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Original Research Article

Aqueous leaf extract of Sutherlandia frutescens attenuates ROS-induced apoptosis and loss of mitochondrial membrane potential in MPP+-treated SH-SY5Y cells

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Abstract

Purpose: *To investigate the neuroprotective activity of the aqueous extract of Sutherlandia frutescens (SF) against 1-methyl-4-phenylpyridinium (MPP^+)-induced toxicity in SH-SY5Y neuroblastoma cells.*
Methods: *SH-SY5Y neuroblastoma cells were divided into different treatment groups: untreated cells, cells treated with MPP^+ alone (2 mM), cells pretreated with SF (20 µg) prior to MPP^+ (2 mM) treatment and cells treated with SF (20 µg) alone. Twenty-four hours after treatment with MPP^+, cell viability was assessed by MTT assay, and changes in cell morphology, intracellular reactive oxygen species (ROS) production, mitochondrial membrane potential (MMP) as well as caspases 3/7 and 9 activities were determined.*
Results: *Treatment of SH-SY5Y cells with MPP^+ alone significantly altered cellular morphology, increased ROS production ($p = 0.005$), induced a significant loss of MMP ($p = 0.0011$) and caused significant apoptotic cell death, via the activation of caspases 3/7 and 9 ($p \leq 0.0359$). These effects were however significantly ($p \leq 0.0359$) attenuated in cells pre-treated with the aqueous leaf extract of SF, indicating the possible neuroprotective activity of the SF extract.*
Conclusion: *The results of this study suggest that the aqueous leaf extract of SF may be neuroprotective against MPP^+-induced toxicity via apoptotic cell death and inhibition of ROS production. Further mechanistic studies are required to validate the results of the present study using additional PD models, different extract preparations and active compounds derived from SF.*

Keywords: *Parkinson's disease, MPP^+, Sutherlandia frutescens, Reactive oxygen species, Apoptosis, Neurodegeneration*

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INTRODUCTION

Parkinson's disease (PD) is the second most common, age-dependent neurodegenerative disorder resulting from continuous loss of dopaminergic neurons in the pars compacta of the substantia nigra of the midbrain (SNpc). Resting tremor, bradykinesia and muscle rigidity are some of the motor symptoms associated with PD, while cognitive and neuropsychiatric disorders constitute the non-motor symptoms [1]. There is currently no cure for PD, hence the

search for effective treatment options capable of halting the death of dopaminergic neurons with little or no side effects, is plausible.

In order to study the mechanisms involved in PD, the standard cellular model of the disease was induced using the neurotoxic compound 1-methyl-4-phenylpyridinium (MPP^+), an active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was used. When MPP^+ is taken up by the dopamine transporter, it causes damage to dopaminergic neurons in a way that is comparable to the mechanisms of the human disease [2]. For instance, MPP^+ inhibits complex I of the mitochondrial electron transport chain (ETC), thus diminishing the production of ATP and increasing the formation of reactive oxygen species (ROS) [2].

Although the etiology and exact molecular mechanisms involved in the neuronal degeneration observed in PD are poorly understood, the involvement of oxidative stress (OS), apoptosis and mitochondrial dysfunction have been reported. Over the years, research has focused more on the pathological importance of OS in PD, owing to its role in aging and aging-related diseases in general. Oxidative stress is known to be caused by the imbalance in the production of ROS and antioxidants and is believed to be a key mechanism that heralds and initiates the death of neurons in PD [3].

Earlier studies have shown substantial evidence of oxidative stress in the brains of animal models of PD [4]. Oxidants are by-products of oxidative phosphorylation, thus making mitochondria the major sites of ROS generation within the cell. In most neurodegenerative diseases characterized by mitochondrial respiratory defects, including PD, the ROS generated by the ETC increases and overwhelms the antioxidant protection mechanisms. This is evidenced by post-mortem reports of complex I deficiency in the SN of individuals with PD, thus establishing a direct link between ROS, mitochondrial dysfunction and the disease [5].

Similarly, the vast majority of *in vivo* and *in vitro* models of PD have been reported to show morphological apoptosis and activation of caspases [6]. In most of these models, the involvement of apoptotic signalling pathways and the attenuation of cell death by caspase inhibitors tend to support suggestions of apoptosis as a mechanism of cell death in PD. Thus, it is plausible to investigate antioxidant-rich medicinal plants for their apoptosis-inhibiting effects as well as their ability to mitigate mitochondrial dysfunction and ROS production in dopaminergic neurons. Plant extracts with such properties could provide potential sources of novel therapeutic agents for the protection or treatment of PD.

Sutherlandia frutescens (SF), also known as 'cancer bush', is a versatile medicinal plant commonly used in South Africa [7]. Its decoctions have been used for many years by traditional healers to treat open wounds, inflammation, fever, eye infections, chicken pox and haemorrhoids [8]. It is also consumed as a herbal remedy by individuals living with HIV/AIDS in South Africa as an immune-booster and to improve general well-being [9].

Various studies have investigated the beneficial effects of SF in cancer [10], diabetes [11] and inflammation [12]. Phytochemical evaluations of this plant have shown that it contains significant amounts of pharmacologically important constituents, including gamma-aminobutyric acid, glycosides, saponins, and L-canavanine. Although SF has been reported to show activity in various disease models, studies on its neuroprotective activity in a Parkinson's disease model have not been reported. This study therefore investigated the mechanisms by which SF could potentially provide neuroprotection against MPP^+-induced cell death in the SH-SY5Y human neuroblastoma cell line used as a model of PD.

EXPERIMENTAL

Preparation of extract

Dry-milled leaf powder of SF (variety *Incana* E. Mey.) [Family: *Fabaceae*] was purchased from Big Tree Health Products (Fish Hoek, South Africa). To prepare the aqueous extract of the plant, 1 kg of the leaf powder was added to boiling water and incubated overnight. The extract was then filtered with Whatman filter paper and freeze-dried to yield the aqueous extract which was stored at -20°C until needed.

Cell culture maintenance

SH-SY5Y cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, USA), supplemented with 10% Fetal bovine serum (FBS) (Life Technologies, USA) and 1% penicillin-streptomycin (Lonza, USA), at 37 °C, 5% CO_2 in a humidified incubator. The culture medium was changed every two days and the cells were trypsinized with 1X trypsin-versene EDTA mixture (Lonza, USA) and transferred to a different dish for

regrowth once a confluency of 70 - 80 % was reached.

Cytotoxicity screening, cell viability and toxicity assay

Cytotoxicity screening was performed to ensure that SH-SY5Y cells were exposed to the correct concentrations of MPP^+ and SF. A fresh stock concentration of MPP^+ was prepared on the day of the experiment and dilutions were made up for final concentration ranges of 0 – 4000 µM. For SF, a fresh stock concentration was prepared on the day of the experiment and dilutions were made up for final concentration ranges of 0 – 60 µg. Cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, St. Louis, MO, USA] assay [13, 14]. SH-SY5Y cells, seeded at a density of 1.0 x 10^4 cells/well in 96-well plates, were allowed to attach for 24 h. Based on the findings from the cytotoxicity screening, cells were pretreated with 20 µg of SF for 1 h and then treated with 2000 µM of MPP^+ for 24 h. Thereafter, 20 µL of the MTT solution was added to each well of the plate and incubated for 3 h. Absorbance was measured at a wavelength of 560 nm using the Glomax Multi Detection System (Promega, USA).

The trypan blue (TB) dye exclusion assay was used to measure toxicity in treated and untreated samples. The TB dye only enters non-viable cells through their damaged cell membranes while healthy viable cells exclude this dye. For this assay, the SH-SY5Y cells were seeded at a density of 1.1 x 10^5 /ml in 60 mm dishes and treated as previously described. Adherent cells were detached by trypsinization, pelleted and re-suspended in fresh culture media. A 20 µL of the cell suspension was added to an equivalent volume of 0.4 % trypan blue dye (Sigma Aldrich, St. Louis, MO, USA) and loaded into a BioRad TC20™ automated cell counter. Toxicity was expressed as the percentage of dead cells relative to the total cell count.

Morphological evaluation of cells

The SH-SY5Y cells were seeded at a density of 1.1 x 10^5 /mL in 60 mm dishes and were allowed to attach for 24 h. The cells were treated as earlier described and morphological changes were observed using a ZEISS Primo Vert (Germany) light microscope.

Determination of reactive oxygen species production

The production of reactive oxygen species was determined using the 2′,7′-dichlorofluorescin diacetate (DCFH-DA, St. Louis, MO, USA) dye. The SH-SY5Y cells were seeded at a density of 1.2 x 10^5 /mL in 100 mm dishes and treated as previously described. After treatment, adherent cells were dislodged with a scrapper and spun using a Bio-Rad tabletop centrifuge at 3000 rpm for 5 min. Cell pellets were resuspended and incubated with 25 µM of DCFH-DA for 45 min. Stained cells were pelleted, resuspended in PBS (500 µL) and thereafter analysed on an Accuri flow cytometer (BD Biosciences Pharmingen, San Diego, CA, USA), with a total of 10000 events acquired for each sample.

Determination of mitochondrial membrane potential

Mitochondrial membrane potential was determined using rhodamine 123 (St. Louis, MO, USA) fluorescence dye. The SH-SY5Y cells were seeded at a density of 1.2 x 10^5 /mL and were treated as previously described. Thereafter, adherent cells were dislodged with a cell scraper and spun at 3000 rpm for 5 min. Cell pellets were resuspended and incubated with 10 µM of rhodamine123 for 30 min. Stained cells were pelleted, resuspended in PBS (500 µL) and thereafter analysed on an Accuri flow cytometer (BD Biosciences Pharmingen, San Diego, CA, USA), with a total of 10000 events acquired for each sample.

Evaluation of Caspase 3/7 and Caspase 9 activities

The ApoTarget™ Caspase-9 Protease Assay (Life Technologies, USA) was used to evaluate the activity of caspase-9. SH-SY5Y cells were seeded at a density of 1.2 x 10^5 cells /mL in 100 mm dishes and treated as earlier described. Adherent cells were displaced, pelleted, re-suspended in lysis buffer and incubated on ice for 10 min. Experiments were performed according to the manufacturer's instructions, and absorbance was detected using a Polarstar Omega plate reader (BMG Labtech, USA) at 405 nm. Also, the Caspase 3/7 assay kit (Promega, USA) was used to evaluate the activity of caspase 3/7. SH-SY5Y cells were seeded at a density of 1.0 x 10^4 cells/well in white-walled 96-well plates and treated as earlier described. After treatment, experiments were performed according to the manufacturer's instructions, and luminescence was measured using the Glomax Multi Detection System.

Statistical analysis

Statistical analyses were done using the GraphPad Prism Software V7

(www.graphpad.com/scientific-software/prism/), and data expressed as mean with standard error of mean (SEM) from three independent experiments. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post-hoc test was used to analyze the relationship between variables. Significance was considered at $p < 0.05$.

RESULTS

Dose-response of MPP^+ and SF toxicity.

Cell viability significantly decreased following a 24-h incubation of the SH-SY5Y cells with increasing concentrations of MPP^+ (0 - 4000 µM). The dose-response investigation was done to determine the MPP^+ concentration which will result in approximately 50 % viable cells (Figure 1A). For SF, there was a significant increase in cell viability after 24-h treatment with increasing concentrations of the SF extract (20 - 60 µg). Consequently, a concentration of 20 µg was chosen for use in further experiments based on the highest absorbance value ($p = 0.0008$, Figure 1 B).

Effect of SF on MPP^+-induced toxicity in SH-SY5Y cells

The MTT assay results (Figure 1C) showed that treatment with MPP^+ alone caused a significant decrease in cell viability ($p < 0.0001$), while pretreatment with SF before exposure to MPP^+ resulted in significant increase ($p = 0.0008$,) in cell viability. In the SH-SY5Y cells treated with SF alone, cell viability was not significantly different ($p = 0.4204$) when compared to the viability of the untreated cells. Similarly, results of the trypan blue dye exclusion assay revealed that treatment with MPP^+ alone caused a significant increase in cell toxicity ($p = 0.0004$), while pretreatment with SF significantly decreased ($p = 0.0016$, Figure 1 D) cell toxicity. When compared to the untreated SH-SY5Y cells, the toxicity observed in SH-SY5Y cells treated with SF alone was not statistically significant ($p = 0.9511$).

Morphology of SH-SY5Y cells following SF pretreatment

Morphological evaluation of the SH-SY5Y cells treated with MPP^+ alone showed shrinkage and rounding up of cell bodies while SF pre-treatment appeared to have prevented these MPP^+-induced morphological alterations. On the other hand, the morphology of cells exposed to SF alone appeared similar to that of the untreated cells (Figure 2).

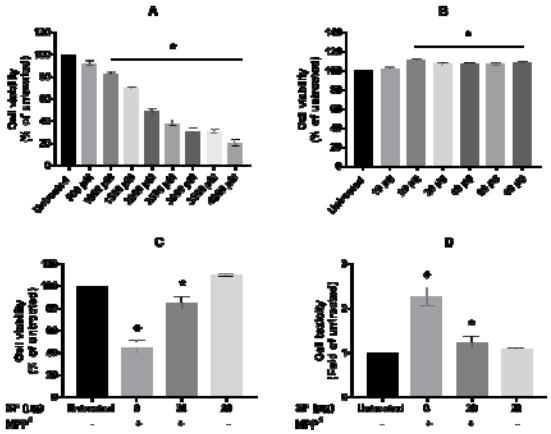

Figure 1: Effect of treatment with SF extract on MPP^+-induced toxicity in SH-SY5Y cells. (A) MPP^+ toxicity screening (B) SF toxicity screening (C) MTT cell viability assay (D) Trypan blue dye exclusion assay. ◆$p < 0.0005$ vs untreated SH-SY5Y cells; *$p < 0.05$ vs SH-SY5Y cells treated with MPP^+ only

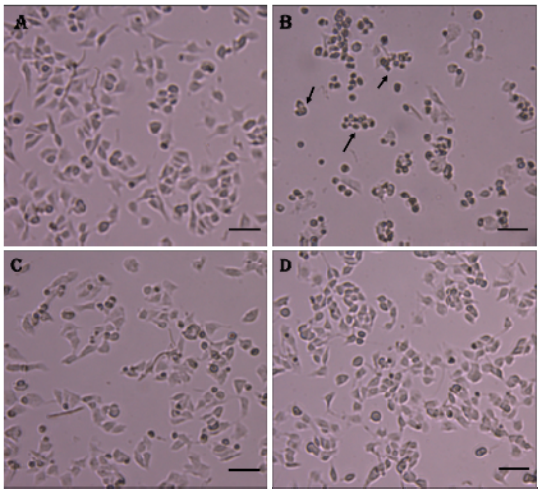

Figure 2: Effects of treatment with SF extract on MPP^+-induced morphological alterations in SH-SY5Y cells. (A) Untreated SH-SY5Y cells (B) SH-SY5Y cells treated with MPP^+ alone (C) SH-SY5Y cells pretreated with SF and then treated with MPP^+ (D) SH-SY5Y cells treated with SF only. Arrows indicate shrinking and contracting SH-SY5Y cells. Scale bar: 5µm

ROS production following SF pretreatment

Whereas ROS levels significantly increased ($p = 0.005$) in SH-SY5Y cells treated with MPP^+ alone, pretreatment with SF significantly reduced ($p = 0.0316$) ROS production. However, there was no significant difference ($p = 0.994$) in the ROS levels in the SH-SY5Y cells treated with SF alone when compared to the untreated cells (Figure 3).

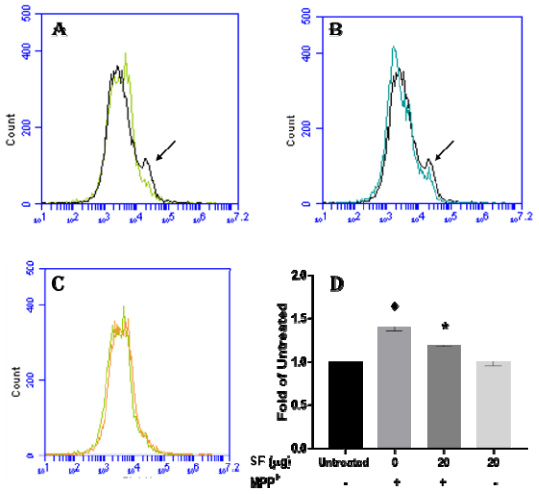

Figure 3: Effects of treatment with SF extract on MPP⁺-induced ROS production. (A) Untreated vs MPP⁺ alone (B) SF pretreated vs MPP⁺ alone (C) Untreated vs SF alone (D) Bar chart showing the effect of SF on ROS production. Arrows indicate fluorescence intensity of cells treated with MPP⁺ only. ◆$p < 0.0005$ vs untreated SH-SY5Y cells; *$p < 0.05$ vs SH-SY5Y cells treated with MPP⁺ only

Mitochondrial membrane potential in SH-SY5Y cells following SF pretreatment

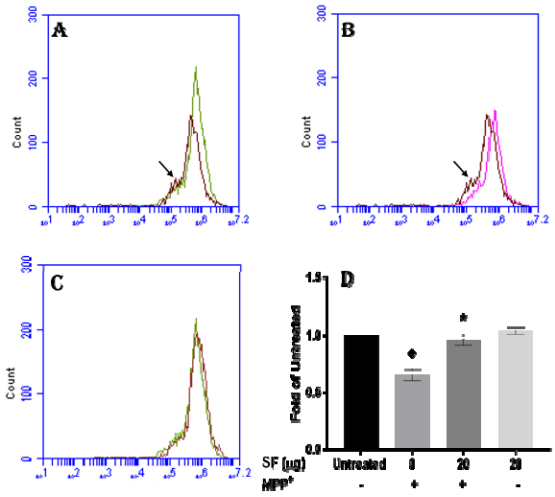

Figure 4: Effects of treatment with SF extract on MPP⁺-induced loss of MMP. (A) Untreated vs MPP⁺ alone (B) SF pretreated vs MPP⁺ alone (C) Untreated vs SF alone (D) Graph showing the effect of SF on MMP. Arrows indicate fluorescence intensity of cells treated with MPP⁺ only. ◆$p < 0.005$ vs untreated SH-SY5Y cells; *$p < 0.005$ vs SH-SY5Y cells treated with MPP⁺ only

The reduction of rhodamine 123 fluorescence intensity signifies a loss of MMP. The graphs in Figure 4 showed that MPP⁺ treatment alone significantly reduced ($p = 0.0011$) the MMP as demonstrated by a shift to the left in the fluorescence intensity while treatment with the SF extract appeared to inhibit ($p = 0.0025$) the MPP⁺-induced reduction in MMP. On the other hand, treatment with SF alone did not significantly affect ($p = 0.8877$) the MMP when compared to untreated SH-SY5Y cells.

Caspase 3/7 and 9 activities following SF pretreatment

The SH-SY5Y cells treated with MPP⁺ alone showed that caspase 3/7 ($p = 0.0006$) and caspase 9 ($p = 0.0011$) activities were significantly increased, which is an indication of the activation of apoptosis (Figure 5). In cells pretreated with SF, there was a significant decrease in the activities of caspase 3/7 ($p = 0.0199$) and caspase 9 ($p = 0.0359$) respectively. Treatment with SF alone did not significantly affect caspase 3/7 ($p = 0.9184$) and 9 ($p = 0.9917$) activity when compared to the untreated SH-SY5Y cells.

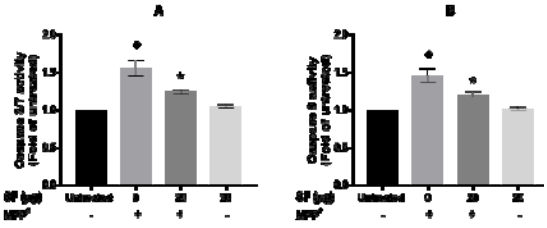

Figure 5: Effects of treatment with SF extract on MPP⁺-induced apoptosis. (A) Caspase 3/7 activity (B) Caspase 9 activity. ◆$p < 0.005$ vs untreated SH-SY5Y cells; *$p < 0.05$ vs SH-SY5Y cells treated with MPP⁺ only

DISCUSSION

This study demonstrated the neuroprotective activity of SF in an *in vitro* model of PD using the neurotoxin MPP⁺ and dopaminergic SH-SY5Y human neuroblastoma cells. Exposure of SH-SY5Y cells to MPP⁺ for 24 h significantly decreased cell viability, increased toxicity, increased intracellular ROS production, induced loss of MMP and activated caspases 3/7 and 9. However, the changes induced by MPP⁺ were significantly inhibited by pretreatment of the SH-SY5Y cells with SF, which suggests that SF may protect the cells from MPP⁺-induced apoptosis and loss of MMP via the regulation of ROS.

Previous studies have indicated that in PD patients, about 50 % of dopaminergic neurons in the nigrostriatal pathway have degenerated in the brain at the time of preliminary diagnosis, and

a substantial portion of the remaining dopaminergic neurons are stressed [15,16]. In a bid to replicate this condition in this model of PD, it was imperative to utilize concentrations of toxins that induced approximately 50 % cell death. Findings from the MTT assay showed that the selected dose of MPP^+ treatment induced approximately 50 % cell death, which is in line with earlier studies indicating that treatment of cells to high concentrations of MPP^+ for 24 h result in a mix of healthy and unhealthy cell population [17,18]. Findings from this study also show that pretreatment of SH-SY5Y cells with SF protected the cells from MPP^+-induced toxicity. From the morphological images, it was observed that the shrinkage of cell bodies and the disappearance of axons were evident in the MPP^+-treated cells, whereas cells pretreated with the SF extract looked similar to the untreated cells.

The pathogenesis of PD has been reported to involve excessive production of ROS and the ability of many medicinal plant extracts to reverse or modulate excess ROS is a known neuroprotection mechanism. MPP^+ is a known ROS-inducing neurotoxin, and overproduction of ROS has been reported to contribute to oxidative damage, mitochondrial dysfunction and apoptotic cell death [19]. One of the mechanisms reported for the excessive production of intracellular ROS by MPP^+ is the inhibition of the mitochondrial electron transport chain complex I [20]. This excessive production of intracellular ROS is believed to be an early event preceding the activation of caspase-9 as well as caspase-3/7, leading to apoptosis [21]. These events show that ROS is a key player in cellular apoptosis induced by MPP^+. The results further revealed that intracellular ROS production significantly increased following treatment of SH-SY5Y cells with MPP^+. Conversely, intracellular ROS production significantly reduced when SH-SY5Y cells were pretreated with SF before MPP^+ treatment.

Mitochondria are susceptible to oxidative stress and when there is sustained generation of ROS, there may be alterations in mitochondrial morphology, which further results in the opening of the mitochondrial transition pore, and a loss of MMP [19]. A reduction in membrane potential is an indicator of early apoptosis, and is consistent with previous findings. The results from the current study showed that SH-SY5Y cells displayed a decline in mitochondrial membrane potential upon treatment with MPP^+. Conversely, pretreatment with SF prevented the loss of MMP which may suggest a neuroprotective effect of SF against MPP^+-induced toxicity.

In the pathogenesis of PD, both the extrinsic (death-receptor) and the intrinsic (or mitochondrial) apoptotic pathways are known to play major roles. Investigations on the events involved in the intrinsic pathway show that cytochrome c binds to the apoptosis protease activating factor 1 (Apaf-1) in the cytoplasm to form an apoptosome (a molecular complex consisting of cytochrome c, Apaf-1, ATP, and procaspase 9). The apoptosome in turn activates caspase 9 (an upstream initiator of apoptosis) which then activates the executioner caspases 3, 6 and 7, eventually resulting in apoptosis [22]. In the present study, the activity of caspases 3/7 and 9 significantly increased in SH-SY5Y cells treated with MPP^+ alone. However, pretreatment of cells with SF significantly inhibited the activity of these caspases, indicating that SF inhibited the activation of the intrinsic apoptotic pathway by MPP^+.

CONCLUSION

The findings of the current study demonstrate the neuroprotective activity of the SF extracts against MPP^+-induced toxicity in SH-SY5Y cells. The SF extracts appeared to inhibit MPP^+-induced overproduction of ROS as well as a loss of MMP. These extracts could attenuate the activation of the intrinsic apoptotic pathway, thus suggesting the potential use of the plant as a neuroprotective and therapeutic agent against PD.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. A.B.E. and S.I.O. performed the experiments and wrote manuscript. OEE designed the experiments and edited the manuscript.

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