

Research Article

Antioxidant and apoptosis-inhibition potential of *Carpobrotus edulis* in a model of parkinson's disease

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Keywords:

Parkinson's disease, *Carpobrotus edulis*, SH-SY5Y cells, 1-methyl-4-phenylpyridinium, reactive oxygen species, apoptosis

ABSTRACT

Background: Parkinson's disease (PD) is a neurological disorder resulting from the progressive loss of dopaminergic neurons. There is currently no known cure for PD, thus the search for complementary and alternative medicines capable of halting the degeneration of dopaminergic neurons is plausible. *Carpobrotus edulis* (CE) is an indigenous plant used in South African traditional medicine used for the treatment of a number of disease conditions including tuberculosis, diabetes mellitus and constipation. It has been suggested that CE contains bioactive compounds which are responsible for its acclaimed medicinal potential. No studies have been reported on the potential benefit of CE to the nervous system. This study was therefore done to evaluate the protective effects of CE against 1-methyl-4-phenylpyridinium (MPP⁺)-induced toxicity in the dopaminergic SH-SY5Y cell line, as well as its underlying mechanism. **Methods:** In this study, SH-SY5Y cells were treated with varying concentrations of CE and MPP⁺ respectively to determine the optimal concentrations of MPP⁺ and CE for further experiments. Thereafter, SH-SY5Y cells were pre-treated with 30 μM of CE before exposure to 2 mM of MPP⁺ to induce cellular damage. Cell viability was evaluated using the MTT assay, intracellular reactive oxygen species (ROS) production was determined using flow cytometry and the Hoechst nuclear staining was used to visualize apoptosis. Caspases 3/7 and 9 activity was assessed using commercially available kits. **Results:** MPP⁺ treatment induced marked cell viability, increased the number of condensed nuclei and apoptotic cells, increased ROS production, initiated caspase 9 and activated caspase 3/7 in SH-SY5Y cells. The observed effects of MPP⁺-induced toxicity were attenuated by the pre-treatment of SH-SY5Y cells with 30 μM of CE. **Conclusion:** The protective effects of CE against MPP⁺-induced toxicity in SH-SY5Y cells may be attributed to its antioxidant and anti-apoptotic properties.

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INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the pars compacta part of the substantia nigra (SNpc) of the midbrain. Its clinical motor symptoms include muscle rigidity, bradykinesia and tremor, while non-motor symptoms include a variety of neuropsychiatric disorders (Magrinelli et al., 2016, Sveinbjornsdottir, 2016). Presently, there is no cure for PD (Hu et al., 2010), as such drug treatments like L-dopa and

carbidopa are only able to alleviate PD symptoms but are unable to halt the degeneration of dopamine neurons or stop the advancement of the disease (Connolly and Lang, 2014). Consequently, the search for new PD therapies has led to widespread research into complementary and alternative medicines of therapeutic value (Doo et al., 2010).

Previous studies have reported the beneficial effects of herbal medicines in preventing dopaminergic neuronal degeneration (Iriti et al., 2010), and the World Health Organization is currently encouraging research on the identification and valorisation of medicinal plants, which are known to be important sources of molecules and/or products in traditional and modern medicine (Rocha et al., 2017). In this regard, herbal plants and their phytochemicals have been proposed as novel

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neuroprotective treatments for several neurodegenerative disorders. A notable plant in this regard is *Carpobrotus edulis* (CE), an edible medicinal plant native to the coast of South Africa (Rocha et al., 2017). CE is known by the name *Igcukuma* among the Xhosa communities of South Africa and is commonly used by the traditional healers to treat diabetes mellitus, constipation, sores, elevated blood pressure, tuberculosis and intestinal worms (Omoruyi et al., 2012). This plant is widely reported to exhibit several biological activities including antioxidant, immunomodulating, antimicrobial; anticholinesterase and antitumoural effects (Martins et al., 2010, Martins et al., 2011, Custódio et al., 2012, Omoruyi et al., 2012). The bioactive molecules already isolated from CE include rutin, oleanolic acid and cactichin (van der Watt and Pretorius, 2001, Martins et al., 2010, Martins et al., 2011).

Cellular neuronal model systems have long been utilized to investigate the mechanisms of neurodegeneration and for the identification of novel neuroprotective agents in PD. In the present study, the human SH-SY5Y neuroblastoma cell line was used based on its reported ability to express tyrosine hydroxylase and to display adequate dopamine- β -hydroxylase activity, which is a peculiar characteristic of dopaminergic neurons (Ross et al., 1983). In addition, the SH-SY5Y cells are known to exhibit some properties of stem cells and to express the dopamine transporter, DAT, which is responsible for the control of dopamine equilibrium via precise uptake and sequestration of dopamine (Takahashi et al., 1994). DAT is the access point for such neurotoxins as 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (MPP⁺) into neurons, consequently making SH-SY5Y cells an appropriate model for studies utilizing toxin-induced PD.

MPP⁺ has been widely utilized as a neurotoxin to investigate PD mechanisms owing to its selective toxicity to dopaminergic neurons (Nakamura et al., 2000). In astrocytes, the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is oxidized by monoamine oxidase (MAO)-B into its active metabolite, MPP⁺ which is then transported into the dopaminergic neurons via the DAT to accumulate in mitochondria. Here, MPP⁺ effectively inhibits complex I of the electron transport chain to trigger a condition that is similar to PD in experimental models (Singer and Ramsay, 1990, Przedborski and Jackson-Lewis, 1998). Evidence in literature suggests that MPP⁺-triggered cell death involves multiple mechanisms (Soldner et al., 1999, Nakamura et al., 2000). For example, MPP⁺ is reported to produce an increase in

reactive oxygen species (ROS) and oxidative damage in various PD models (Cassarino et al., 1997). Also, studies on animal models of PD showed that MPP⁺-induced oxidative damage was inhibited by the overexpression of antioxidant enzymes, while another study showed that antioxidant molecules attenuated MPP⁺ toxicity in *in vitro* PD models (Przedborski et al., 1992, Lai et al., 1993). In the same light, MPP⁺-triggered cell death has been found to exhibit features of apoptosis (Marini et al., 1989, Dipasquale et al., 1991). Although MPP⁺-induced apoptotic cell death is mediated by the initiation of caspase 9 and 8, as well as the activation of caspase 3, it is attenuated by the caspase inhibitors (Marini et al., 1989). Therefore, this study aims to evaluate the neuroprotective activity of CE against MPP⁺-induced oxidative damage and apoptosis in SH-SY5Y neuroblastoma cells.

METHODS

Preparation of extract

Small twigs of CE were obtained from the University of the Western Cape nature reserve, air-dried at room temperature and the dried plant parts then reduced to powder and extracted overnight with 1 litre of de-ionized boiling water. The resultant extract was then filtered and freeze-dried to give a powdered aqueous extract.

Cell culture maintenance

SH-SY5Y neuroblastoma cells, generously donated by Dr AM Serafin (Division of Radiobiology, Stellenbosch University, South Africa), 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 and 5% CO₂ in a humidified incubator. The culture media was replaced every 2-3 days and cells were trypsinized (1X trypsin-versene EDTA mixture, Lonza, USA) and transferred to another dish for regrowth once it attained confluency of approximately 70-80%.

Cytotoxicity screening

To ensure that SH-SY5Y cells were exposed to the right concentration of MPP⁺ and CE for the appropriate exposure duration, cytotoxicity screening was performed using the MTT assay. A fresh 50mM stock concentration of MPP⁺ was prepared in un-supplemented DMEM, and dilutions were made in culture medium for final concentrations ranging between 0 and 5000 μ M. To evaluate the neuroprotective effects of CE, a fresh 5 mg/ml stock

concentration of the extract was prepared in 1X Phosphate buffered saline solution (PBS, Lonza, USA) and dilutions were made up in culture media for final concentrations ranging between 0 and 50 µg.

Evaluation of cell viability

Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, St. Louis, MO, USA) assay, which indicates the functional integrity of the mitochondria and of cellular viability (Slater et al., 1963, Van de Loosdrecht et al., 1994). SH-SY5Y cells were seeded at a density of 1.0×10^4 cells/well in 96-well plates and were allowed to attach for 24 hours. Cells were thereafter pre-treated with complete culture media supplemented with 30 µM concentrations of CE for 1 hour and then treated with 2 mM of MPP⁺ for 24 hours. These concentrations and exposure durations were selected based on findings from prior cytotoxicity screening. After treatment, 20µl of the MTT solution was added to each well of the 96-well plate for 4 hours, and the Glomax Multi Detection System (Promega, USA) was used to detect the absorbance at a wavelength of 560nm.

Evaluation of cell morphology

SH-SY5Y cells were seeded at a density of 1.1×10^5 /ml in 60 mm dishes and allowed to attach for 24 hours after which the cells were treated as previously described and cell morphology was done using a ZEISS Primo Vert (Germany) light microscope.

Nuclear staining with Hoechst

Hoechst 33342 nucleic acid stain (Life Technologies, USA) was used to demonstrate apoptosis in the treated samples. SH-SY5Y cells were seeded at a density of 1.1×10^5 /ml in 60mm dishes and were allowed to attach for 24 hours under standard incubation conditions. After treatment, experiments were performed according to the manufacturer's instructions and 20µl of cells in the staining solution was placed on a glass slide, followed by cover-slipping and viewing with a Zeiss Axio-plan 2 fluorescent microscope (Zeiss, Germany).

Evaluation of Caspase 9 activity

The ApoTarget™ Caspase-9 Protease Assay (Life Technologies, USA) kit was used to evaluate the activity of caspase-9 in treated and untreated SH-SY5Y cells seeded at a density of 1.2×10^5 /ml in 100mm dishes. The cells were allowed to attach for 24 hours after treatment, and adherent cells were displaced with a cell scraper, transferred to a tube on ice, pelleted in ice-cold PBS at 3000 rounds per minute (rpm) for 5

minutes, re-suspended in 50µL of chilled cell lysis buffer and incubated on ice for 10 minutes. Experiments were performed according to the manufacturer's instructions, and absorbance was detected using a Polarstar Omega plate reader (BMG Labtech, USA) at 405nm.

Evaluation of Caspase 3/7 activity

The Caspase 3/7 assay kit (Promega, USA) was used to determine the activity of caspase 3/7 in treated and untreated samples. SH-SY5Y cells were seeded at a density of 10000 cells/well in white-walled 96-well plates and allowed to attach for 24 hours. After treatment, subsequent experiments were performed according to the manufacturer's instructions, and luminescence was detected using the Glomax Multi Detection System.

Evaluation of reactive oxygen species

The reagent 2',7'-Dichlorofluorescein diacetate (DCFH-DA, St. Louis, MO, USA) was used to evaluate intracellular ROS production in SH-SY5Y cells seeded at a density of 1.2×10^5 /ml in 100mm dishes. Cells were allowed to attach for 24 hours under standard incubation conditions and after treatment, adherent cells were displaced with a cell scraper, spun down using a Bio-Rad table top centrifuge at 3000 rpm for 5 minutes, and cell pellets were resuspended and incubated with DMEM containing 25 µM of DCFH-DA for 45mins. Afterwards, cells were centrifuged for 5 minutes at 3000 rpm, and the pellets were re-suspended in 500µl PBS. Stained cells were acquired and analysed on an Accuri flow cytometer (BD Biosciences Pharmingen, San Diego, CA, USA) and a total of 10000 events were collected for each sample.

Statistical analysis

GraphPad Prism Software V7 was used for all statistical analyses (www.graphpad.com/scientific-software/prism/) and data was expressed as means 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 performed to analyze the relationship between variables. Level of statistical significance was considered at $p < 0.05$.

RESULTS

Dose-response of MPP⁺ and CE toxicity

In order to determine the concentration of MPP⁺ that induced approximately 50% cell death in the SH-SY5Y cells, increasing concentrations of MPP⁺ (1000 µM – 5000 µM) were added to the cells and allowed to

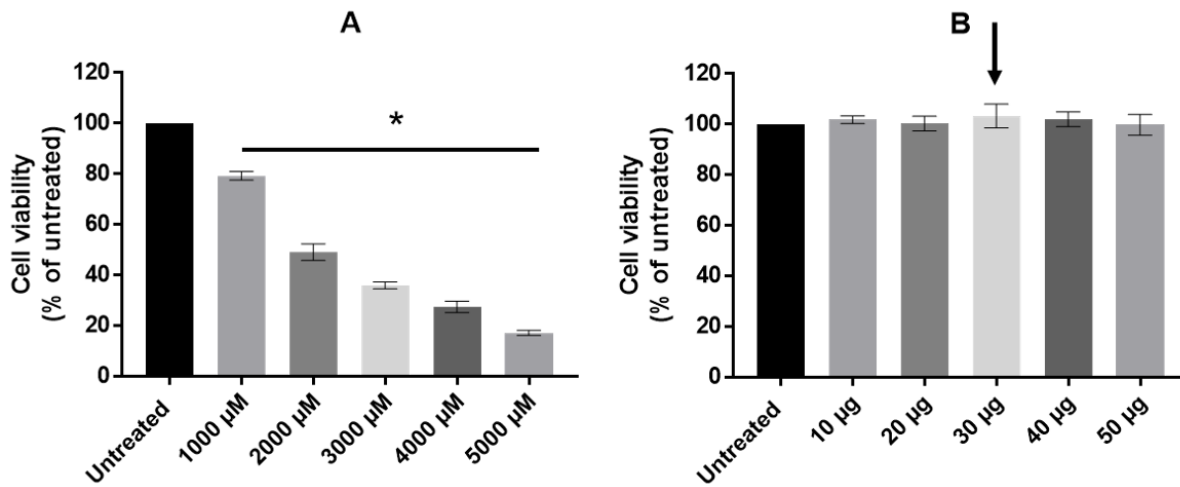


Fig. 1: Cytotoxicity screening in SH-SY5Y cells. (A) MPP⁺ and (B) CE. **p* < 0.0001 vs untreated SH-SY5Y cells.

incubate for 24 hours. Results obtained show significant decrease (*p* < 0.0001, Figure 1A) in cell viability which was concentration-dependent. Approximately 50% cell death occurred at the MPP⁺ concentration of 2000 µM and this concentration was chosen for use in further experiments. On the other hand, exposure of SH-SY5Y cells to increasing concentrations of CE (10 µg - 50 µg) showed no effect on cell viability after 24 hours incubation. Based on the highest absorbance value, a concentration of 30 µg of CE (*p* = 0.9734, Figure 1B) was chosen for further experiments.

CE inhibits MPP⁺-induced reduction in cell viability

To investigate the protective activity of CE, cells were pre-treated with CE before being exposed to MPP⁺. Results showed that whereas treatment with MPP⁺ significantly decreased cell viability (*P* = 0.0001), pre-treatment with CE significantly improved (*p* = 0.0024, Figure 2) cell viability. Also, there was no significant difference (*p* > 0.05) following treatment of SH-SY5Y cells with CE only.

CE prevents MPP⁺-induced changes in morphology

Following visualisation of SH-SY5Y cells under the light microscope, morphological changes linked to cell death in MPP⁺-treated SH-SY5Y cells, were observed including cell contraction/shrinkage and rounding up of cell bodies. Pre-treatment with CE however, significantly inhibited the morphological alterations induced by MPP⁺, and the morphology of cells exposed to CE only, was similar to that of the untreated cells (Figures 3A and 3D respectively).

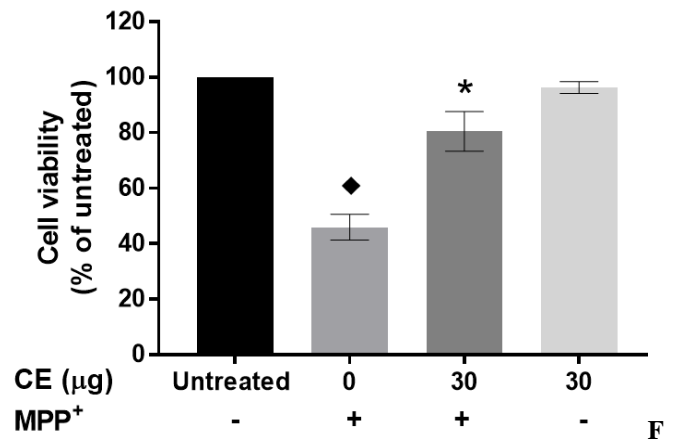


Fig. 2: CE protects cell viability in MPP⁺-treated SH-SY5Y cells ♦ *P* < 0.0005 vs untreated SH-SY5Y cells; **p* < 0.005 vs SH-SY5Y cells treated with MPP⁺ only.

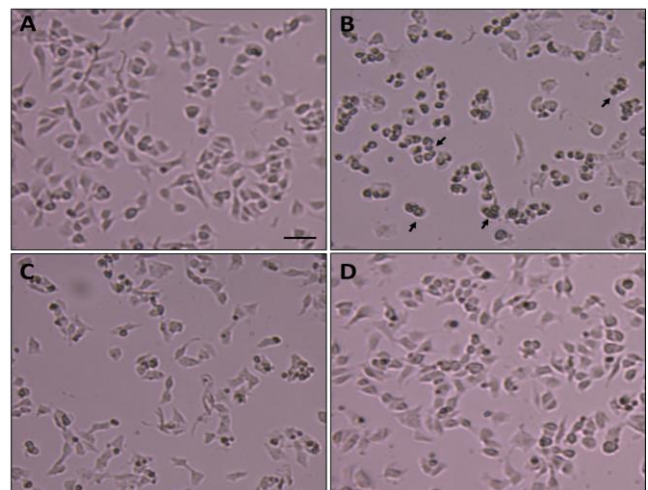


Fig. 3: Morphological changes in SH-SY5Y cells. (A) Untreated SH-SY5Y cells (B) SH-SY5Y cells treated with MPP⁺ only (C) SH-SY5Y cells pre-treated with CE and then treated with MPP⁺ (D) SH-SY5Y cells treated with CE only. Arrows indicate degenerating and shrinking SH-SY5Y cells. Scale bar: 5 µm.

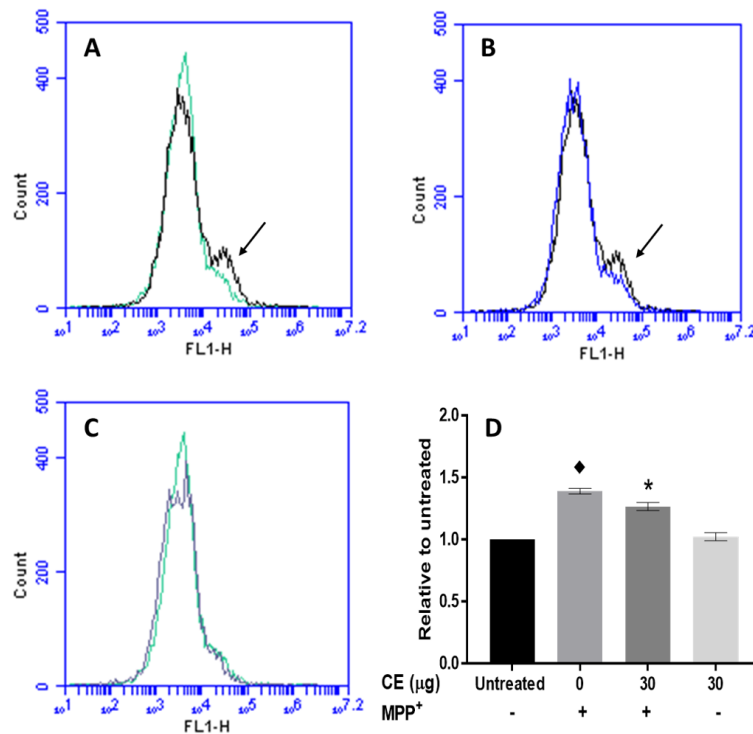


Fig. 4: CE inhibits accumulation of ROS following MPP⁺ toxicity. SH-SY5Y cells treated with MPP⁺ only vs (A) untreated (B) CE pre-treated (C) untreated vs CE (D) ROS production in SH-SY5Y cells. Arrows indicate intensity of cells treated with MPP⁺ only. ♦ $p < 0.0001$ vs untreated SH-SY5Y cells; * $p < 0.05$ vs SH-SY5Y cells treated with MPP⁺ only.

CE mitigates MPP⁺-induced overproduction of ROS

Reports indicate that excessive production of ROS is a principal mechanism for MPP⁺-induced apoptosis and cell death (Pariyar et al., 2017). *CE mitigates MPP⁺-induced overproduction of ROS*

Reports indicate that excessive production of ROS is a principal mechanism for MPP⁺-induced apoptosis and cell death (Pariyar et al., 2017). Accordingly, the possible involvement of antioxidant mechanisms in the protective activity of CE against MPP⁺-induced toxicity was investigated. Results showed that exposure to MPP⁺ significantly increased ($p < 0.0001$, Figure 4) intracellular ROS levels in SH-SY5Y cells, and pre-treatment with the selected CE concentration significantly ($p = 0.0376$) mitigated MPP⁺-induced accumulation of intracellular ROS. Treatment of SH-SY5Y cells with CE only did not significantly ($p > 0.05$) alter intracellular ROS levels when compared to the untreated cells (Figure 4D).

CE inhibits MPP⁺-induced apoptosis in SH-SY5Y cells

To further examine the protective effects of CE on MPP⁺-induced toxicity, morphological changes in cell nuclei were investigated in the presence or absence of

CE using the Hoechst 33342 nucleic acid stain. Results showed that the cells undergoing apoptosis (a form of cell death) in the MPP⁺-treated alone group had more condensed nuclei when compared to the untreated cells. Conversely, in the cells pre-treated with CE, fewer condensed nuclei were observed (Figure 5), and no nuclear condensation was observed in cells treated with CE only.

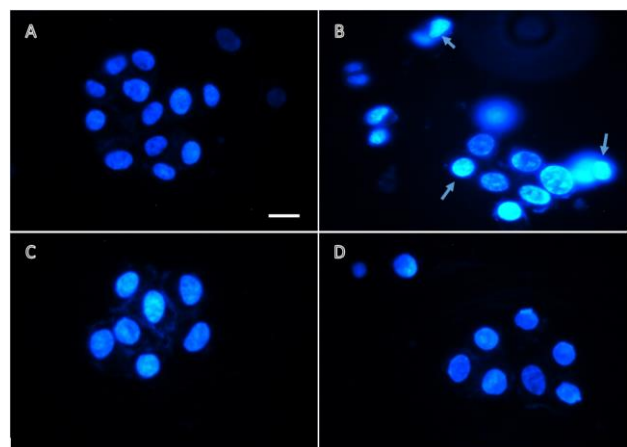


Fig. 5: CE inhibits nuclear damage following MPP⁺ toxicity. SH-SY5Y cells were (A) untreated (B) treated with MPP⁺ only (C) pre-treated with CE and then MPP⁺ (D) treated with CE only. Scale Bar: 100 µm

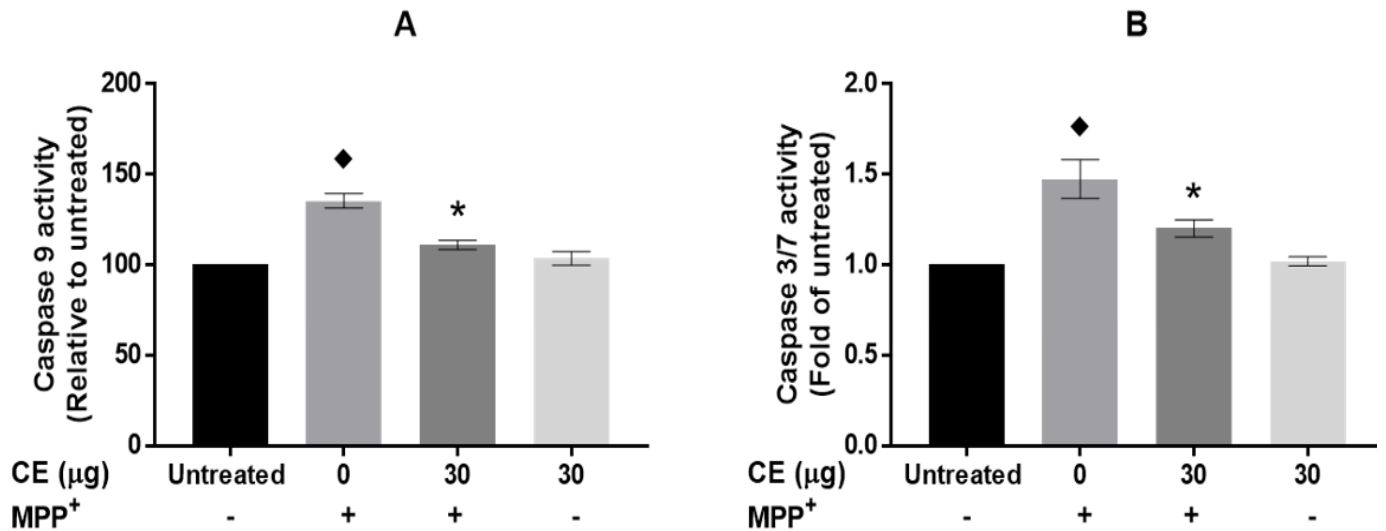


Fig. 6: CE inhibits MPP⁺-induced apoptosis in SH-SY5Y cells. (A) Caspase 9 activity (B) Caspase 3/7 activity. ♦ $p < 0.005$ vs untreated SH-SY5Y cells; * $p < 0.05$ vs SH-SY5Y cells treated with MPP⁺ only.

CE inhibits Caspase 3/7 and 9 activities in SH-SY5Y cells treated with MPP⁺

Apoptosis is moderated through caspases which initiate a proteolytic cascade when triggered, thereby resulting in cell death (Halonen, 2015). To determine the role of caspases in this model of PD, the activities of caspase 9 (an initiator caspase) and caspase-3/7 (a key executioner of apoptosis) were investigated. Results showed that following treatment with MPP⁺ only, there was a significant increase in the activities of caspases 9 and 3/7 respectively, while pre-treatment with CE significantly decreased the activity of caspase 9 and 3/7. Also, there was no significant difference ($p > 0.05$) in the activities of the caspases in cells treated with CE only when compared to untreated cells. These findings indicate that although treatment with CE alone had no significant effects, pre-treatment of SH-SY5Y cells with CE protected the cells from apoptosis induced by MPP⁺. Apoptosis is moderated through caspases which initiate a proteolytic cascade when triggered, thereby resulting in cell death (Halonen, 2015). To determine the role of caspases in this model of PD, the activities of caspase 9 (an initiator caspase) and caspase-3/7 (a key executioner of apoptosis) were investigated. Results showed that following treatment with MPP⁺ only, there was a significant

increase in the activities of caspases 9 and 3/7 respectively, while pre-treatment with CE significantly decreased the activity of caspase 9 and 3/7. Also, there was no significant difference ($p > 0.05$) in the activities of the caspases in cells treated with CE only when compared to untreated cells. These findings indicate that although treatment with CE alone had no significant effects, pre-treatment of SH-SY5Y cells with CE protected the cells from apoptosis induced by MPP⁺.

DISCUSSION

PD is a complex disorder pathologically characterized by age-dependent degeneration of dopaminergic neurons in the SNpc of the midbrain. Several PD models have been utilized for the investigation of mechanisms involved in PD, however, over the years, MPP⁺-treated SH-SY5Y cells have become an important *in-vitro* model for investigating mechanisms of neurodegeneration in PD. This study aimed at evaluating the neuroprotective activity and possible mechanisms of CE on MPP⁺-induced toxicity in SH-SY5Y neuroblastoma cells. In the present study, we provided the first evidence that CE attenuates MPP⁺-induced toxicity in SH-SY5Y neuroblastoma cells. CE inhibited MPP⁺-induced ROS generation, improved viability, prevented

nuclear condensation, inhibited the initiation of caspase-9 activity and prevented the activation of caspase-3/7. The investigated into the cellular and molecular mechanisms responsible for the activity of CE have not been previously reported, and our findings demonstrate possible mechanisms that may underlie the neuroprotective activity of CE.

One of the possible mechanisms for the neuroprotective activity of CE is its antioxidant effect. Previous reports demonstrate that the oxidative damage occurring in the brains of PD patients may be responsible for the degeneration of dopaminergic neurons (Lo et al., 2012). Excessive production of ROS and an impairment in the cellular antioxidant activities lead to oxidative stress. It has been reported that excessive ROS is produced in the brains of PD patients during dopamine metabolism, further aggravated by reduced levels of glutathione and increased calcium and iron levels in the SNpc (Jenner, 2003). Various studies have demonstrated that herbal products with antioxidant properties are capable of protecting dopaminergic neurons in various toxin-induced models of PD. For instance, the aqueous extract of *Panax ginseng* was evaluated for its protective activity against MPP⁺-induced cytotoxicity in SH-SY5Y cells, and the authors reported the inhibition of ROS production as a possible mechanism for the protective effects (Hu et al., 2011). Similarly, *Gastrodia elata* extracts were found to significantly prevent MPP⁺-induced reduction in viability of SH-SY5Y cells possibly through the inhibition of ROS generation (An et al., 2010). In another study, the dried root extract of *Polygala tenuifolia* was found to significantly mitigate 6-OHDA-induced damage to PC12 cells via the amelioration of ROS production and caspase-3 activity (Choi et al., 2011). As previously mentioned, our findings from this study agree with these studies showing that MPP⁺ treatment resulted in a significant increase in ROS production (Figure 4), and that CE effectively attenuated this effect and thus prevented the deleterious effect associated with excessive ROS production.

Caspases play a primary role in the process of apoptotic cell death (Markus, 2000); two prominent pathways involved in apoptosis include the intrinsic or mitochondrial pathway and

extrinsic or death receptor pathway (Elmore, 2007). Either of the extrinsic or intrinsic pathways activate caspase 8, 9 and 10 (initiator caspases), and congregate on caspase 3 to trigger caspase 6 and 7 (effector caspases), thus leading to apoptosis (Halonen, 2015). Caspase-3/7 and 9 have been reported to participate in MPP⁺-induced apoptosis, and it is believed that excessive production of ROS triggers these activities. In this study, treatment of SH-SY5Y cells with MPP⁺ resulted in an increase in the activities of caspase-3/7 and 9 respectively, which is in line with previous studies (Kost et al., 2012, Li et al., 2017). However, pre-treatment with CE successfully inhibited MPP⁺-induced activation of caspase-3/7 and 9 (Figure 6). The inhibition of MPP⁺-induced activation of caspase-3/7 by CE indicates that it may act to prevent the initiation of caspase 9 in order to prevent apoptosis. Also, in line with reports in literature on MPP⁺-induced condensation of nuclei (Wang and Xu, 2005, Shen et al., 2017), findings from the present study showed that CE prevented nuclear condensation and further decreased the number of apoptotic cells in MPP⁺-SH-SY5Y cells (Figure 5), thus providing additional proof for the anti-apoptotic properties of CE.

In conclusion, the results from the present study indicate that CE prevents MPP⁺-induced toxicity in SH-SY5Y neuroblastoma cells. Its antioxidant and anti-apoptotic properties render this plant potentially protective against the deleterious effects of PD. The neuroprotective mechanisms of CE observed in this study are possibly as a result of the bioactive compounds contained in the plant. The bioflavonoid, rutin (already isolated from this plant), is reported to be effective in the inhibition of 6-OHDA-induced toxicity in animal and cellular models of PD (Khan et al., 2012, Magalingam et al., 2013). Based on the findings from this study, further investigation on the neuroprotective mechanisms of CE in other experimental models of PD is recommended, to increase the volume of evidence on the potential candidacy of this plant for the development of neuroprotective and neurotherapeutic agents.

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