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Original Article

Pharmacology Section

Evaluation of Chlorogenic Acid in Parkinson's Disease Model: An In-vitro Study in 6-OHDA Induced SH-SY5Y Cell Line

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ABSTRACT

Introduction: Parkinson's Disease (PD) is the most common neurodegenerative movement disorder. Mitochondrial dysfunction is a key pathological driver of PD. Pharmacological agents used in PD are not disease-modifying therapies and do not influence mitochondrial activity. Diets rich in polyphenols have been shown to prevent pathologies associated with ageing.

Aim: To evaluate Chlorogenic Acid (CGA), a phenolic acid, for its neuroprotective effect against PD in a 6-Hydroxydopamine (6-OHDA) induced SH-SY5Y cell line using in-vitro assays.

Materials and Methods: An in-vitro study was conducted at the Department of Pharmacology, Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India, from September 2023 to November 2023. The human neuroblastoma cell line SH-SY5Y was induced using 6-OHDA. The activity of CGA against cytotoxicity in the 6-OHDA induced SH-SY5Y cell line was evaluated using the MTT assay with different concentrations (3.125 µM, 6.25 µM, 12.5 µM, 25 µM and 50 µM). Mitochondrial Membrane Potential (MMP) was evaluated using flow cytometry and antiapoptotic activity was assessed using Cytochrome C immunofluorescence by fluorescence microscopy. One-way Analysis of Variance (ANOVA) with Tukey's posthoc analysis was used to identify statistical significance at p-value <0.05.

Results: The CGA exhibited the maximum protective concentration on the 6-OHDA induced SH-SY5Y cell line at 25 µM, with a restoration of cell viability of 43.04%. CGA also improved the mitochondrial membrane potential and reduced the levels of cytochrome C release thus maintaining the mitochondrial membrane integrity.

Conclusion: The findings provide evidence that CGA may exert an anti-PD effect through its action on mitochondrial dysfunction and the release of pro-apoptotic factors. The observed action on neuronal cells makes CGA a potential candidate for anti-PD treatment.

Keywords: 6-Hydroxydopamine, Cytochrome C, Mitochondrial membrane potential

INTRODUCTION

Parkinson's disease is the most prevalent neurodegenerative movement disorder, occurring in individuals aged between 55 and 65 years, with a mean age of onset around 60 years [1,2]. The prevalence of PD is approximately 5 million and is expected to double by 2030 [1,3]. The early, substantial loss of dopamine in the substantia nigra pars compacta (SNpc) and the widespread presence of the intracellular protein alpha-synuclein (aSyn) are the hallmarks of PD. A deficiency of dopamine in the basal ganglia causes characteristic motor symptoms of PD, such as tremor, bradykinesia, postural instability and rigidity. Non motor components, including cognitive impairment, olfactory dysfunction, psychiatric symptoms, sleep disorders, autonomic dysfunction, pain and fatigue, may appear more than a decade before motor symptoms [4]. As the disease progresses, these non motor symptoms become increasingly challenging [5]. Despite significant progress in both medical and surgical treatments for PD, a definitive disease-modifying therapy is still unavailable [1,3,5,6].

Mitochondria, a vital and dynamic organelles that provide energy for the cell, have long been implicated in the pathogenesis of PD [7,8]. Mitochondrial dysfunction is a central hallmark of PD. Evidence from various studies underscores mitochondrial dysfunction as a significant driver of both idiopathic and familial PD. Key attributes of the pathogenesis include impairment of mitochondrial Complex I (CI), disturbed mitochondrial quality control mechanisms, increased oxidative stress and bioenergetic deficiency [8]. CI deficiency in PD may be associated with the primary disease process [3,9]. However, the drugs commonly used to manage PD are not known to influence complex I activity [10].

6-Hydroxydopamine (6-OHDA) is a widely used neurotoxin that serves as an inducing agent in PD models. 6-OHDA enters dopaminergic and noradrenergic neurons via specific transporters, accumulates in the cytosol and induces oxidative stress [11,12]. It also causes mitochondrial complex I inhibition [13], reactive oxygen species production, protein misfolding and apoptosis, ultimately leading to the degeneration of dopaminergic neurons [11,12].

Modelling PD is challenging with mature neurons due to their non dividing nature; therefore, immortalised cell lines have been established to serve as cell culture models [11,14]. The SH-SY5Y cell line, derived from human neuroblastoma, is the most commonly used immortalised cell line in PD research [15]. This subline was established using bone marrow from a four-year-old female with metastatic neuroblastoma [16]. SH-SY5Y cells are widely employed to model PD and evaluate the neuroprotective role of various biologically active compounds. These cells display characteristics similar to neurons found in the substantia nigra [6,11]. Additionally, they can be differentiated into a more mature dopaminergic phenotype for advanced studies. Together, these features make SH-SY5Y an effective in-vitro model for PD research [15].

In recent years, the study of natural products has garnered significant global attention, offering promising opportunities for therapeutic advancement in the treatment of PD. Studies have shown that both Mediterranean and Asian diets play a role in preventing various age-related diseases. Notably, the polyphenols present in these diets are strongly associated with a significant reduction in neurodegeneration [17]. One such polyphenol is CGA, predominantly found in coffee. Studies in in-vivo and in-vitro models have revealed that CGA exhibits strong antioxidant properties and

effectively alleviates oxidative stress across diverse disease models [18]. However, research on the influence of phenolic acids in PD and other neurodegenerative diseases remains scarce. The present study examined the potential benefits of CGA on SH-SY5Y cells injured by 6-OHDA and investigated its potential influence on complex I activity, aiming to uncover the underlying mechanisms of its neuroprotective action and its potential role in PD.

MATERIALS AND METHODS

The present in-vitro study was conducted in the Department of Pharmacology and Central Research Facility at the Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai, India, from September 2023 to November 2023, after obtaining ethical approval (CSP-MED/23/SEP/92/212).

Study Procedure

Chemicals: Dulbecco's Phosphate Buffered Saline (DPBS), diphenyl tetrazolium bromide (MTT) dye, Foetal Bovine Serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) - High Glucose, were obtained from HiMedia (Mumbai, India). Dimethyl Sulfoxide (DMSO), Anti-Cytochrome C primary antibody, 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), 4′, 6-Diamidino-2-Phenylindole Dihydrochloride (DAPI) dye and 6-hydroxydopamine (6-OHDA) were procured from Sigma Aldrich (USA). CGA was obtained from Sisco Research Laboratories, India.

Cell culture: The human bone marrow neuroblastoma cell line SH-SY5Y, purchased from the National Centre for Cell Science, India, was maintained in DMEM high glucose media supplemented with 10% FBS and 1% antibiotic-antimycotic solution. The cells were incubated in an atmosphere of 18-20% O_2 and 5% CO_2 in a CO_2 incubator at 37ºC and sub-cultured every third day [19].

Treatment of cells: A 96-well plate was seeded with approximately 20,000 cells. After a 24-hour incubation at 37°C, the spent media was aspirated. Following this, the cells were treated for an additional 24 hours with various concentrations of CGA.

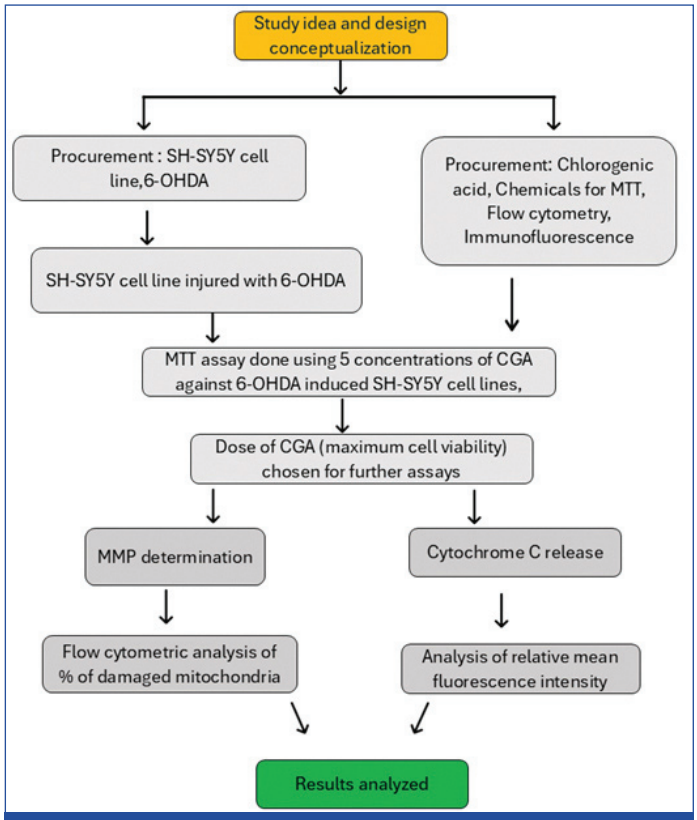
MTT assay: To determine how CGA affected the viability of 6-OHDA induced SH-SY5Y cells, approximately 20,000 cells were cultured for 24 hours in a 96-well plate. The SH-SY5Y cells were subsequently exposed to multiple doses of CGA (3.125, 6.25, 12.5, 25 and 50 µM) for a period of one hour and then treated with 100 µM of 6-OHDA for 24 hours. One group was treated with only 100 µM of 6-OHDA, while the untreated group was not exposed to either 6-OHDA or CGA. After treatment, the cells were subjected to MTT (0.5 mg/mL in DMEM) for one hour in a CO_2 incubator at 37°C to evaluate cell viability. Following the removal of the MTT solution, the dye crystals were dissolved in 100 µL of DMSO and measured at 570 nm using an ELISA reader [20]. The cell viability percentage was estimated using the following equation based on the observed absorbance values.

$$\% \text{ cell viability} = \frac{\text{Mean absorbance value of treated cells}}{\text{Mean absorbance value of untreated cells}} \times 100 \text{ [21]}$$

Determination of mitochondrial membrane potential: The MMP/ ΔΨm is pivotal in sustaining the electrochemical gradient and its impairment may lead to neuronal cell death [22]. JC-1, a lipophilic fluorochrome, is employed to assess the status of ΔΨm. JC-1 is the first J-aggregate-forming cationic dye and is responsive to ΔΨm. In functioning mitochondria, JC-1 aggregates and emits red fluorescence. Conversely, in dead cells with low MMP, the dye remains in a monomeric form, emitting green fluorescence.

Post-treatment with CGA, the 6-OHDA induced SH-SY5Y cells were harvested and centrifuged for five minutes in polystyrene tubes. After washing with PBS, each pellet was incubated for 10-15 minutes with JC-1 working solution (0.5 mL). The supernatant was removed after two washes with assay buffer and the cells were resuspended in 0.5 mL of assay buffer. Flow cytometric analysis was performed using FL1 and FL2 channels, with Fluorescence-Activated Cell Sorting Diva (FACSDiva) software utilised for histogram generation [23].

Cytochrome C immunofluorescence: Cytochrome C immunofluorescence serves as a method to qualitatively measure apoptotic cells, as its release into the cytoplasm marks a pro-apoptotic event [24]. Following treatment with CGA, cells were harvested in 2 mL Eppendorf tubes and treated with fluorescence-labelled cytochrome C antibody for 10 minutes. After removal of the staining solution and excess antibody, the cells were counterstained with DAPI. Subsequently, 50 µL of the cell suspension was spread on a glass slide with mounting medium and observed under fluorescence using the LSM 880 live cell imaging confocal system (Carl Zeiss). The images were combined in ImageJ Software v1.48 using Regions of Interest (ROI)-based Intensity Quantification to determine the Mean Fluorescence Intensity [25]. [Table/Fig-1] depicts an overview of the study flowchart.



[Table/Fig-1]: Overview of the study flowchart.

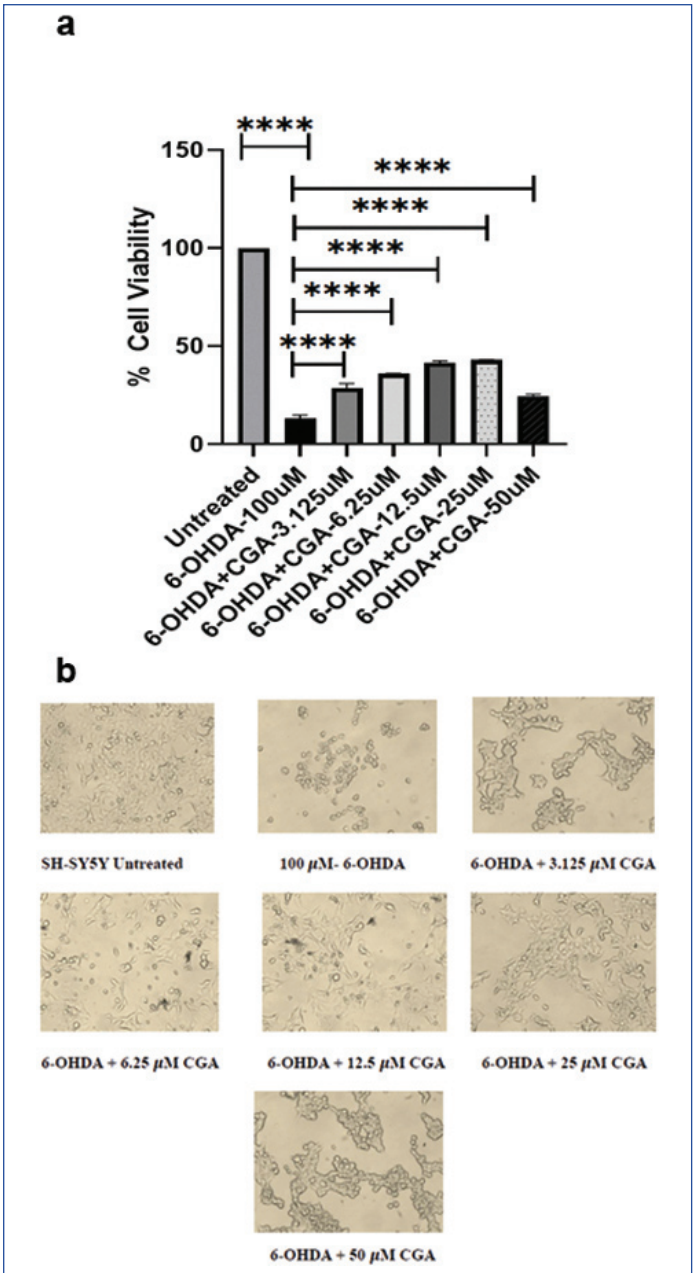
STATISTICAL ANALYSIS

The data was expressed as Mean±Standard Deviation (SD). Statistical analyses were conducted using GraphPad Prism 8. To determine the significance of differences, a one-way ANOVA with Tukey's posthoc test was utilised, with significance considered at a p-value of less than 0.05. Each experiment was performed in triplicate.

RESULTS

Effect of CGA on Cell Viability

To analyse the CGA-mediated effect on cell viability, an MTT assay was performed on the 6-OHDA-induced SH-SY5Y cell line. As illustrated in [Table/Fig-2a-c], treatment with 6-OHDA (100 µM) led to a substantial loss of cell viability (13.10±0.74%) compared to the control. In contrast, treatment with CGA restored cell viability, with a maximum restoration percentage of (43.04±0.19%) observed at 25 µM ($p<0.001$). Cell morphology, visualised under a bright field microscope, revealed that treatment with various concentrations of CGA salvaged cell viability compared to 6-OHDA [Table/Fig-2b].



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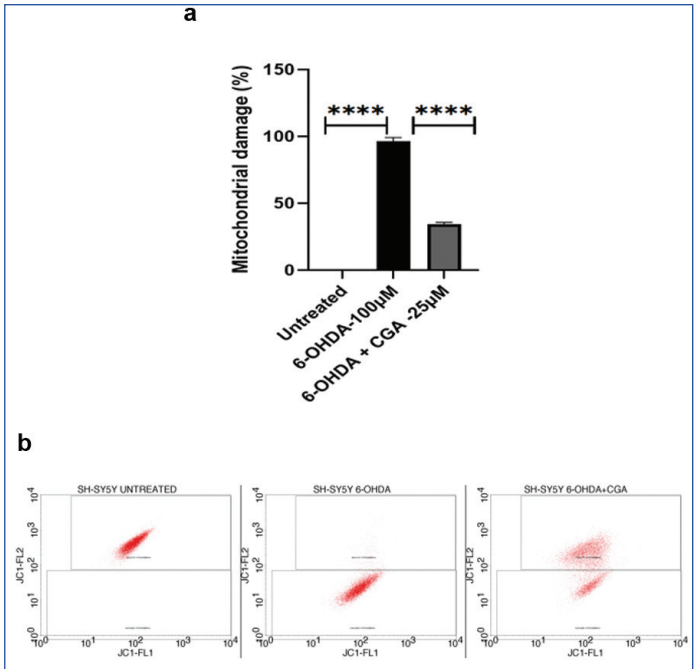
Culture condition	% of cell viability
Untreated	100.00±0
6-OHDA-100 µM	13.10±0.74
6-OHDA+CGA-3.125 µM	28.64±0.90
6-OHDA+CGA-6.25 µM	36.06±0.03
6-OHDA+CGA-12.5 µM	41.51±1.00
6-OHDA+CGA-25 µM	43.04±0.19
6-OHDA+CGA-50 µM	24.75±0.34

[Table/Fig-2]: a) Effect of CGA on the viability of 6-OHDA induced SH-SY5Y cells (n=3). Significant difference was expressed as (****p<0.001) compared to the untreated group and as (****p<0.001) compared to CGA treated group; b) Images from the bright field microscope depicting cell viability for various treatment groups; c) The cell viability using MTT assay.

Effect of CGA on Mitochondrial Membrane Potential

As depicted in [Table/Fig-3a,c], exposure to 6-OHDA depolarised the mitochondria in neuronal cells, resulting in mitochondrial damage (96.65±1.06%). However, treatment with CGA preserved healthy mitochondria in the cells against 6-OHDA toxicity (34.74±0.41%) (p<0.001). Depolarised ΔΨm is indicated by JC-1 fluorescing in the FL-1 channel while not exhibiting fluorescence in the FL-2 channel. The upward shift in cell population towards FL-2 indicates improved membrane polarisation following treatment with CGA [Table/Fig-3b].



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Culture condition	% of mitochondrial damage
Untreated	0.07±0.02
6-OHDA-100 µM	96.65±1.06
6-OHDA+CGA-25 µM	34.74±0.41

[Table/Fig-3]: a) Effect of CGA on 6-OHDA induced MMP changes in SH-SY5Y cells. % of mitochondrial damage alleviated with the use of CGA. (****p<0.001); b) FACS dot plot showing treatment with CGA significantly reduced the membrane potential damage caused by 6-OHDA. Upper quadrant- healthy mitochondria, Lower quadrant- Damaged mitochondria; c) % of mitochondrial damage.

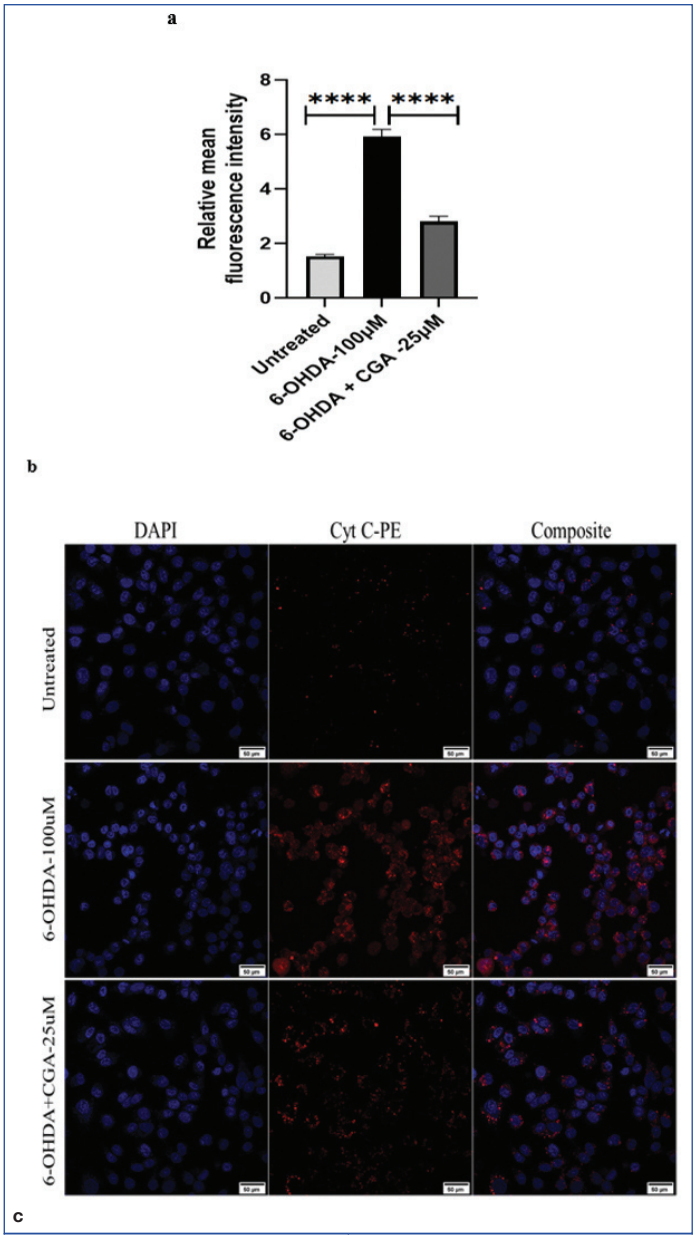
Effect of CGA on Cytochrome C Immunofluorescence

Exposure to 6-OHDA increased cytochrome C release in neuronal cells, as evidenced by the diffuse red staining and nuclear changes in blue. In contrast, treatment with CGA restored nuclear morphology and mitochondrial integrity, as indicated by the punctate red staining and intact blue nuclei in the presence of 6-OHDA toxicity [Table/Fig-4a,c]. The relative mean fluorescence intensity of the 6-OHDA group was 5.9±0.2; however, when treated with CGA, the intensity reduced to 2.81±0.19.

DISCUSSION

Over the years, the concept of disease-modifying treatment has been widely explored in neurodegenerative disorders such as PD [26]. Various mechanisms attributed to the pathophysiology of PD include α-synuclein aggregation, oxidative stress, mitochondrial impairment, dysfunction in cellular clearance pathways, autophagy, alterations in the microbiome and a number of genetic contributors [2]. Developing disease-modifying therapies for PD has proven to be exceptionally challenging due to these complex underlying mechanisms, making a drug that addresses all these domains crucial for effective management.

The CGA is a phenolic acid found abundantly in coffee [18]. Multiple studies conducted on human cell lines and animal models have demonstrated the protective function of CGA in combating oxidative stress in brain tissue [27-29]. CGA has the potential to scavenge superoxide radicals ($\bullet O_2^-$), 2,2'-azino-bis (3-ethylbenothiazoline-6-sulfonic acid) cation radicals (ABTS•+), hydroxyl radicals (•OH) and 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH•) [18,25]. In a study conducted on the 6-OHDA-treated SH-SY5Y cell line, CGA alleviated ROS production and endoplasmic reticulum stress and restored 6-OHDA-induced cytotoxicity. It also reversed motor deficits in 6-OHDA lesioned rats and enhanced antioxidant enzyme activity in the striatum of the rat brain [30]. Moreover, studies have revealed that CGA possesses an antiapoptotic effect as well [27,31].

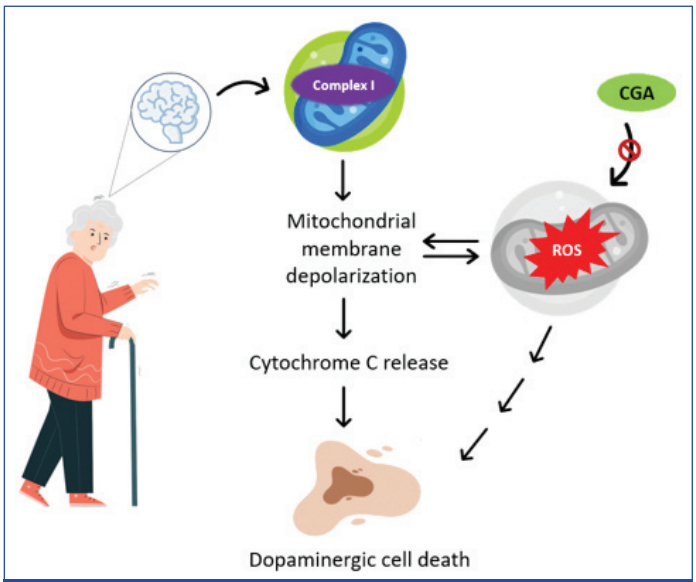


Culture condition	Relative mean fluorescence intensity
Untreated	1.528±0.029
6-OHDA-100 µM	5.932±0.257
6-OHDA+CGA-25 µM	2.810±0.194

[Table/Fig-4]: a) Relative mean fluorescence intensity reveals that treatment with CGA reduced the cytochrome C release (****p<0.001); b) Fluorescence microscope images of SH-SY5Y stained with Cytochrome C antibody and counter stained with DAPI; c) Relative mean fluorescence intensity of cytochrome C.

In a study conducted by Teraoka M et al., on α-synuclein-related cytotoxicity in catecholaminergic PC12 cells, CGA demonstrated an inhibitory effect against dopamine oxidation, the interaction of oxidised dopamine with α-synuclein, as well as α-synuclein oligomerisation [32]. CGA also inhibited the accumulation of α-synuclein and the degeneration of dopaminergic neurons, improving motor ability via autophagy induction in Caenorhabditis elegans [33]. Furthermore, CGA has been shown to modulate intestinal inflammation, thus regulating the gut-brain axis [34].

In the present study, the MTT assay revealed that CGA reduced the cytotoxicity caused by 6-OHDA and restored the viability of SH-SY5Y cells. The effects of reactive oxygen species and mitochondrial membrane impairment are well-documented pathophysiological mechanisms in PD. The function of the mitochondrial Permeability Transition Pore (mPTP), located in the inner mitochondrial membrane, is regulated by changes in MMP and is generally closed. However, due to the charge difference between the mitochondrial matrix and the cytosol, mitochondrial membrane depolarisation can lead to the prolonged opening of the mPTP, resulting in the release of cytochrome C, a pro-apoptotic factor. This release triggers the activation of the caspase cascade, ultimately leading to cell death [24]. In the present study, CGA at a dose of 25 µM alleviated mitochondrial membrane depolarisation and reduced cytochrome C release by maintaining mitochondrial membrane integrity [Table/Fig-5].



[Table/Fig-5]: Schematic illustration of the role of CGA in mitochondrial complex I impairment. Created by Sunprakathi using Freepik.

Research suggests that CGA and its metabolites can traverse the Blood-Brain Barrier (BBB), potentially exerting neuroprotective effects on brain tissue. A study conducted by Cropley V et al., on a healthy older population, who were administered decaffeinated coffee enriched with CGA, showed improvements in mood and cognition [35]. However, these findings were not consistently reproducible in other studies [27,36]. In research conducted by Lardeau A and Poquet L using an in-vitro BBB model, very low rates of BBB permeation were observed, leading to the conclusion that CGA cannot be considered for its direct action on the central nervous system [37]. A brain distribution and plasma pharmacokinetic study comparing intravenous and intranasal CGA in Charles-Foster rats concluded that intranasal CGA provided rapid absorption, increased penetration and higher drug targeting efficiency compared to the intravenous route, offering a promising approach for treating neurodegenerative disorders [38].

The findings of the present study indicate that CGA reduced the cytotoxicity caused by 6-OHDA and curtailed cytochrome C release by maintaining mitochondrial membrane integrity in the SH-SY5Y cell line, owing to its antioxidant effects.

Limitation(s)

The assays used in the present study focus primarily on mitochondrial dysfunction. However, further studies are crucial to assess the effects of CGA on various factors and genes implicated in the pathophysiology of PD. Additionally, in-vivo studies involving appropriate animal models are necessary to validate these findings.

CONCLUSION(S)

The results of the present study conclude that CGA, at a dose of 25 µM, protected the SH-SY5Y cell line from 6-OHDA toxicity by mitigating the effects of complex I impairment. This was achieved by improving cell viability, maintaining MMP and minimising the release of cytochrome C. The findings of the present study contribute to the holistic potential of CGA as a viable candidate for the management of PD.

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Authors' contribution: All authors contributed equally to this work. The authors confirm their contribution to the paper as follows: SAP-study conception and design; KKK-Data analysis and interpretation of results; GV-Draft preparation and KR- Manuscript guidance. All authors reviewed the results and approved the final version of the manuscript.

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