011; 1: 32-74.
- 4) Pringsheim T, Jette N, Frolkis A, Steeves TDL. The prevalence of Parkinson's disease: A systematic review and meta-analysis. *Mov Disord* 2014; 29: 1583-1590.
- 5) Ritz B, Lee PC, Hansen J, Lassen CF, Ketzler M, Sørensen M, Raaschou-Nielsen O. Traffic-Related Air Pollution and Parkinson's Disease in Denmark: A Case-Control Study. *Environ Health Perspect* 2016; 124: 351-356.
- 6) Moustafa AA, Chakravarthy S, Phillips JR, Gupta A, Keri S, Polner B, Frank MJ, Jahanshahi M. Motor symptoms in Parkinson's disease: A unified framework. *Neurosci Biobehav Rev* 2016; 68: 727-740.
- 7) Dissanayaka NNNW, White E, O'Sullivan JD, Marsh R, Pachana NA, Byrne GJ. The clinical spectrum of anxiety in Parkinson's disease. *Mov Disord* 2014; 29: 967-975.
- 8) Takamatsu Y, Fujita M, Ho GJ, Wada R, Sugama S, Takenouchi T, Waragai M, Masliah E, Hashimoto M. Motor and Nonmotor Symptoms of Parkinson's Disease: Antagonistic Pleiotropy Phenomena Derived from α -Synuclein Evolvability? *Parkinsons Dis* 2018; 2018: 1-6.
- 9) Langston J, Ballard P, Tetrud J, Irwin I. Chronic Parkinsonism in humans due to a product of me-

- peridine-analog synthesis. *Science* 1983; 219: 979-980.
- 10) Przedborski S, Tieu K, Perier C, Vila M. MPTP as a Mitochondrial Neurotoxic Model of Parkinson's Disease. *J Bioenerg Biomembr* 2004; 36: 375-379.
 - 11) Przedborski S, Jackson-Lewis V. Mechanisms of MPTP toxicity. *Mov Disord* 1998; 13: 35-38.
 - 12) Zheng M, Chen M, Wang W, Zhou M, Liu C, Fan Y, Shi D. Protection by rhynchophylline against MPTP/MPP⁺-induced neurotoxicity via regulating PI3K/Akt pathway. *J Ethnopharmacol* 2020; 8: 113568.
 - 13) Fabbrini G, Brotchie JM, Grandas F, Nomoto M, Goetz CG. Levodopa-induced dyskinesias. *Mov Disord Off J Mov Disord Soc* 2007; 22: 1379-1389.
 - 14) Graça VC, Ferreira ICFR, Santos PF. Phytochemical composition and biological activities of *Geranium robertianum* L.: A review. *Ind Crops Prod* 2016; 87: 363-378.
 - 15) Catarino MD, Silva AMS, Cruz MT, Cardoso SM. Antioxidant and anti-inflammatory activities of *Geranium robertianum* L. decoctions. *Food Funct* 2017; 8: 3355-3365.
 - 16) Gębarowska E, Politowicz J, Szumny A. Chemical composition and antimicrobial activity of *geranium robertianum* L. essential oil. *Acta Pol Pharm* 2017; 79: 699-705.
 - 17) Ilić MD, Marčetić MD, Zlatković BK, Lakušić BS, Kovačević NN, Drobac MM. Chemical Composition of Volatiles of Eight *Geranium* L. Species from Vlasina Plateau (South Eastern Serbia). *Chem Biodivers* 2020; 17: e1900544.
 - 18) Amaral S, Mira L, Nogueira JMF, da Silva AP, Helena Florêncio M. Plant extracts with anti-inflammatory properties—A new approach for characterization of their bioactive compounds and establishment of structure-antioxidant activity relationships. *Bioorg Med Chem* 2009; 17: 1876-1883.
 - 19) Neagu E, Paun G, Constantin D, Radu GL. Cytostatic activity of *Geranium robertianum* L. extracts processed by membrane procedures. *Arab J Chem* 2017; 10: S2547-S2553.
 - 20) Paun G, Neagu E, Litescu C, Rotinberg P, Radu L. Membrane processes application on the *Symphytum officinale* and *Geranium robertianum* extracts concentration to obtain high antioxidative activity compounds. *J Serbian Chem Soc* 2012; 77: 1191-1203.
 - 21) Graça VC, Barros L, Calhelha RC, Dias MI, Carvalho AM, Santos-Buelga C, Santos PF, Ferreira ICFR. Chemical characterization and bioactive properties of aqueous and organic extracts of *Geranium robertianum* L. *Food Funct* 2016; 7: 3807-3814.
 - 22) de Rus Jacquet A, Tambe MA, Ma SY, McCabe GP, Vest JHC, Rochet JC. Pikuni-blackfeet traditional medicine: neuroprotective activities of medicinal plants used to treat parkinson's disease-related symptoms. *J Ethnopharmacol* 2017; 206: 393-407.
 - 23) de Rus Jacquet A, Timmers M, Ma SY, Thieme A, McCabe GP, Vest JHC, Lila MA, Rochet JC. Lumbee traditional medicine: Neuroprotective activities of medicinal plants used to treat Parkinson's disease-related symptoms. *J Ethnopharmacol* 2017; 206: 408-425.
 - 24) Montalván M, Peñafiel MA, Ramírez J, Cumbicus N, Bec N, Larroque C, Bicchi C, Gilardoni G. Chemical Composition, Enantiomeric Distribution, and Sensory Evaluation of the Essential Oils Distilled from the Ecuadorian Species *Myrcianthes myrsinoides* (Kunth) Grifo and *Myrcia mollis* (Kunth) DC. (Myrtaceae). *Plants* (Basel) 2019; 8: 511.
 - 25) Faraone I, Rai DK, Russo D, Chiummianto L, Fernandez E, Choudhary A, Milella L. Antioxidant, Antidiabetic, and Anticholinesterase Activities and Phytochemical Profile of *Azorella glabra* Wedd. *Plants* 2019; 8: 265.
 - 26) Rabiei Z, Solati K, Amini-Khoei H. Phytotherapy in treatment of Parkinson's disease: a review. *Pharm Biol* 2019; 57: 355-362.
 - 27) Dias V, Junn E, Mouradian MM. The Role of Oxidative Stress in Parkinson's Disease. *J Parkinsons Dis* 2013; 3: 461-491.
 - 28) Bose A, Beal MF. Mitochondrial dysfunction in Parkinson's disease. *J Neurochem* 2016; 139: 216-231.
 - 29) Venkatesh Gobi V, Rajasankar S, Swaminathan Johnson WM, Prabu K, Ramkumar M. Antiapoptotic role of *Agaricus blazei* extract in rodent model of Parkinson's disease. *Front Biosci* 2019; 11: 12-19.
 - 30) Blum D, Torch S, Lambeng N, Nissou MF, Benabid AL, Sadoul R, Verna JM. Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. *Prog Neurobiol* 2001; 65: 135-172.
 - 31) Xicoy H, Wieringa B, Martens GJM. The SH-SY5Y cell line in Parkinson's disease research: a systematic review. *Mol Neurodegener* 2017; 12: 10.
 - 32) Xie HR, Hu L Sen, Li GY. SH-SY5Y human neuroblastoma cell line: In vitro cell model of dopaminergic neurons in Parkinson's disease. *Chin Med J (Engl)* 2010; 123: 1086-1092.
 - 33) Cheung YT, Lau WKW, Yu MS, Lai CSW, Yeung SC, So KF, Chang RCC. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. *Neurotoxicology* 2009; 30: 127-135.
 - 34) Encinas M, Iglesias M, Liu Y, Wang H, Muhaisen A, Ceña V, Gallego C, Comella JX. Sequential Treatment of SH-SY5Y Cells with Retinoic Acid and Brain-Derived Neurotrophic Factor Gives Rise to Fully Differentiated, Neurotrophic Factor-Dependent, Human Neuron-Like Cells. *J Neurochem* 2002; 75: 991-1003.
 - 35) Jämsä A, Hasslund K, Cowburn RF, Bäckström A, Vasänge M. The retinoic acid and brain-derived neurotrophic factor differentiated SH-SY5Y cell line as a model for Alzheimer's disease-like tau

- phosphorylation. *Biochem Biophys Res Commun* 2004; 319: 993-1000.
- 36) Li H, Yahaya BH, Ng WH, Yusoff NM, Lin J. Conditioned Medium of Human Menstrual Blood-Derived Endometrial Stem Cells Protects Against MPP⁺-Induced Cytotoxicity in vitro. *Front Mol Neurosci* 2019; 12: 80.
- 37) Santos NAG, Martins NM, Sisti FM, Fernandes LS, Ferreira RS, Queiroz RHC, Santos AC. The neuroprotection of cannabidiol against MPP⁺ -induced toxicity in PC12 cells involves trkA receptors, upregulation of axonal and synaptic proteins, neurogenesis, and might be relevant to Parkinson's disease. *Toxicol Vitro* 2015; 30: 231-240.
- 38) Jiang D, Peng Y. The protective effect of decoction of *Rehmanniae* via PI3K/Akt/mTOR pathway in MPP⁺ -induced Parkinson's disease model cells. *J Recept Signal Transduct* 2021; 41: 74-84.
- 39) Liu N, Liu J, Wen X, Bai L, Shao R, Bai J. TRPV4 contributes to ER stress: Relation to apoptosis in the MPP⁺-induced cell model of Parkinson's disease. *Life Sci* 2020; 261: 118461.
- 40) Küçükdoğru R, Türkez H, Arslan ME, Tozlu ÖÖ, Sönmez E, Mardinoğlu A, Cacciatore I, Di Stefano A. Neuroprotective effects of boron nitride nanoparticles in the experimental Parkinson's disease model against MPP⁺ induced apoptosis. *Metab Brain Dis* 2020; 35: 947-957.
- 41) Kowall NW, Hantraye P, Brouillet E, Beal MF, McKee AC, Ferrante RJ. MPTP induces alpha-synuclein aggregation in the substantia nigra of baboons. *Neuroreport* 2000; 11: 211-213.
- 42) Cheng W, Huang A, Zhang L, Feng D, Sun X, Xu H, Sun Q, Li X. Isoflavone Attenuates the Nuclear Transcription Factor Kappa B (NF-κB) Activation on MPP⁺ - Induced Apoptosis of PC12 Cells. *J Behav Brain Sci* 2020; 10: 191-199.
- 43) Zhou S, Zhang D, Guo J, Chen Z, Chen Y, Zhang J. Deficiency of NEAT1 prevented MPP⁺-induced inflammatory response, oxidative stress and apoptosis in dopaminergic SK-N-SH neuroblastoma cells via miR-1277-5p/ARHGAP26 axis. *Brain Res* 2021; 1750: 147156.
- 44) Garg Neeraj K, Sharad M, Tejram S, Abhinav M, Vyas Suresh P, Tyagi Rajeev K. Evaluation of anti-apoptotic activity of different dietary antioxidants in renal cell carcinoma against hydrogen peroxide. *Asian Pac J Trop Biomed* 2011; 1: 57-63.
- 45) Zhi Y, Jin Y, Pan L, Zhang A, Liu F. Schisandrin A ameliorates MPTP-induced Parkinson's disease in a mouse model via regulation of brain autophagy. *Arch Pharm Res* 2019; 42: 1012-1020.
- 46) Filograna R, Beltramini M, Bubacco L, Bisaglia M. Anti-Oxidants in Parkinson's Disease Therapy: A Critical Point of View. *Curr Neuropharmacol* 2016; 14: 260-271.
- 47) Park H, Seol GH, Ryu S, Choi IY. Neuroprotective effects of (-)-linalool against oxygen-glucose deprivation-induced neuronal injury. *Arch Pharm Res* 2016; 39: 555-564.
- 48) Karakaya S, Yilmaz SV, Özdemir Ö, Koca M, Pınar NM, Demirci B, Yıldırım K, Sytar O, Turkez H, Baser KHC. A caryophyllene oxide and other potential anticholinesterase and anticancer agent in *Salvia verticillata* subsp. *amasiaca* (Freyne & Bornm.) Bornm. (Lamiaceae). *J Essent Oil Res* 2020; 32: 512-525.
- 49) Pagoni A, Marinelli L, Di Stefano A, Ciulla M, Turkez H, Mardinoglu A, Vassiliou S, Cacciatore I. Novel anti-Alzheimer phenol-lipoyl hybrids: Synthesis, physico-chemical characterization, and biological evaluation. *Eur J Med Chem* 2020; 186: 111880.
- 50) Cacciatore I, Fornasari E, Marinelli L, Eusepi P, Ciulla M, Ozdemir O, Tatar A, Turkez H, Di Stefano A. Memantine-derived drugs as potential antitumor agents for the treatment of glioblastoma. *Eur J Pharm Sci* 2017; 109: 402-411.
- 51) Vasquez V, Mitra J, Hegde PM, Pandey A, Sengupta S, Mitra S, Rao KS, Hegde ML. Chromatin-Bound Oxidized α-Synuclein Causes Strand Breaks in Neuronal Genomes in in vitro Models of Parkinson's Disease. *J Alzheimers Dis* 2017; 60: S133-S150.
- 52) Türkez H, Arslan ME, Sönmez E, Açikyıldız M, Tatar A, Geyikoğlu F. Synthesis, characterization and cytotoxicity of boron nitride nanoparticles: emphasis on toxicogenomics. *Cytotechnology* 2019; 71: 351-361.
- 53) Turkez H, Cacciatore I, Arslan ME, Fornasari E, Marinelli L, Di Stefano A, Mardinoglu A. Histidyl-Proline Diketopiperazine Isomers as Multipotent Anti-Alzheimer Drug Candidates. *Biomolecules* 2020; 10: 737.
- 54) Marinelli L, Fornasari E, Di Stefano A, Turkez H, Arslan ME, Eusepi P, Ciulla M, Cacciatore I. (R)-α-Lipoyl-Gly-1-Pro-1-Glu dimethyl ester as dual acting agent for the treatment of Alzheimer's disease. *Neuropeptides* 2017; 66: 52-58.
- 55) Aydin E, Türkez H, Keleş MS. The effect of carvacrol on healthy neurons and N2a cancer cells: Some biochemical, anticancerogenicity and genotoxicity studies. *Cytotechnology* 2014; 66: 149-157.
- 56) Turkez H, Nóbrega FRD, Ozdemir O, Bezerra Filho CDSM, Almeida RN, Tejera E, Perez-Castillo Y, Sousa DP. NFBTA: A Potent Cytotoxic Agent against Glioblastoma. *Molecules* 2019; 24: 2411.