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Research Article

Preliminary investigation of the neuroprotective potentials of *Crossyne guttata* in MPP$^+$-induced toxicity in SH-SY5Y

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ABSTRACT

Background: Parkinson's disease (PD) is a movement disorder caused by the gradual and sustained loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). There is presently no permanent treatment for PD, thus the need for more investigations into complementary and alternative medicine capable of inhibiting neuronal damage. This study investigates the potential neuroprotective activity of *Crossyne guttata*, a plant commonly found in the western and southern Cape of South Africa in 1-methyl-4-phenylpyridinium (MPP$^+$)-induced, *in vitro* model of PD, using the SH-SY5Y neuroblastoma cells. **Methodology:** The optimal concentration of *Crossyne guttata* aqueous extract (CGE) that showed no toxicity on cells and the concentration of MPP$^+$ that reduced cell viability to about 50% was determined using the 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Following this SH-SY5Y cells were pretreated with 10 µg/ml of CGE for 2 hours before the addition of 2000 µM of MPP$^+$ and cell survival was determined. Furthermore, morphological changes associated with treatments were observed under the light microscope. **Results:** Results show that CGE did not reduce cell viability in the cells at all concentrations tested, while MPP$^+$ showed a dose-dependent reduction in cell viability. Also, pretreatment of cells with CGE extract improved cell survival as well as cell morphology by inhibiting toxicity induced by MPP$^+$. **Conclusion**: Findings from this study suggest that CGE may be a potential neuroprotective agent in PD and thus make a case for further investigation into the mechanism(s) of action as well as bioactive components of the plant eliciting such effects.

INTRODUCTION

Neurodegenerative diseases (NDDs) play roles in mortality and morbidity especially among older people. NDDs are a heterogeneous group of diseases, defined by the gradual reduction of neuronal cell viability as well as quantity of these cells (Pentón-Rol and Cervantes-Llanos, 2018). One of the common NDDs is Parkinson's diseases among old people with about 1-2% of older population over the demonstrating some signs of PD (Wood et al., 2010). Parkinson's disease (PD) is a neurodegenerative disorder that involves a loss of neurons in a region of the midbrain referred to as the substantia nigra pars compacta (SNpc) leading to motor and cognitive impairments (Dauer and Przedborski, 2003, Wirdefeldt et al., 2011, Dexter and Jenner, 2013). A neuropathological hallmark of PD is the presence of Lewy bodies in neurons, cytosolic inclusions rich in fibrillar forms of the presynaptic protein α-synuclein (aSyn) (Yasuda and Mochizuki, 2010, Del Tredici and Braak, 2012, Macphee and Stewart, 2012).

At the molecular level, PD is characterized by an impairment of the activity of mitochondria complex I, an enzyme critical for the progression of the mitochondria electron transport chain (Winklhofer and Haass, 2010, Choi et al., 2011). Following the inhibition of complex, there is increased loss of ATP and generation of reactive oxygen species (ROS) which results in accumulation of aSyn aggregates around neurons and this in turn, lead to cell death (Büeler, 2010, Perier et al., 2012). This is particularly heightened as dopaminergic neurons have been reported to have high basal amount of ROS (Betarbet et al., 2000).

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Several cellular models have been used to investigate PD both in vitro and *in vivo* with the aid of neurotoxins (Beal, 2001, Sherer et al., 2003, Bové et al., 2005). A notable neurotoxin is the 1-methyl-4-phenylpyridinium (MPP^+), which is the toxic metabolite of 1-methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP), a by-product in the synthesis of 1-methyl-4-phenyl-4-propionoxy-piperidine (MPPP) (Wolf, 2013). MPPP which is a synthetic analogue of a commonly abused substance heroin, triggers parkinsonism in humans when injected intravenously (Langston et al., 1983). MPTP is converted to MPP^+ by monoamine oxidase B in non-neuronal cells, such as glial cells and astrocytes and thereafter, MPP^+ induces loss of dopaminergic neurons via a cascade of events leading to ROS generation and ultimately, cell death (Ransom et al., 1987, Brooks et al., 1989, Lotharius and O'Malley, 2000).

There is presently no permanent cure for PD as current therapies including L-dopa and carbidopa act by controlling disease symptoms but do not put a stop to underlying degeneration of dopaminergic neurons or reverse this process (Connolly and Lang, 2014, Zigmond and Smeyne, 2014). Despite the breakthroughs recorded in the development of synthetic drugs for NDDs, traditional herbal medicine is still of value in most part of the world for the treatment of disease conditions, owing to their ready availability and cheap cost (Kong et al., 2003, Bandaranayake, 2006, WHO, 2005)

Medicinal plants provides good source of treatment for several illnesses such as neurodegenerative diseases, diabetes mellitus and cancers (Ekor, 2014). Africa, especially Southern Africa has a rich diversity of plants; approximately 25 % of the total number of plants in the world, hailing from sub-Saharan Africa (Van Wyk, 2008). South Africa has a rich flora and a lot of people rely on traditional herbal medicine as alternative therapy due their accessibility. Many plants used as traditional herbal medicine contain compounds such as polyphenols, flavonoids, alkaloids and terpenes which are capable of scavenging free radicals (Rates, 2001, Tiwari, 2008). It is therefore important for medicinal plants to be investigated for their potential treatment of NDDs, especially in most African countries.

Crossyne guttata is an indigenous plant of the Cape Province belonging to the Amaryllidaceae family. The leaf margins are completely fringed with straight short. To the best of our knowledge, there is so far, no report on the biological activities of this plant hence we provide first evidence on the possible biological effects of the plant. This study therefore aims to investigate the potential neuroprotective effects of *Crossyne guttata* in an in vitro MPP^+-SH-SY5Y cellular model of PD.

METHODS

Sample collection and extract preparation

Fresh leaves of *Crossyne guttata* were harvested, washed and air-dried to remove moisture content. Thereafter, the dried leaves were reduced to powder and about 1 kg of the powdered leaves were soaked in boiling water and left overnight for extraction. The resultant extract was thereafter filtered with Whatmann filter paper and the filtrate evaporated in a freeze dryer to obtain a dried powder aqueous *Crossyne guttata* extract (CGE) which was stored at 8 °C until needed. A portion of CGE was weighed and dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis MO, USA) to give a stock solution of 40 mg/ml from which dilutions in media were made for treatment of cells each day of experiment.

Cell culture

The human neuroblastoma SH-SY5Y cells were generously donated by the Blackburn Laboratory, University of Cape Town. Cells were grown 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), 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza Group Ltd., Verviers, Belgium). Cultures were incubated at 37 °C in humidified air with 5% CO_2 with a medium change every three days. Cells were sub-cultured when they attained 70 to 80 percent confluency using a solution of of 0.25 % trypsin EDTA (Lonza Group Ltd., Verviers, Belgium).

Cytotoxicity screening

The MTT assay was performed to determine the optimal concentration of CGE that had no adverse effect on cell viability and the concentration of MPP^+ (Sigma-Aldrich, St Louis MO, USA) that induced significant toxicity to SH-SY5Y cells. SH-SY5Y cells were plated at a density of 100 cells/well and exposed to increasing concentrations of CGE ranging from 10, 20, 30, and 40 µg/ml and vehicle (amount of DMSO in the highest concentration of extract) treated cells served as control. For MPP^+, a 50 mM stock solution was prepared in un-supplemented DMEM and further dilutions were made in supplemented growth medium to arrive at final concentrations of 1000 to 2500 µM which were introduced to cells, and cells not exposed to MPP^+ served as control. All treatments lasted for 24 hours.

Cell viability

Cell viability was evaluated after treatments as stated above using the MTT (Sigma-Aldrich, St Louis MO, USA) assay. This assay determines mitochondrial

function of living cells on the basis of their ability to reduce the yellow MTT tetrazolium salt into a purple formazan crystal mainly by the mitochondrial dehydrogenases. For neuroprotection experiment, cells were plated in 96 well plates at a density of 10 000 cells/well and were left overnight to attach. Thereafter, cells were pretreated for 2 hours with 10 µg/ml of CGE followed by the addition of 2000 µM MPP^+. These concentrations were obtained from the cytotoxicity screening and after 24 hours, 20 µL of the MTT stock solution (5mg/mL in PBS) was added to each well and left to incubate at 37°C for 4 hours in the dark. At the end of the incubation, the medium containing the MTT dye was removed and the resulting purple formazan formed was solubilized with 100 µL of DMSO. After that, absorbance was measured using a microplate reader (BMG Labtech Omega® POLARStar) at a wavelength 570 nm and percentage cell survival was calculated relative to control.

Cell morphology

Cells were plated and treated as stated above in the neuroprotection experiment. At the end of the treatment, cell morphology was analyzed in the various treatment conditions using the Zeiss inverted light microscope with 10X objective lens. Images were captured using the Zeiss software version 2.3.

Statistical analysis

Data generated from this study were analyzed using GraphPad Prism 6 and expressed as means and standard error of means of three independent experiments performed in quadruplicate wells. One-way analysis of variance (ANOVA) was used to determine level of significance and data set with p value less than 0.05 was considered significant.

RESULTS

Effect of CGE on cell survival of SH-SY5Y cells

The effects of CGE on cell survival was determined using the MTT assay by exposing SH-SY5Y cells to increasing concentrations of extract (10 to 40 µg/ml) for 24 hours. The results show that at all concentrations tested, CGE had no effect on cell viability (**Fig. 1**); however, the 10 µg/ml concentration was chosen for further studies as it improved cell viability slightly above the control treated cells albeit not significant. Taken together, CGE had no adverse effect on SH-SY5Y cell survival at the concentrations tested.

Cytotoxicity of MPP^+ on SH-SY5Y cells

To ascertain the effective concentration of MPP^+ that induced less than 50% neuronal cell death, cells were

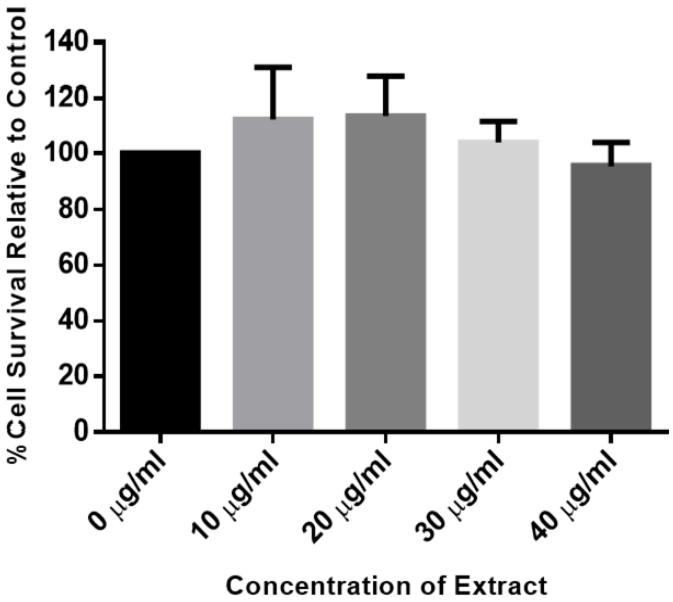

Fig. 1. Effect of CGE on SH-SY5Y cells. Cells were exposed to concentrations of 10 µg/ml, 20 µg/ml, 30 µg/ml and 40 µg/ml for 24 hours. Percentage cell survival was determined using the MTT assay and expressed relative to the control. Data represent the mean ± SEM of at least three independent experiments.

Cytotoxicity of MPP^+ on SH-SY5Y cells

To ascertain the effective concentration of MPP^+ that induced less than 50% neuronal cell death, cells were treated with 500, 1000, 1500, 2000 and 2500 µM of MPP^+ for 24 hours. Results obtained shows that at the lowest concentration of MPP^+ (500 µM), there was no significant change in cell viability of SH-SY5Y cells when compared to control. In contrast, at higher concentrations (1000-2500 µM), MPP^+ significantly decreased cell viability when compared to control (Fig. 2). However, 2000 µM of MPP^+ was selected for further experiments as it reduced cell survival to approximately fifty percent (50%).

Crossyne guttata extract mitigate MPP^+-induced toxicity in SH-SHY5Y cells

To investigate the neuroprotective effects of CGE in MPP^+ induced neuronal toxicity, SH-SY5Y cells were pretreated with 10 µg/ml of CGE for 2 hours followed by the addition of 2000 µM of MPP^+ for 24 hours. Fig. 3 shows that pretreatment of SH-SY5Y cells with CGE mitigated against MPP^+-induced toxicity. Indeed MPP^+ reduced cell survival to 47.3%, while pretreatment with CGE reversed the effect of MPP in the cells as cell survival rose to 78.1%. As expected, cell viability of CGE treated cells was similar to control. Together, these findings suggest that CGE may be effective in protecting neuronal cells from MPP^+-induced toxicity.

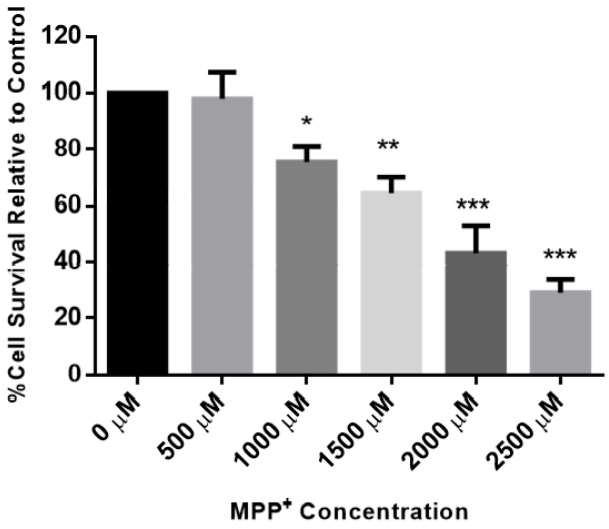

Fig. 2. MPP^+ induce cytotoxicity on SH-SY5Y cells. Cells were exposed to concentrations of 500 μM, 1000 μM, 1500 μM, 2000 μM and 2500 μM for 24 hours. Percentage cell survival was determined using the MTT assay and expressed relative to the control. Data represent the mean ± SEM of at least three independent experiments.

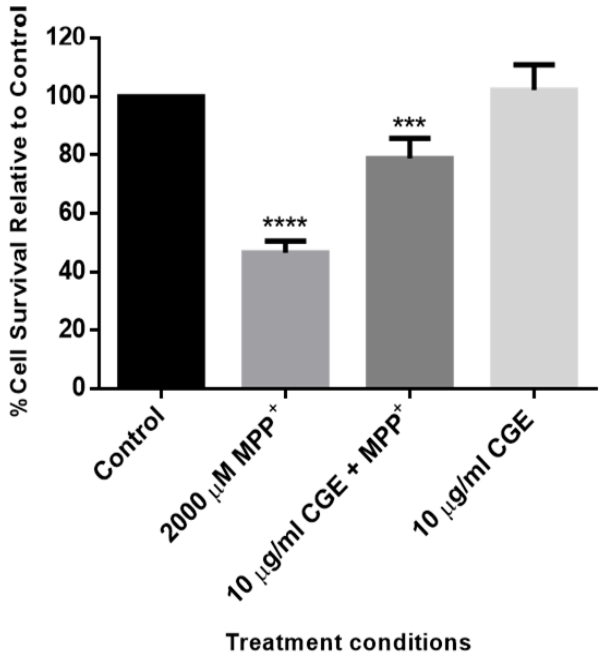

Fig. 3. CGE protects SH-SY5Y cells from MPP^+-induced toxicity. Cells were pretreated with 10 μg/ml for 2 hours followed by the introduction of 2000 μM MPP^+ as well as 10 μg/ml of CGE for 24 hours. Percentage cell survival was determined from MTT assays and expressed relative to the control. Each bar represents mean ± SEM of at least three independent experiments performed in quadruplicates.

survival rose to 78.1%. As expected, cell viability of CGE treated cells was similar to control. Together, these findings suggest that CGE may be effective in protecting neuronal cells from MPP^+-induced toxicity.

CGE inhibits morphological changes induced by MPP^+

In order to visualize morphological changes in SH-SY5Y cells following treatments, cells were treated as stated above for neuroprotection and viewed under the light microscope. Images obtained show that compared to control, MPP^+ led to changes in cell morphology which could be reminiscent of a cell undergoing programmed cell death (Figs 4 A and B). These cellular changes include: cell shrinkage as well as loss of neuronal projections; however, following pretreatment with CGE, an improvement of cell morphology was evident (Fig. 4 C). As expected cells exposed to CGE alone showed no remarkable change in morphology when compared to control (Fig. 4 D). These findings suggest that CGE protects SH-SY5Y cells from morphological changes induced by MPP^+.

DISCUSSION

Parkinson's disease is the second most common neurodegenerative disorder next to Alzheimer's disease (Elbaz et al., 2016). The exact cause of Parkinson's disease is unknown, but it is assumed to be the result of a combination of environmental influences superimposed on genetic predisposition or susceptibility (Lees et al., 2009). Since PD is clinically incurable, due to the multiple pathologies associated with the disease,if untreated, approximately 80% of patients diagnosed of the disease become severely disabled after 20 years of onset (Iranzo et al., 2013, Connolly and Lang, 2014, Elbaz et al., 2016). Thus, it is quite important to develop strategies capable of delaying the onset of the disease. The present study investigated the neuroprotective activity of CGE on MPP^+-induced neuronal toxicity on SH-SY5Y cells.

The MPP^+-SH-SY5Y cells *in vitro* PD model is widely used for investigating potential neuroprotective agents and mechanism of neurodegeneration (Aymerich et al., 2016, Zhu et al., 2018, Chanthammachat and Dharmasaroja, 2019). MPP^+, upon transportation into cells by the dopamine transporter, is concentrated in the mitochondria, where it binds NADH dehydrogenase to inhibit the electron transport chain (Ramsay et al., 1991). MPP^+ selectively damages dopaminergic neurons in the substantia nigra of the brain due to its inhibitory effect on mitochondria complex I, which in turn, reduces ATP synthesis and accumulation of ROS (Trist et al., 2019). These cascade of events in the dopaminergic neurons eventually lead to cell death which shows in clinical settings as PD (Büeler, 2010, Perier et al., 2012).

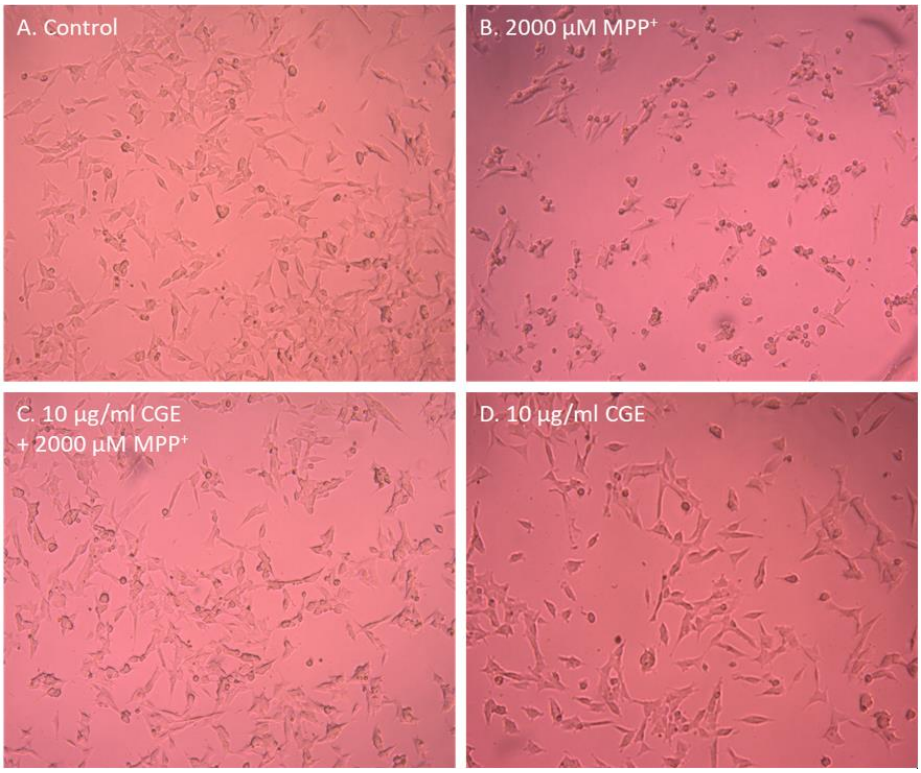

Fig. 4. Morphological changes associated with treatment. (A) Untreated cells (B) Cells treated with 2000 µM MPP^+ (C) Cells pretreated with 10 µg/ml CGE and exposed to 2000 µM MPP^+ (D) Cells exposed to 10 µg/ml CGE alone. Images were visualized under the light microscope using the 10X objective.

In the current study, we show for the first time that CGE mitigates MPP^+-induced cytotoxicity in SH-SY5Y cells and also restores cell morphology in the cells exposed to the toxin. Morphological changes in MPP^+ treated cells could be reminiscent of cells undergoing apoptosis but this needs to be confirmed with assays investigating apoptosis; however, induction of apoptosis is widely known as one of the mechanisms of action of MPP^+-induced neuronal toxicity in PD models (Zhao et al., 2016, Jovanovic-Tucovic et al., 2019, Shishido et al., 2019).

While the mechanisms of action of CGE was not characterized in this study, it is however worthy to note that plant extracts with antioxidant activities have been reported to attenuate MPP^+-induced toxicities in PD models (Abushouk et al., 2017). *Zizyphus spina-christi* fruits, *Psephellus pyrrhoblepharus* and ginseng extract have been reported to inhibit MPP^+-induced ROS generation and apoptosis in SH-SY5Y cells (Hu et al., 2011, Singh et al., 2018, Taştan et al., 2019).

CONCLUSION

This study investigates the potential neuroprotective activity of CGE extract in MPP^+-SH-SY5Y *in vitro* model of PD. Findings suggest that CGE may be effective in the protection of neuronal cells exposed to MPP^+; however, the potential mechanism(s) of action of CGE on PD models should be investigated and the possible bioactive compounds promoting neuroprotection elucidated.

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