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In vitro neuroprotective effects of boophone disticha, brunsvigia bosmaniae and strumaria truncata extracts in SH-SY5Y cells

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ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. The pathological hallmarks of PD are defined by the loss of dopaminergic neurons in the substantia nigra pars compacta of the midbrain, with its characteristic clinical motor and non-motor symptoms. Current treatments for PD are mainly palliative; hence, novel neuroprotective and therapeutic approaches are needed. This study investigated the potential neuroprotective effects of three Amaryllidaceae plant extracts, *Boophone disticha, Brunsvigia bosmaniae*, and *Strumaria truncata,* in an *in vitro* model of PD involving the exposure of SH-SY5Y cell lines to the MPP^+ neurotoxin. The protective effects of the three medicinal plants on cell viability were evaluated. The mechanism of the induced neuroprotection was investigated by measuring intracellular reactive oxygen species (ROS) levels, nitric oxide (NO) levels, adenosine triphosphate (ATP) levels, caspase 3 activity, and autophagy levels in the SH-SY5Y cells. The results showed that treatment with the extracts was protective against the toxicity induced by MPP^+ in the SH-SY5Y cells. This was demonstrated by the significant increase in cell viability in the MPP^+-treated cells, attenuation of intracellular ROS and NO levels, caspase 3 activity, and increased ATP activity. These findings suggest that the three extracts could be sources of novel bioactive agents with possible neuroprotective activities owing to their anti-apoptotic and antioxidant activities. Further studies are recommended to characterise the constituent compounds in these extracts to support their potential as drug discovery and development candidates.
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1. Introduction

Parkinson's disease (PD) is characterised by the loss of dopaminergic neurons in the substantia nigra pars compacta in the midbrain and is the second most common neurodegenerative disease (NDD) after Alzheimer's disease (AD), affecting approximately 10 million people worldwide (Ferreira and Romero-Ramos, 2018; Boyd et al., 2022; Ou et al., 2021). PD has also been reported to be more prevalent in males than females (Lewin, 2019; Rizek et al., 2016). Its symptoms vary from motor to non-motor, with the non-motor symptoms often preceding the motor deficits (Goldman and Postuma, 2014).

PD's pathophysiology involves protein aggregation, excitotoxicity, neuroinflammation, mitochondrial dysfunction, genetic mutations, and oxidative stress (Michel et al., 2016; Tansey et al., 2007). Mitochondrial dysfunction caused by genetic mutations and environmental toxins leads to compromised mitochondrial membrane potential, decreased ATP levels, and disruption of calcium homeostasis, among other effects, all resulting in elevated caspase activity and apoptosis, neurodegeneration, and eventually PD (Golpich et al., 2017; Rego and Oliveira, 2003; Yang et al., 2020). From several theories, the process of neurodegeneration leading to Parkinson's Disease can be attributed to seven main factors, which include oxidative stress (ROS, free radicals, NO), proteolysis defects, protein misfolding (α-synuclein protein), immunological reactions, iron metabolism disorders (toxic iron accumulation), mitochondrial dysfunction and apoptosis (Abramov et al., 2020; Amro et al., 2018; Gurunnaselage Don, 2016; Harischandra et al., 2019; Koziorowski et al., 2021; Sian-Hulsmann and Riederer, 2021; Stykel and Ryan, 2022).

Abbreviations: AD, Alzheimer's disease; ATP, adenosine triphosphate; DCFDA, dichlorodihydrofluorescein diacetate; MPP^+, 1-methyl-4-phenylpyridinium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays; NDD, neurodegenerative disease; NO, nitric oxide; PD, Parkinson's disease; ROS, reactive oxygen species

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To date, no disease-modifying treatments are available for PD, as current treatments only offer symptomatic relief of the symptoms associated with the disease. For over 40 years, levodopa has remained the most widely used oral treatment for relieving motor symptoms of PD. Still, its usage over time also induces Parkinson-like symptoms (Hauser, 2009; Poewe et al., 2010; Salat. Unfortunately, most available treatments offer minimal clinical benefits regarding the non-motor manifestations of PD (Zahoor et al., 2018).

In most parts of the world, there has been interest in the use of phytotherapy for neurological and NDDs, mainly because most plant extracts or plant-derived bioactive compounds and nutraceuticals have been reported to have potent neuroprotective effects (Kumar and Khanum, 2012; Da Costa et al., 2020). South Africa has a wide variety of medicinal plants, and most rural communities have relied on herbs to treat different ailments for hundreds of years. The availability of medicinal plants in South Africa and their folkloric use have elicited renewed interest in their putative use as sources of potent plant-derived medicines for treating neurological, neurodegenerative, and psychiatric disorders (Enogieru et al., 2018).

Although widely distributed around the world and consisting of about 800 species and 80 genera, some of the Amaryllidaceae species are endemic to the southern part of Africa, with the Western Cape province of South Africa having higher numbers of these plant species (Conrad, 2008; Goldblati, 1978; Ibrakaw et al., 2020). They have been reported to have a wide range of biological activities, including anti-cancer, antiviral, cytotoxicity, anti-hypertensive, antioxidant, anti-inflammatory, anti-tumour, anti-microbial, anticonvulsive, anti-rheumatic, anti-depressive and antiparasitic (Adewusi et al., 2012; Bastida et al., 2006; Nair and Van Staden, 2013; Castilhos et al., 2007; Da Silva et al., 2006; Farinon et al., 2017; Kornienko and Evidente, 2008; Reis et al., 2019). Furthermore, our laboratory has previously reported on the neuroprotective activities of some species of this plant family, which include *Boophoone haemathiodes, Crossyne flava, Crossyne gutata* and *Nerine humilis* (Ibrakaw et al., 2020; Omoruyi et al., 2019, 2020, 2021). *Strumaria* truncata (commonly known as Cape snowflake) is second only to *Cyrtanthus* regarding biological species' rarity amongst the Southern African Amaryllidaceae and has few or no reported studies. *Brunsvigia* plants are extensively distributed in the Drakensberg, KwaZulu-Natal province of South Africa, and their traditional uses are mainly centred around local diviners to enhance the accuracy of their readings (Dee Snijman, 2005). The San people used the bulbs to influence and affect the behaviour and mind of the person to whom they were administered (Rust, 2009).

In this study, the extracts of the Amaryllidaceae plants *Boophone disticha* (BD), *Brunsvigia bosmaniae* (BB) and *Strumaria truncata* (ST) were evaluated for their neuroprotective effects in an *in vitro* model of PD. We show for the first time the neuroprotective activities of ST and BB in an *in vitro* model of PD and also report the activity of BD in the MPP^+ model of PD.

2. Materials and methods

2.1. Preparation of plant material

The bulbs of BD, BB and ST extracts were weighed, and a mass of 200 g for each plant bulb was dissolved in methanol. Extraction was then carried out at room temperature for 48 h, after which the plant material was filtered and evaporated under reduced pressure at 45 °C then eventually allowed to dry. Once the extracts were solvent-free, they were stored in Eppendorf tubes at 4 °C for later use.

2.2. Cell culture and maintenance

The neuronal cell line SH-SY5Y was obtained as a gift from the Blackburn Laboratory, University of Cape Town and stored at −80 °C until use. Once thawed, cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Life Technologies Corporation, Paisley, UK), supplemented with 10 % Fetal Bovine Serum (FBS, Gibco, Life Technologies Corporation, Paisley, UK) and 1 % Penicillin/Streptomycin (Lonza Group Ltd., Verviers, Belgium) in a water-jacketed CO_2 incubator at 37 °C with 5 % CO_2 (Labtec, Germany) until 70–80 % confluence was attained. Cells were subcultured before each experiment after reaching about 70 to 80 % confluency with 1 mL of trypsin EDTA (Lonza Group Ltd., Verviers, Belgium).

2.3. Treatments

To determine the optimum concentration of plant extracts to be used for neuroprotection assay, stock solutions of 40 mg/mL of the BD, BB and ST extract were made in DMSO (Sigma-Aldrich, St Louis MO, USA) from which further dilutions were made in growth medium to obtained desired concentrations. For dose-response of extracts, trypsinised cells were seeded in 96-well plates at a density of 10,000 cells/well and incubated overnight to allow attachment. After that, cells were exposed to increasing concentrations of 2.5, 5, 7.5 and 10 μg/mL for BD, BB and ST for 24 h, and cell viability was obtained afterwards. In addition, the concentration of MPP^+ required to reduce cell viability to about 50 % was also determined by exposing SH-SY5Y cells to increasing concentrations of 500 to 2500 μM, and after that, cell viability was determined, and the 2000 μM MPP^+ was selected for further studies.

For neuroprotection studies, 10,000 cells of the SH-SY5Y cells were plated and incubated overnight, and, after that, the cells were exposed to 2.5, 5, and 10 μg/mL of either the BB, BD, or ST extract for 2 h before the addition of 2000 μM of MPP^+. All treatments lasted for 24 h, and cell viability was determined afterwards.

2.4. Cell viability assays

The MTT [3-(4, 5-dimethylthiazol-2-yl)−2, 5-diphenyltetrazolium bromide] dye was used to measure cell viability. To achieve this, 10 % of the volume per well of the MTT working solution (5 mg/mL) was added to each well then incubated for 4 h, after which the growth medium was aspirated, and the formed formazan crystals were solubilised by adding 100 μL of DMSO to each well before reading the results with a PolarStar Omega (BMG Labtech Omega® POLARStar) microplate reader at an absorbance of 570 nm, to quantify the intensity of the dissolved formazan crystals (purple colour). Upon obtaining absorbance, cell viability was expressed as percentages of the control:% of cell viability = Absorbance of treated cells X 100 Absorbance of control

2.5. Trypan blue exclusion assay

This assay was also carried out to determine the effects of various treatments on cell viability. Due to compromised cell membrane integrity, Trypan blue dye selectively enters only the dead cells, binds to intracellular proteins, and stains cells blue. On the other hand, live cells do not pick up the dye and can be distinguished from dead cells (Aslantürk, 2018). For this assay, cells were seeded at a density of 2.5×10^5/dish in 35 mm dishes and treated for 24 h, with the selected concentrations of all three extracts (BD, BB and ST), as was done for the neuroprotection studies. After treatment, cells were detached by trypsinisation, centrifuged, and the pellet resuspended in a fresh growth medium. From the cell suspension, 20 μL was transferred to an Eppendorf tube, to which an equivalent volume of 0.4 % trypan blue dye (Sigma-Aldrich, St Louis MO, USA) was added and loaded onto the counting chambers of a haemocytometer after which the percentage of live cells was determined.

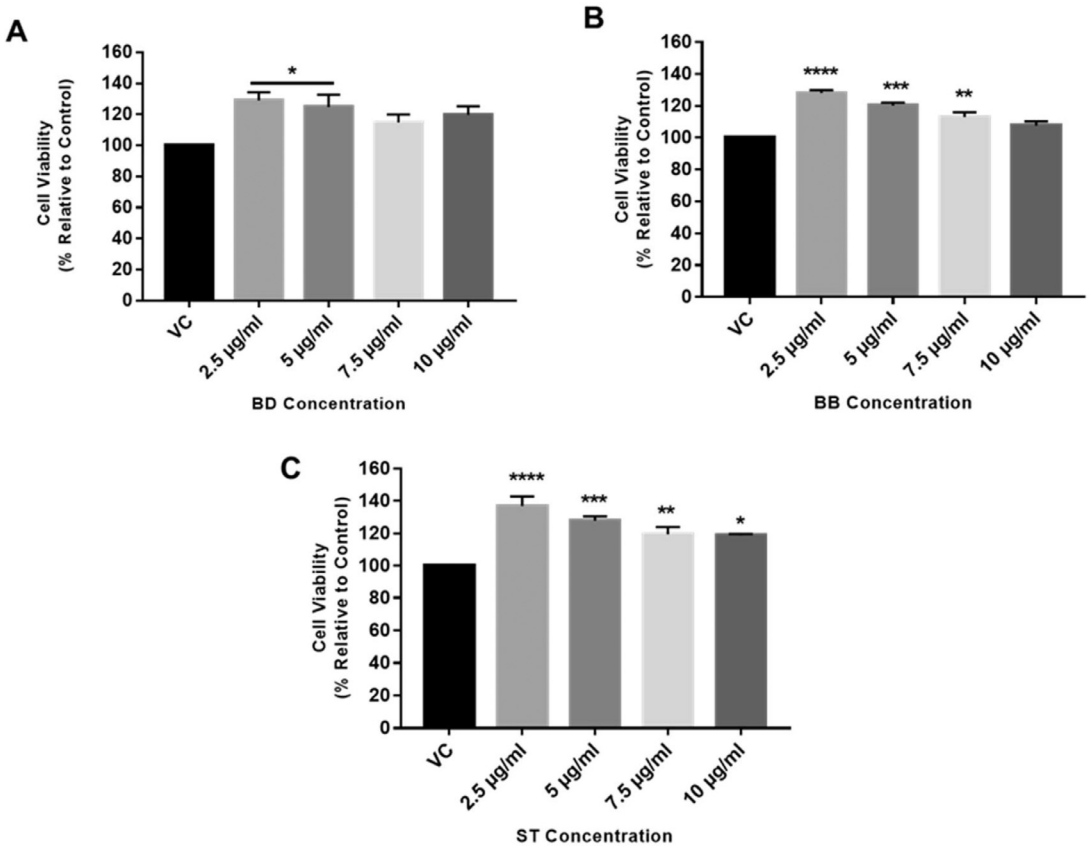


Fig. 1. Dose-response of plant extracts. SH-SY5Y cells were exposed to concentrations 2.5, 5, 7.5 and 10 μg/mL of BD (A), BB (B) and ST (C), and cell viability was determined after 24 h using the MTT assay. Each bar represents the mean ± SEM of three experiments (n = 3), and statistical significance was denoted by asterisks (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$). VC = vehicle control.

2.6. Changes in cell morphology

To investigate any changes in cell morphology, the SH-SY5Y cells were seeded in 96-well plates and treated with the selected concentrations of all three extracts and that of MPP^+as per neuroprotection assay. Post-treatment, the cells were visualised using the 10X objective lens of a Zeiss inverted light microscope and the Zeiss software Version 2.3 was used to obtain the images of the control and treated cells.

2.7. Measurement of intracellular ROS production

To measure the levels of intracellular ROS, the cell-permeable molecular probe dichlorodihydrofluorescein diacetate (DCFDA; Sigma-Aldrich, St Louis MO, USA) was used, and the protocol by Ibrakaw and colleagues was followed, with slight modifications (Ibrakaw et al., 2020). Cells were seeded at 10,000 cells/well in a black 96-well microplate and treated for 24 h as described in the neuroprotection section. After treatment, hydrogen peroxide (10 % H_2O_2) was added to the positive control cells for 10 min. After that, cells were washed with PBS before adding 100 μL of 20 μM DCFH-DA to each well and incubated for an hour at 37 °C. Post incubation, cells were washed gently with PBS, followed by measurement of the dye fluorescence intensity using a microplate reader (BMG Labtech Omega® POLARStar) at excitation wavelength 485 nm and emission wavelength 538 nm and ROS production was expressed as a percentage of control.

2.8. Measurement of nitric oxide production

Nitric oxide production was investigated using the Griess reagent system kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cells were seeded at 10,000 cells/well and treated as described in the neuroprotection section. Cell-free

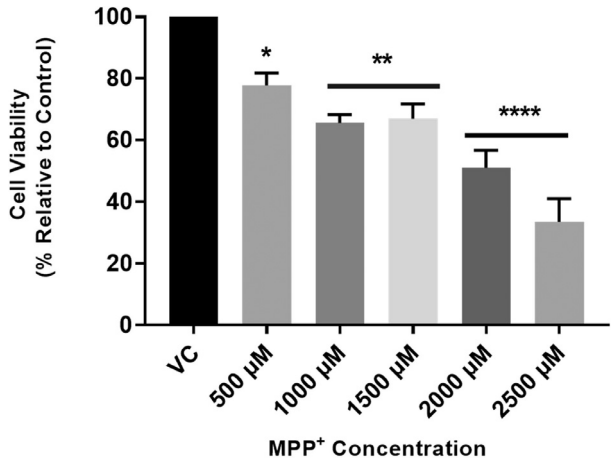


Fig. 2. Cytotoxicity of MPP+. The toxicity of MPP^+ on the SH-SY5Y cells was evaluated using the MTT assay following a 24-hour exposure. The control cells were left untreated, and the graph was presented as mean ± SEM of three experiments (n = 3) using GraphPad Prism 7 statistical software. The statistically significant changes are denoted by asterisks (* $p < 0.05$; ** $p < 0.01$, and ****$p < 0.0001$).

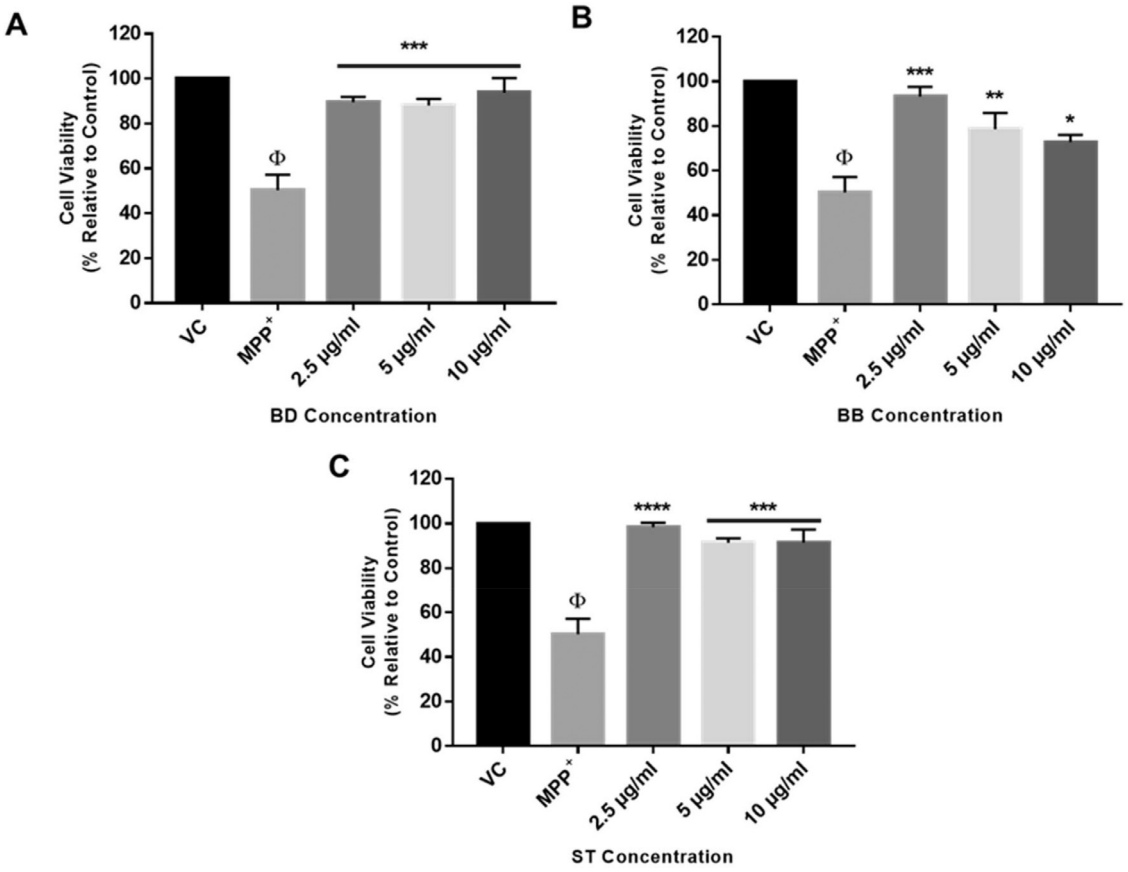


Fig. 3. Evaluation of the neuroprotective capabilities of the plant extracts (A) BD, (B) BB, and (C) ST on the viability of the SH-SY5Y cells following a 24-hour exposure and evaluated using the MTT cell viability assay. Each bar represents the mean ± SEM of three experiments (n = 3). The statistically significant changes are denoted by asterisks (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$) when extracts were compared with MPP^+-treated cells and Φ when control cells were compared with MPP^+-treated cells.

supernatants from all experimental samples were then transferred to a new plate in triplicates at a volume of 50 μL/well, after which plates were handled carefully according to the manufacturer's manual, and measurement of absorbance was done using a microplate reader (BMG Labtech Omega® POLARStar) at a wavelength of 540 nm.

2.9. Measurement of adenosine triphosphate levels

A Mitochondrial ToxGlo ATP assay kit (Promega, Madison, WI, USA) was used according to the manufacturer's instructions to investigate the changes in cellular ATP levels. Briefly, cells were seeded at 10,000 cells/well in a white 96-well microplate and treated for 24 h as described in the neuroprotection section. After that, cells were handled according to the manufacturer's manual and luminescence intensity was measured using a microplate reader (BMG Labtech Omega® POLARStar) at a wavelength of 520 nm.

2.10. Caspase 3/7 apoptosis assay

The caspase 3/7 assay kit (Promega, Madison, WI, USA) was used to investigate apoptosis in the cells, according to the manufacturer's instructions. Briefly, cells were seeded at 10,000 cells/well in a white 96-well microplate and treated as described in the neuroprotection section for 24 h. With the wells having a total volume of 200 μL, 100 μL was discarded, and an equal volume of the Caspase-Glo 3/7 reagent solution was added per well. The microplate was placed on a plate shaker for 30 s at 300–500 rpm to mix the contents gently and then incubated at room temperature for 1 hour, after which luminescence was measured using a microplate reader (BMG Labtech Omega® POLARStar). Values were expressed as percentages of control.

2.11. Autophagy assay

The autophagy assay kit (Sigma-Aldrich, St Louis, MO, USA) was used according to the manufacturer's instructions to investigate autophagy in the cells. Briefly, cells were seeded at 10,000 cells/well in a black 96-well microplate and treated for 24 h, as described in the neuroprotection section. After that, the cell growth medium was discarded and replaced with 100 μL/well of the autophagosome detection reagent working solution. The plate was incubated for 1 hour at 37 °C, after which cells were gently washed thrice. Fluorescence intensity was measured using a microplate reader (BMG Labtech Omega® POLARStar) at an excitation wavelength of 360 nm and an emission wavelength of 520 nm.

2.12. Statistical analysis

All experiments in this study were performed in triplicates, and data obtained were presented as means ± standard error of the mean (SEM). Statistical analyses were done with a one-way analysis of variance test followed by a post hoc analysis (Turkey's multiple comparison tests) using GraphPad Prism 7 statistical software GraphPad Software, Inc, San Diego, California. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxicity screening of plant extracts to determine the optimum concentration

To determine the optimum concentrations of extracts that will confer neuroprotection, the cytotoxicity effects of extracts were evaluated on the SH-SY5Y cells using the MTT assay after 24 h of

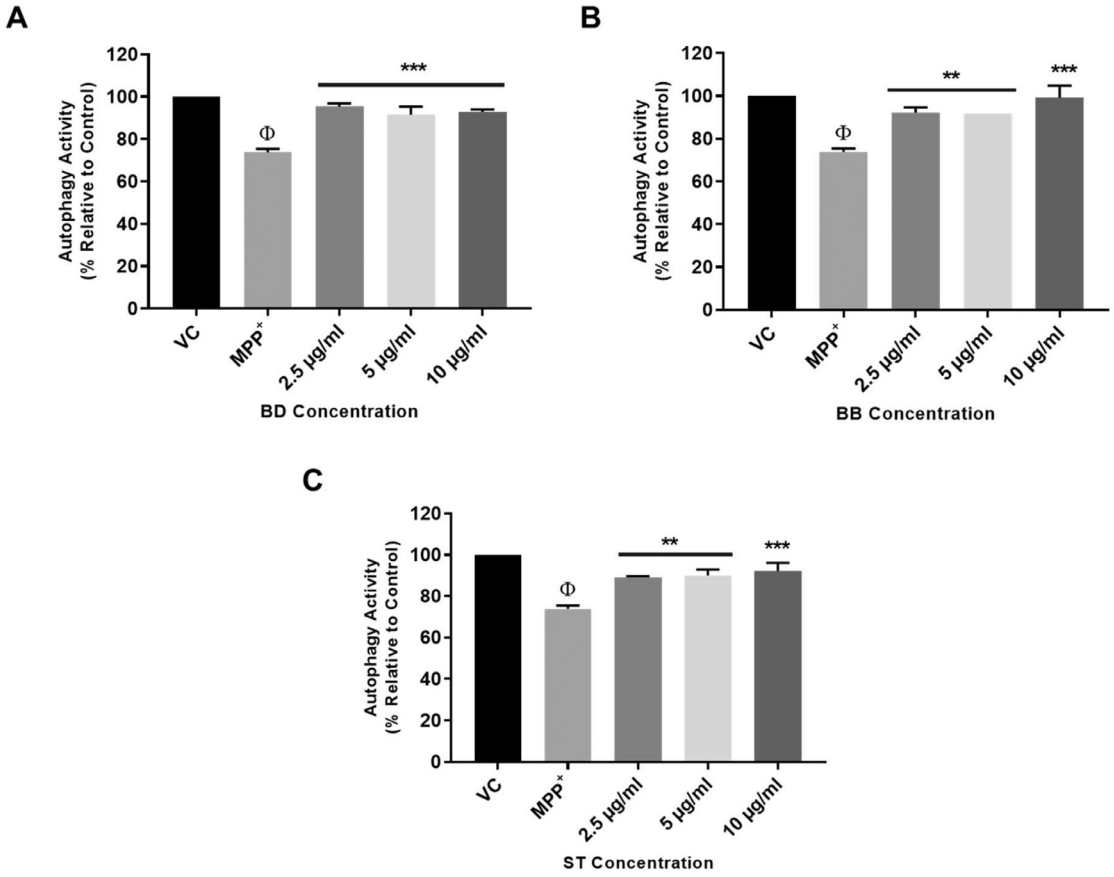


Fig. 10. Impact of treatments on autophagy. The cells were pre-treated with BD (A), BB (B) and ST (C) before exposure to 2000 μM MPP^+, and autophagy was determined using the autophagy detection kit. The graphs were prepared as mean ± SEM of three experiments ($n = 3$) using GraphPad Prism 7 statistical software. The statistically significant changes are denoted by asterisks (** $p < 0.01$, and *** $p < 0.001$) when extracts were compared with MPP^+-treated cells and Φ when controls were compared with MPP^+-treated cells.

Declaration of competing interest

The authors declare there are no competing interests.

CRediT authorship contribution statement

Tusekile S. Kangwa: Writing – original draft, Investigation, Formal analysis. **Donavon C. Hiss:** Resources, Project administration. **Ahmed A. Hussein:** Resources. **Okobi E. Ekpo:** Conceptualization, Writing – review & editing, Supervision. **Sylvester I. Omoruyi:** Conceptualization, Methodology, Formal analysis, Validation, Writing – review & editing, Supervision.

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