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Research Article

Assessment of Protective Effects of *Celastrus paniculatus* Seed Extract against MPP$^+$ induced Damage in SH - SY5Ycells

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ABSTRACT

In the Indian system of traditional medicine, *Celastrus paniculatus* seeds and oil have been recognized for their therapeutic efficacy in treating cognitive dysfunctions. This study unveils the protective effect of *Celastrus paniculatus* seed extract (CPME) against the neurotoxin 1-methyl - 4-phenlypyridinium (MPP$^+$) induced damage in SH-SY5Y human neuroblastoma cell line. The protective effect of CPME was assessed by MTT assay by pretreating the SH-SY5Y cells with CPME at two different concentrations (10 and 25 µg/mL) prior to MPP$^+$ exposure (250µM) and the percentage of viable cells was determined. Significant improvement in cell viability was observed with CPME pretreatment and the effect was well pronounced at 25µg/mL concentration. In addition, MPP$^+$ treated cells with or without CPME pretreatment were analyzed for apoptotic cell death by different staining techniques like acridine orange/ethidium bromide (AO/EB), propidium iodide (PI) and 4' 6 - diamidino - 2 - phenyl indole dihydrochloride (DAPI). Same way, changes in the mitochondrial membrane potential and intracellular ROS production were assessed by staining respectively with rhodamine 123 and 2', 7' - dichlorofluorescin diacetate (DCFDA). Quercetin served as a positive control in this study. Observations recorded in fluorescence microscopic image analysis indicated that pretreatment with CPME caused appreciable protective effects against MPP$^+$ induced cell death by alleviating apoptotic changes such as cell shrinkage, condensation/fragmentation of nuclear material along with restoring ROS levels and mitochondrial membrane potential. Altogether, results from this study points to the protective effects of CPME against MPP$^+$ induced cell death by regulating the cellular production of ROS.

Introduction

Parkinson's disease (PD) is the second most common chronic neurodegenerative disease that affects 6 million people worldwide.[1] PD's major clinical symptoms are slowness of movement (bradykinesia), resting tremors, rigidity, and postural instability, cognitive & autonomic dysfunction, depression, anxiety, sleep disturbance, etc.[2] These manifestations are mainly due to progressive loss of dopaminergic neurons that project from the substantia nigra pars compacta (SNpc) region to the striatum that controls voluntary movement.[3] It has been suggested that several cellular and molecular mechanisms may contribute to the pathogenesis of this disease which includes proteasomal dysfunction, unfolded protein response (UPR), ER stress, mitochondrial damage, oxidative stress inflammation, and apoptotic cell death.[4-6] Also, intracellular accumulation of insoluble proteinaceous Lewy bodies comprising α-synuclein (α-syn) is considered a specific pathological hallmark of PD.[7,8] The current treatment approaches for PD aim largely at alleviating the symptoms but do not address the underlying causes.

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Plants, in the form of herbs, spices, and foods, constitute an unlimited source of molecules available for improving human health. In recent years, much research has focused on traditional herbs since plants have long been documented as an opulent source of potentially useful drugs.[9] Also, several medicinal plants may possess therapeutic potential without serving a nutritional role in the human diet. One such plant is *C. paniculatus*, which has long been used in *the Ayurvedic medicine system to treat cognitive dysfunctions and improve* memory.[10] The seeds and the seed oil of this plant have been reported for neuroprotective effect against various neurological disorders, including Alzheimer's disease,[11] Huntington's chorea,[10] and various cognitive disabilities.[12]

Among the various experimental models of PD, the human neuroblastoma cell line-SH-SY5Y that possess morphological and biochemical characteristics of dopaminergic neurons has been widely used as an ideal cellular model for studying Parkinson's disease *in vitro*.[13,14]

The neurotoxic compound MPP^+ (1-methyl-4-phenyl pyridinium) is an active metabolite of MPTP and has been widely used to induce symptoms closely resembling PD in animal and different cellular models.[15]

The present study is designed to assess the protective effect of methanolic extract of *C. paniculatus* seeds (CPME) against MPP^+ induced damage in SH-SY5Y cells, and quercetin was used as a positive control. The cell viability was determined by MTT assay. Changes in the intracellular ROS and mitochondrial membrane potential were assessed by staining respectively with 2', 7'- dichlorofluorescein diacetate (DCFDA).and rhodamine123. MPP^+ induced apoptotic changes were assessed by staining the cells with acridine orange/ethidium bromide (AO/EB), 4' 6-diamidino-2-phenyl indole dihydrochloride (DAPI), and propidium iodide (PI).

Materials and Methods

Materials:

The *C. paniculatus* seeds were purchased online from Attar Ayurveda, Rajasthan. Authentication of seeds were obtained from Siddha Central Research Institute, Arumbakkam Chennai (Authentication code: C02122001P). MPP^+ was purchased from *Sigma-aldrich*, Bangalore, India. All other chemicals and reagents used in this work were analytical or cell culture grades and purchased from Hi-Media, India.

Cell Culture

The human neuroblastoma cell line SH-SY5Y was procured from NCCS, Pune, India. The SH-SY5Y cells were cultured in DMEM: HAM F12 medium supplemented with 10% FBS, 100U/mL of penicillin and 100ug/mL of streptomycin and the cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Methods

Preparation of Celastrus paniculatus Seed Extract

The methanolic extract of *Celastrus paniculatus* seeds (CPME) was prepared by cold maceration of coarsely powdered seeds with methanol,[16] and the mixture was left for 3 days. The solvent extract was separated by filtration and was concentrated using a rotary flash evaporator.

Determination of Cell Viability by MTT Asay

The MTT (3-(4,5dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay is a widely used quantitative colorimetric technique to determine the metabolic status of the cells, particularly the activity of mitochondria.[17] This technique is based on the reduction of tetra ring of MTT by mitochondrial dehydrogenases with NADH in healthy cells, yielding a blue formazan product that can be measured using a spectrophotometer at 570 nm. To assess the potential detrimental effects of *Celastrus paniculatus* seeds on SH-SY5Y cells, MTT assay was used in this study. Briefly, exponentially growing SH-SY5Y cells were seeded in 96-well plates at a density of 10 $x10^3$ cells/well and treated with different concentrations of CPME, namely 10, 25, 50, and 100 µg/mL in 0.2% DMSO and incubated for 24 hours following which MTT was added to each well and further incubated for 3 hours in the dark. After incubation, the formazan crystals formed were dissolved in 100 µL of DMSO, and the absorbance was measured at 570 nm using a multi-well ELISA microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA.[18] A 0.2% DMSO was used as control, and the results are expressed as % viable cells.

$$\text{Percentage of cell viability} = [\text{absorption (test)} / \text{absorption (control)}] \times 100$$

Determination of Protective Effect of CPME Against MPP^+ Induced Damage in SH-SY5Y Cells by MTT Assay

If any, the protective effect of CPME against MPP^+ mediated, cellular damage in SH-SY5Y cells was assessed by MTT assay. Cells were pretreated with two different concentrations of CPME (10 and 25 µg/mL) in 0.2% DMSO and incubated for 2 hours. Subsequently, cells were treated with MPP^+ (250µM/well)[19] incubated for 24hr and the percentage cell viability was determined at 8, 16, and 24 hours respectively by MTT assay. Similarly, cells were pretreated with 100 µM of quercetin which served as a positive control,[20] and the percentage cell viability was determined at 24 hours by MTT assay.

Observation of Cytomorphology

(i) Analysis of Morphological Changes in MPP+ Treated Cells

SH-SY5Y cells seeded in 6 well tissue culture plates of (1×10^5 cells/well) following pretreatment with 2 different concentrations (10 and 25 µg/mL) of CPME for 2 hours were subjected to treatment with MPP^+ (250 µM) and further incubated for 24 hours. The cells were then observed for

morphological changes under inverted phase-contrast microscope (Olympus, Japan) at 200X magnification.

(ii) Evaluation of Intracellular Reactive Oxygen Species (ROS)

The SH-SY5Y cells seeded in a tissue culture plate of 6 well (1 x 10^5 cells/well) were pretreated with 2 different concentrations (10 and 25 µg/mL) of CPME in 0.2% DMSO for 2 hours followed by addition of 250 µM MPP^+ and 24 hours incubation along with the untreated control cells. Following incubation, 1X PBS was used to wash the cells and stained in the dark with a non-fluorescent dye DCFH-DA (15µM) for 40 min at 37°C. Again, cells were washed with 1X PBS (2x) and recorded under the fluorescence microscope (Olympus, Japan) at 200X magnification. Further, the fluorescence of DCF in cells due to ROS generation was also measured by spectrofluorimeter in blue filter with excitation maxima (488 nm) and emission maxima (525) nm.[21]

(iii) Determination of Mitochondrial Membrane Potential

The SH-SY5Y cells seeded in a tissue culture plate of 6 well (1 x 10^5 cells/well) were pretreated with 2 different concentrations (10 and 25 µg/mL) of CPME in 0.2% DMSO for 2 hours followed by the addition of 250µM MPP^+ and 24 hours incubation along with the untreated control cells. Cells were then washed with 1X PBS, stained with Rhodamine 123 (10 µg/mL, 50 µL), incubated for about 30 minutes, and washed with 1X PBS again and monitored using Olympus, Japan, fluorescence microscope for identifying loss of membrane potential due to apoptosis at 200X magnification. The fluorescence intensity was evaluated by spectrofluorimeter in blue filter with excitation maximum (503 nm) and emission maximum (535) nm.[22]

(iv) Detection of Apoptotic Cells Death by Staining with Acridine Orange/Ethidium Bromide (AO/EB)

In order to determine MPP+ induced apoptotic changes, double staining technique using acridine orange/ ethidium bromide was performed. The SH-SY5Y cells seeded in 6 well tissue culture plate (1 x 10^5 cells/well) were pre-incubated with 2 different concentrations of CPME (10 and 25 µg/mL) for 2 hours, followed by exposure to MPP^+ (250 µM) for 24 hours. Following incubation, cells were washed with 1X PBS (pH 7.2), treated with AO/EB mixture (10 mg/mL in 1X PBS, 1 mL), and incubated in the dark 2 minutes. Cells were then washed again with 1X PBS and examined under a fluorescence microscope (Olympus, Japan) at 200X magnification using a blue filter with 480-490 nm of excitation maximum and 617 nm of emission maximum.[23]

(v) Determination of Nuclear Morphology by DAPI Staining

To assess the nuclear morphological changes associated with apoptosis, such as condensation and fragmentation of nucleus, 4', 6-diamidino-2 phenylindole (DAPI) staining assay was done. In this assay, The SH-SY5Y cells seeded in a tissue culture plate of 6 well (1 x 10^5 cells/well) were pretreated with 2 different concentrations (10 and 25 µg/mL) of CPME in 0.2% DMSO for 2 hours followed by addition of 250µM MPP^+ and 24h incubation along with the untreated control cells. After incubation, paraformaldehyde (4%) and ethanol (70%) were used to permeabilize and fix the cells. After fixation 1X PBS was used to wash the cells and stained with DAPI (2mg/mL). Later, it was maintained in the dark for about 20 min and wash again with 1X PBS. The cells stained with DAPI were recorded in a fluorescence microscope (Olympus, Japan) in UV filter with excitation maxima (358 nm) and emission maxima (461 nm) for identifying apoptotic cell death at 200X magnification.[24]

(vi) Determination of DNA Integrity by PI Staining

Another technique used to assess apoptosis-associated nuclear morphological changes was propidium iodide (PI) staining. SH-SY5Y cells seeded in a 6 well (1 x 10^5 cells/well) plate were pretreated with 2 different concentrations (10 and 25 µg/mL) of CPME in 0.2% DMSO for 2 hours followed by addition of 250 µM MPP^+ and subsequently incubated for 24 hours along with untreated control cells. After incubation, 1X PBS was used to wash the cells. Following that, paraformaldehyde (4%) and ethanol (70%) were used to permeabilize and fix the cells and further both treated and untreated control cells were washed and stained with PI (50 µg/mL), incubated for 20 min. Following another 1X PBS wash the cells were examined under Olympus, Japan, fluorescence microscope in green filter with excitation maximum (535 nm) and emission maximum (617) nm for identifying apoptotic cell death at 200X magnification.[25]

Statistical Analysis

Statistical evaluation of results was carried out by applying one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, and results were represented as mean ± SEM (Standard Error) of different phytochemicals. Statistical significance of results was considered at $p < 0.05$, 0.01, 0.001 and 0.0001 etc. All statistical analyses have been performed by using *Graph Pad Prism 5 software.*

Results

CPME Treatment did not Induce Toxic Effects on SH-SY5Y Cells

To investigate the cytotoxic effects of CPME if any on SH-SY5Y cells, the cells were treated with increasing concentrations of CPME (10, 25, 50, and 100 µg/mL in 0.2% DMSO) for 24 hr and the cell viability was evaluated by MTT assay. Data obtained in this study indicated that CPME did not induce any toxic effect on SH-SY5Y cells wherein the cell viability was found to be 94 ± 3.1, 92 ± 0.65, 81 ± 2.8 and 81 ± 4.5 respectively for 10, 25, 50, and 100 µg/mL of CPME "Fig. 1". Based on these results, two different

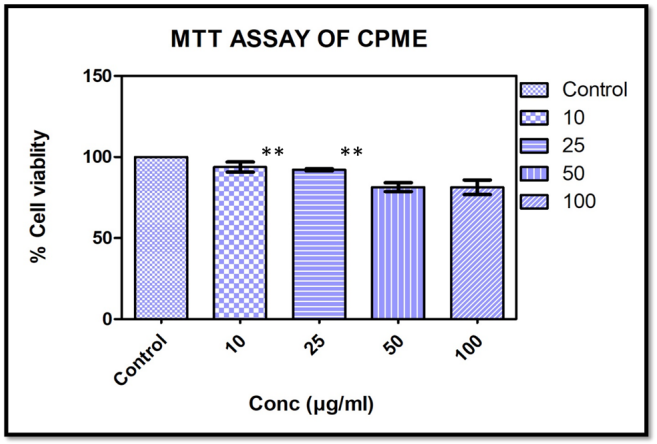

Fig. 1: The percentage of cell viability of different concentrations of CPME namely 10, 25, 50 and 100 µg/ml as compared to control Statistical significance: **p < 0.05, n = 3, as compared to control. Values were presented as mean ± SEM.

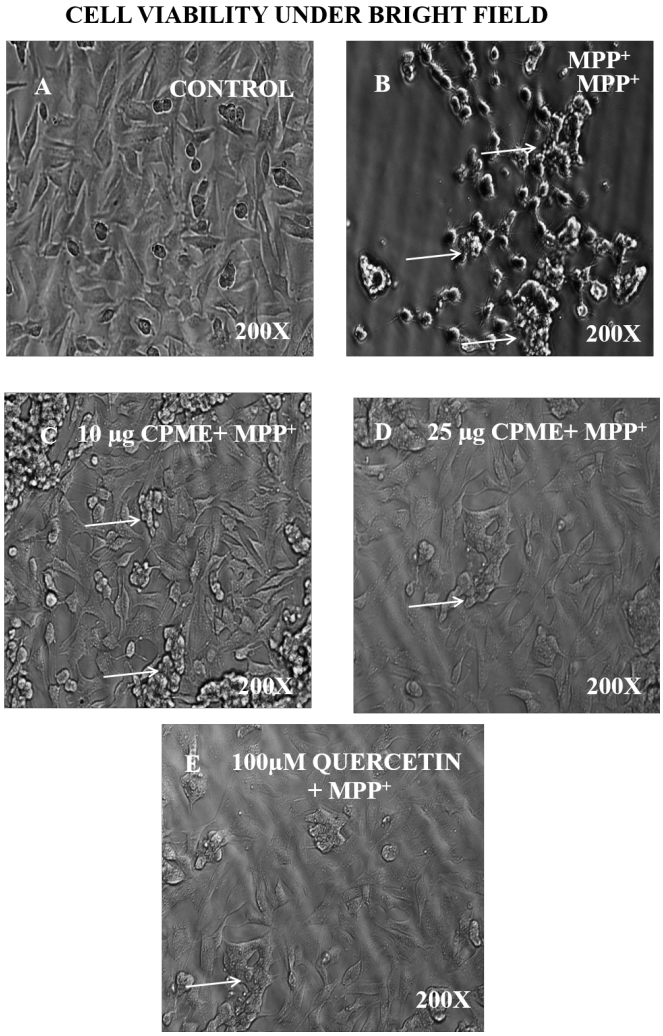

Fig. 2: The cell viability under bright field
A - Control, B - 250 µM MPP^+ showing increased cell death as compared to vehicle control cells, C- Pre - treatment with 10 µg/mL of CPME against 250 µM MPP^+, D - pretreatment with 25 µg/mL of CPME against 250 µM MPP^+ and E-positive Control (pretreatment with 100 µM of quercetin against 250 µM MPP^+).

concentrations of CPME i.e., 10 and 25 µg/mL were selected for further studies.

CPME attenuated MPP^+- Induced Cytotoxicity in SH-SY5Y cells

The influence of CPME against MPP^+ induced damage in SH-SY5Y cells was determined by pretreating the cells with two different concentrations (10 and 25 µg/mL) of CPME prior to exposure to MPP^+ (250 µM).

10 and 25 µg/mL of CPME treated cells showed decreased cell death as compared to MPP^+ induced cells. Morphological changes such as cell shrinkage, membrane blebbing and granulation were observed in MPP^+ treated cells "Fig. 2B" as compared to control (Fig. 2A). However, these morphological changes were found to be less pronounced in cells pretreated with CPME at 10 µg/mL (Fig. 2C) and 25 µg/mL "Fig. 2D" concentrations.

Also, time and dose dependent effect of CPME (10 and 25 µg/mL) against with MPP^+ (250µM) challenge was recorded at 8, 16 and 24hr of treatment. Data obtained indicated that, cells exposed to MPP^+ alone showed significant ($p<0.0001$) reduction in percent viability (49 ± 2.2) after 24hr as compared to control (Fig.3B). On the other hand, cells pretreated with 25 µg/mL CPME showed significant ($p < 0.05$) improvement in viability (74 ± 5.5) at 24hr as compared to MPP^+ alone treated cells (Fig. 3B). Also, the protective effect exhibited by 25 µg/mL CPME was found to be better than that of positive control - quercetin (66 ± 5). However, 10 µg/mL CPME pretreatment did not show significant improvement (59 ± 6.7) in cell viability at 24 hours as compared to MPP^+ alone treated cells (Fig. 3B). Also, 10 µg/mL CPME was comparatively less effective than 25 µg/mL CPME against MPP^+ challenge at 8 and 16 hr incubation. Taken together, it was recorded

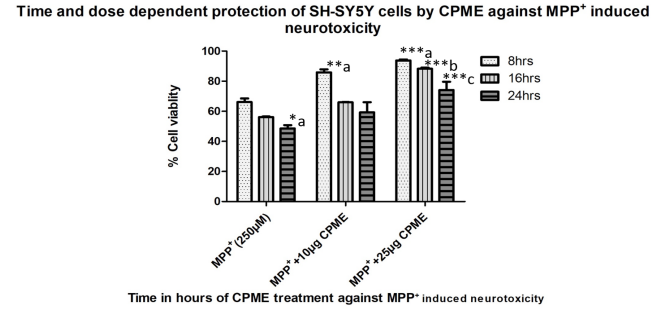

Fig. 3A: Time and Dose-dependent Protection of SH-SY5Y Cells by CPME Against MPP^+ Induced Neurotoxicity
The cells pretreated with CPME (10 and 25 µg/mL) were challenged with 250µM of MPP^+ and the cell viability was measured at 8hr, 16hr and 24hr by MTT assay.
Values are expressed as mean ± SEM, n = 3
One way ANOVA followed by Tukey's multiple comparison test.
[a]Values with superscript *, ** and *** differ significantly at $p < 0.05$, $p < 0.01$ $p < 0.0001$as compared to 8 hours exposed MPP^+ cells
[b]Values with superscript *** differ significantly at $p < 0.0001$as compared to 16 hours exposed MPP^+ cells
[c]Values with superscript *** differ significantly at $p < 0.0001$as compared to 24 hours exposed MPP^+ cells.

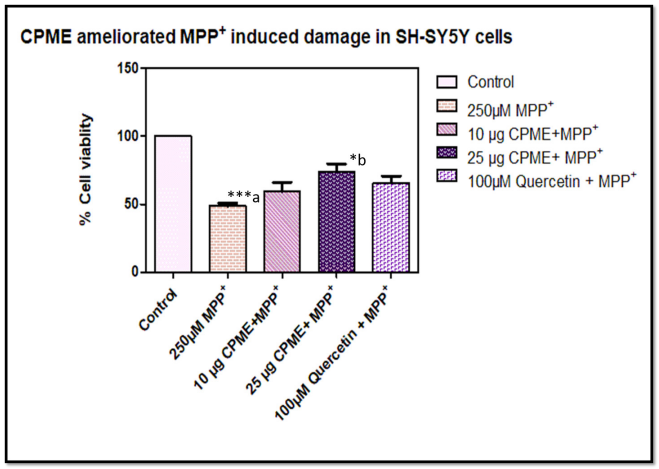

Fig. 3B: CPME Ameliorated MPP^+ Induced Damage in SH-SY5Y Cells
The cells pretreated with CPME (10 and 25 µg/mL) and quercetin (100 µM) were challenged with 250µM of MPP^+, and the cell viability was measured at 24 hours by MTT assay.
Values are expressed as mean ± SEM, n = 3
One-way ANOVA followed by Tukey's multiple comparison test.
[a]Values with superscript *** differ significantly at p <0.0001 as compared to control
[b]Values with superscript * differ significantly at p <0.05 as compared to MPP^+ alone treated cells

that among the two doses of CPME used in this study, significantly improved cell viability was observed with 25 µg/ml of CPME at 24hr.

CPME Alleviated MPP^+ Induced Increase in Intracellular Reactive Oxygen Species (ROS) in SH-SY5Y Cells

To correlate the changes in intracellular ROS levels and apoptotic changes observed in MPP+ treated cells, the ROS generated was determined by fluorescence microscopy with DCFH-DA staining. Th DCFH-DA is a non-fluorescent dye that gets converted to a green fluorescent compound in peroxides.

Cells treated with MPP^+ alone exhibited strong green fluorescence compared to control "Fig. 4A, Fig. 4B" indicating significantly increased intracellular ROS levels. These observations suggest that ROS generation in MPP^+ treated cells as a contributory factor for induction of cell death (Fig. 4). On the other hand, cells pretreated with 10 and 25 µg/mL of CPME or with quercetin (100 µM) showed significant reduced fluorescence intensity (Figs 4C, 4D, Fig.4E) as compared to MPP^+ alone treated cells. Moreover, pretreatment with 25 µg/mL CPME showed distinguishable inhibitory effects against ROS generation better than that of 10 µg/mL CPME as well as 100 µM quercetin indicating its free radical scavenging potential.

CPME Mitigated MPP^+ Induced Mitochondrial Membrane Damage in SH-SY5Y Cells

As MPP^+ is known to specifically affect the mitochondrial respiratory complex I, the protective effect of CPME against MPP^+ induced changes in the mitochondrial

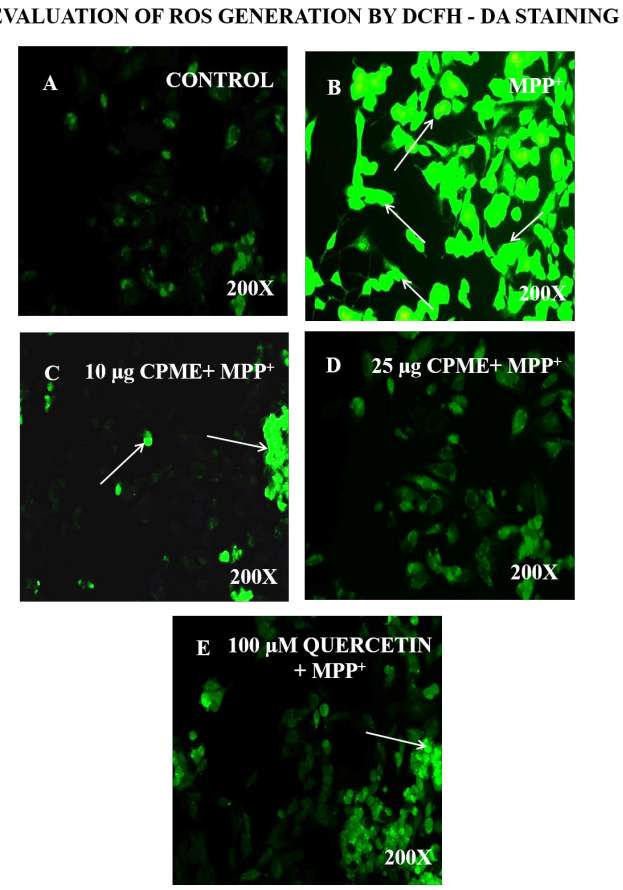

Fig. 4: Evaluation of ROS Generation by DCFH-DA Staining
A-vehicle control, B-MPP^+ (250 µM) shows exhibited strong green fluorescence indicating significantly increased levels of intracellular ROS as compared to vehicle control cells, C-pretreatment with 10 µg/mL of CPME against MPP^+, D-pretreatment with 25 µg/mL of CPME MPP^+ and E-pretreatment with 100 µM of quercetin against MPP^+ displayed lesser green fluorescence indicating a decreased level of ROS as compared to MPP^+ (250 µM) induced cells.
Magnification - 200X.

membrane potential was assessed in this study by using Rhodamine 123 a fluorescent dye. The lipophilic Rhodamine 123 upon entering intact mitochondria, bind to the inner mitochondrial membrane and emit a bright green fluorescence. Any reduction in the fluorescence intensity designates mitochondrial membrane damage. In this study, control cells showed bright green fluorescence indicating intact mitochondrial membrane "Fig. 5A". While MPP^+ alone treated cells showed significantly reduced fluorescence "Fig. 5B", those cells were subjected to pretreatment with CPME (10 and 25 µg/mL) and 100 µM quercetin before MPP^+ treatment showed substantially enhanced fluorescence intensity. In particular, cells treated with 25 µg/mL CPME exhibited more prominent green fluorescence (Fig. 5D) than those treated with 10 µg/mL CPME (Fig. 5C) and positive control (100 µM quercetin) (Fig. 5E) which indicated the potential of CPME at 25 µg/mL dose in protecting against MPP^+ induced mitochondrial membrane damage.

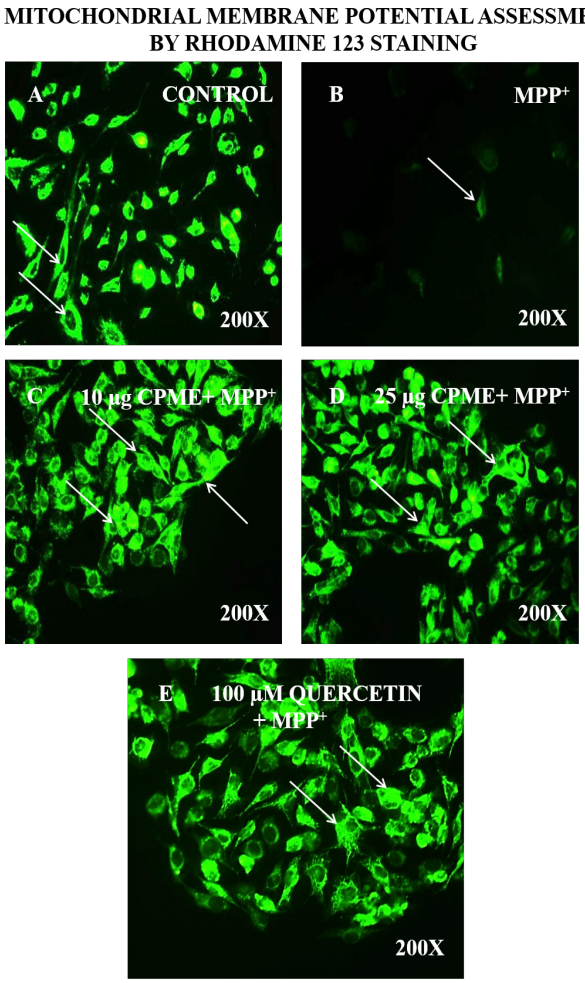

Fig. 5: Mitochondrial Membrane Potential Assessment by Rhodamine 123 Staining
A -vehicle control, B-MPP^+ (250 µM) shows lesser green fluorescence indicating loss of membrane potential as compared to vehicle control cells, C-pretreatment with 10 µg/mL of CPME against MPP^+, D-pretreatment with 25 µg/mL of CPME MPP^+ and E-pretreatment with 100 µM of quercetin against MPP^+ displayed bright green fluorescence.as compared to MPP^+ (250 µM) induced cells. Magnification-200X.

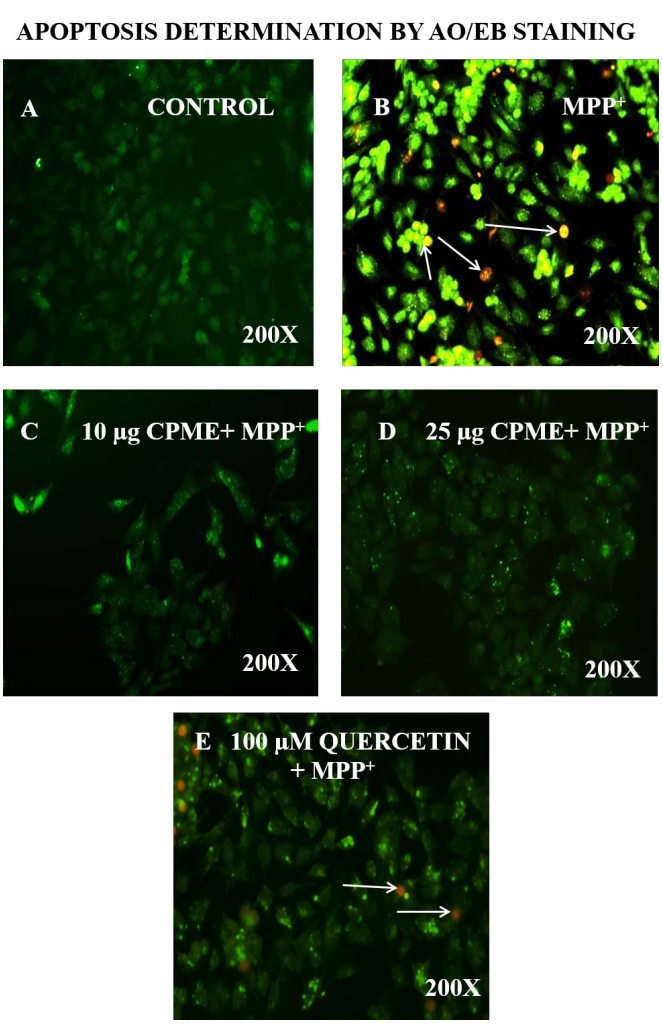

Fig. 6: Apoptosis Determination by AO/EB Staining
A - Control, B - 250 µM of MPP^+ shows granular yellow-green and orange colored apoptotic bodies with condensed nuclei indicating early-stage and late apoptosis. C-pretreatment with 10 µg/mL of CPME against MPP^+, D-pretreatment with 25 µg/mL of CPME MPP^+ and E-pretreatment with 100 µM of quercetin against MPP^+ reduced the number of granular yellow-green and orange colored apoptotic bodies as compared to MPP^+ alone treated cells. Magnification - 200X.

CPME Showed Protective Effect against MPP^+ Induced Apoptotic Changes in SH-SY5Y Cells

To detect apoptosis-associated nuclear changes in cells treated with MPP^+ with and without CPME pretreatment, three different staining techniques, namely AO/EB, DAPI, and PI, were used in this study. In the AO/EB staining method, MPP+ alone treated cells displayed granular yellowish green and orange colored apoptotic bodies with condensed nuclei (Fig. 6B), indicating apoptosis's onset compared to control cells (Fig. 6A). Remarkably, cells that were pretreated with 25µg/mL CPME, exhibited a significantly reduced level of orange-colored apoptotic bodies (Fig. 6D) than those cells treated with 10 µg/ml CPME (Fig. 6C) as well as those treated with Quercetin (Fig. 6E). In DAPI staining technique, control cells showed intact nuclei with enriched fluorescence intensity of DAPI "Fig. 7A" while cells treated with MPP^+ alone showed distinct nuclear damage such as nuclear condensation, margination, and cell shrinkage "Fig. 7B". In this technique also, cells subjected to pretreatment with CPME showed a substantial reduction in the extent of nuclear damage, and this protective effect of CPME was more marked in those cells treated with 25 µg/mL CPME (Fig.7D) as compared to cells treated with 10 µg/mL CPME "Fig. 7C" or 100 µM quercetin (Fig. 7E).

In the PI staining technique, cells treated with MPP+ exhibited increased red fluorescence, which indicated induction of apoptosis and associated nuclear changes like chromatin condensation "Fig. 8B" compared to untreated cells (Fig. 8A). In contrast, those cells subjected to pretreatment with CPME (10 and 25 µg/mL) and 100

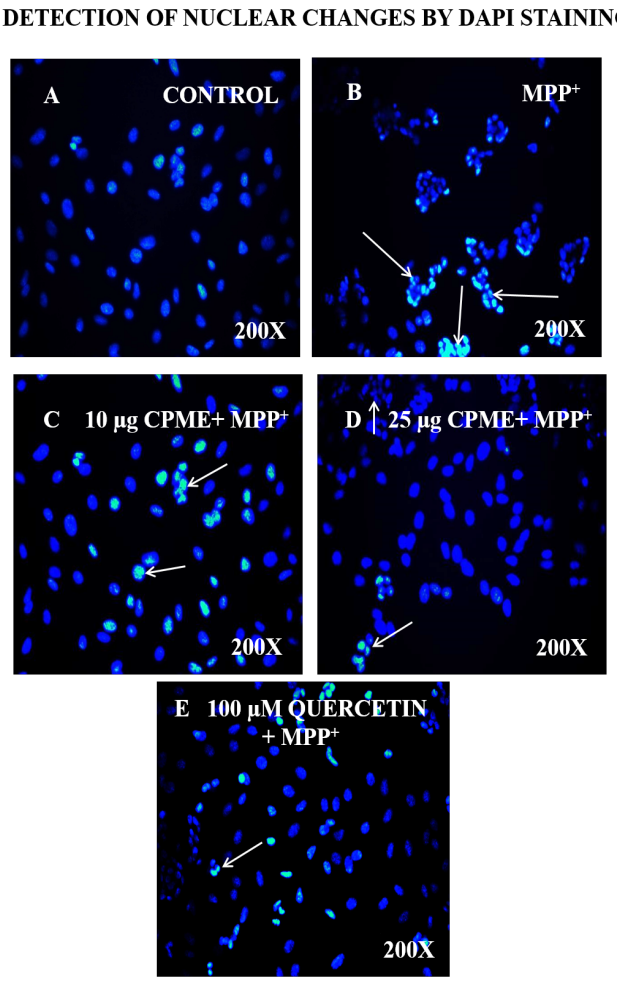

Fig. 7: Detection of nuclear changes by DAPI staining
A -Vehicle control, B - MPP^+ (250 µM) shows increased nuclear damage such as nuclear condensation, margination and cell shrinkage as compared to vehicle control cells, C-pretreatment with 10 µg/mL of CPME against MPP^+, D-pretreatment with 25 µg/mL of CPME MPP^+ and E-pretreatment with 100 µM of quercetin against MPP^+ showed reduced nuclear condensation as compared to MPP^+ (250 µM) induced cells. Magnification-200X.

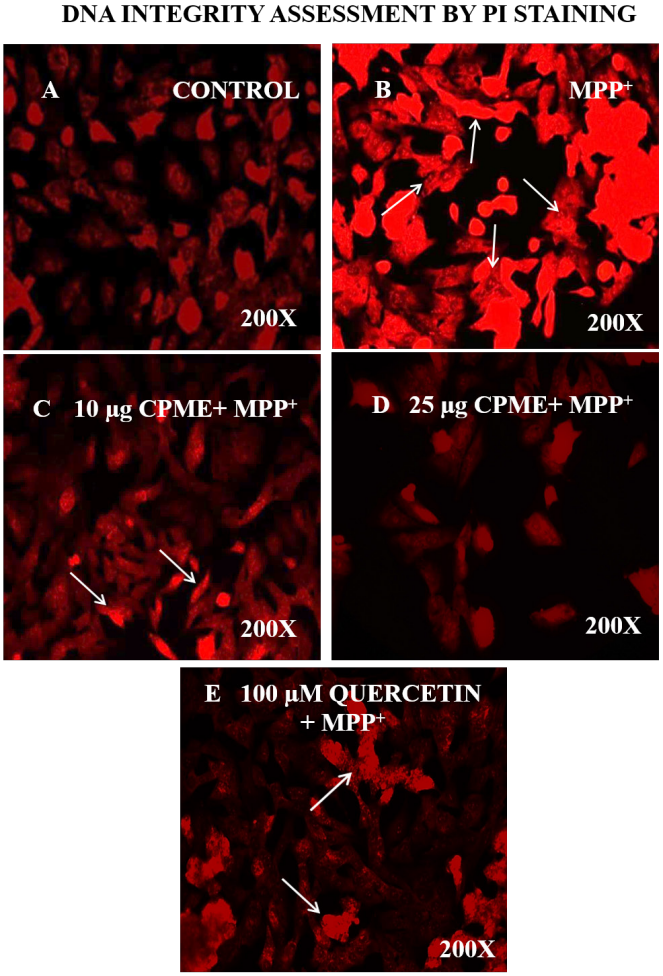

Fig. 8: DNA integrity assessment by PI staining
A -Vehicle control, B - MPP^+ (250 µM) shows increased red fluorescence as compared to vehicle control cells suggesting induction of apoptosis and associated nuclear changes like chromatin condensation, C-pre-treatment with 10 µg/mL of CPME against MPP^+, D-prereatment with 25 µg/mL of CPME MPP^+ and E - Pre - treatment with 100 µM of quercetin against MPP^+ showed decreased red fluorescence as compared to MPP^+ (250 µM) induced cells. Magnification - 200X.

µM quercetin prior to challenging with MPP^+ showed decreased red fluorescence (Fig. 8C, Fig. 8D) and "Fig. 8E". Here also, the protective effect of CPME was particularly more evident with cells treated with 25 µg/mL CPME "Fig. 8D". Altogether, based on the above observations, it may be assumed that CPME at 25 µg/mL concentrations exhibited superior protective effects against MPP^+-induced apoptotic changes.

Discussion

The current strategies adopted for treating Parkinson's disease provide only symptomatic relief. Some of the commonly used anti-Parkinson's drugs, such as dopamine agonists or monoamine oxidase inhibitors, apart from producing severe side effects, become less effective on prolonged use. Hence, incessant efforts are being made by biomedical scientists worldwide for identifying new drug candidates that can alleviate the progressive degeneration of dopaminergic neurons.

As plant-based therapeutic approaches offer several health benefits without much side effects, increased attention on herbal medicines to identify of potent phytochemical constituents has been recorded in recent years. As each plant possesses several bioactive molecules, attribution of their health-promoting effects of medicinal herb/plant food to one or two phytocompounds may become inappropriate. Diverse groups of phytochemicals likely produce synergistic effects *in vivo*, thus amplifying (or reducing/inhibiting) their activities.[26]

Human SH-SY5Y neuroblastoma cells treated with MPP^+ have been widely used as an established model to study the neurodegenerative events that occur in PD.[27] In

this study, efforts are taken to demonstrate the protective effects of methanolic extract of CPME against MPP^+ induced changes in undifferentiated SH-SY5Y cells by pretreatment with two different concentrations of CPME namely 10µg and 25µg/mL and 100 µM quercetin as positive control prior to exposure to the neurotoxin MPP^+. It has been reported that MPP^+ accumulates in mitochondria within dopaminergic neurons and selectively inhibit the activity of complex I of the electron transport chain.[28] Inhibition of complex I guide the flow of electrons through complex-II, which results in increased generation of ROS and thus mitochondrial damage and subsequent cell death.[29]

In the present study, loss in viability was observed in MPP^+ treated SH-SY5Y cells while treatment with CPME alone at different concentrations did not affect the viability of SH-SY5Y cells. Remarkably, pretreatment with CPME attenuated the MPP^+ induced loss in cell viability. This observation can be corroborated with the findings made in an earlier study where H_2O_2 induced loss in cell viability in SH-SY5Y cells was found to be restored to normal upon treatment with curcumin at 20 µM concentration.[30]

Increased ROS can lead to mitochondrial oxidative damage and subsequent activation of neuronal apoptosis.[31-33] Unabated ROS levels can also trigger the oxidation of various cellular components such as lipids, enzymes, and DNA, thereby altering signaling pathways that culminate in cell death.[34] Reports are available on the damaging effect of oxidative stress at the mitochondrial and DNA level in the autopsy PD brain samples and the CSF of live PD patients.[35] There is increasing evidence that oxidative stress plays a key role in the pathogenesis of PD.[36] In the present study also, increased ROS levels were recorded in MPP^+ alone treated SH-SY5Y cells, and interestingly cells subjected to pretreatment with CPME at 25 µg/mL concentration displayed significantly reduced ROS levels. This ROS quenching effect of CPME (25µg/ml) was comparatively better than that of positive control quercetin used in this study.

It has been reported that the loss of mitochondrial membrane potential increases the mitochondrial permeability and results in the release of cytochrome c, which triggers the activation of caspase 9/3 and ultimate cell death.[37] In the present study, a significant reduction in the mitochondrial membrane potential was observed in MPP+ alone treated SH-SY5Y cells that specified mitochondrial membrane damage. Strikingly, those cells pretreated with 25 µg/mL CPME showed appreciable restoration of mitochondrial membrane potential than those treated with 100 µM quercetin or 10 µg/mL CPME.

Previous reports have indicated that unrestrained ROS levels induce apoptotic changes in various types of cells including neuronal cells by activation of caspase 3 like proteases.[38-40] Induction of apoptosis in target cells is normally associated with specific morphological changes such as dynamic membrane blebbing, loss of adhesion to neighboring cells, chromatin condensation, DNA fragmentation by specific endonucleases (CAD), cell shrinkage, formation of apoptotic bodies etc. [41]. Reports are available on the possible interplay of ROS in the apoptotic mechanism of MPP^+-mediated neurotoxicity.[42,43] In the present study, phase-contrast microscopic analysis indicated cytomorphological changes such as cell shrinkage, membrane blebbing and detachment of cells in MPP^+ treated SH-SY5Y cells. In order to further investigate the changes in nuclear morphology characteristic of apoptotic cells, experiments were performed using AO/EB, DAPI, and PI staining methods in SH-SY5Y cells with and without CPME pretreatment. The commonly used dual staining method with Acridine orange/ethidium bromide (AO/EB) stains helps distinguish cells in different apoptosis stages.[44] Acridine orange is a cationic dye which can enter normal cells as well as early apoptotic cells with intact cell membranes and emits green fluorescence upon binding to DNA. On the other hand, ethidium bromide is an intercalating agent that can enter only into cells that have lost their membrane integrity, such as late apoptotic and dead cells, and emit orange-red fluorescence upon binding to concentrated DNA fragments or apoptotic bodies.[45] Therefore, this staining technique is used widely to distinguish normal cells from early or late apoptotic cells where the appearance of yellow-green color represents early apoptosis; orange color indicates late apoptosis and dark red color denote necrosis.

In the present study, while control cells emitted green fluorescence with intact nuclear architecture, MPP^+ treated cells were found to display yellow-green as well as orange-colored apoptotic bodies with condensed nuclei indicating early and late apoptosis. However, CPME pretreatment at 25 µg/ml showed conspicuous protective effects against MPP^+ induced apoptotic changes by reducing the number of granular yellow-green colored and orange-colored apoptotic bodies. Moreover, this effect was more evident than the positive control group (100 µM quercetin), which exhibited few orange-colored apoptotic bodies with condensed nuclei.

During apoptosis progression, the nuclear material undergoes a phase change from a genetically active chromatin network to an inert and highly condensed form. The DAPI staining technique performed in this study also indicated that MPP^+ alone treated SH-SY5Y cells displayed increased fluorescence intensity of the nuclear stain DAPI suggesting nuclear condensation/fragmentation. On the other hand, CPME pretreatment (25µg/mL) resulted in reduced nuclear condensation and cell shrinkage and this effect was better than that observed in quercetin and 10 µg/mL CPME pretreated cells. Similar findings have been observed in an earlier study with alpha mangostin treatment wherein MPP^+ induced cell death and apoptotic nuclear changes in SH-SY5Y cells were alleviated by co-treatment with 10 µM alpha mangostin.[46] The other technique used in this study to detect nuclear condensation was Propidium Iodide (PI) which is an intercalating agent

that can enter permeabilized or fixed cells, bind to DNA, and emit red fluorescence. In this study, the MPP^+ alone treated SH-SY5Y cells exhibited increased red fluorescence confirming the induction of apoptosis and associated nuclear changes.

On the other hand, cells pretreated with 25µg/mL CPME showed a substantial reduction in the fluorescence intensity indicative of their protective effects, and this effect was more pronounced than that observed with quercetin (positive control) as well as 10µg/mL CPME pretreatment. Similar protective effects have been observed with Ginsonide Rd (GSRd), which is one of the active compounds from the medicinal plant Panax ginseng against the MPP^+ induced cell death in SH-SY5Y cells.[47] Altogether, findings from this study substantiate the neuro-protective effects of CPME and among the two doses of CPME used in this study, 25 µg/mL of CPME exhibited remarkable potential.

In brief, our results indicated that pretreatment with CPME protected SH-SY5Y cells from MPP^+ induced cell death by ameliorating the ROS production, mitochondrial dysfunction and nuclear damage. Data obtained from this work denote the neuroprotective effects of methanolic seed extract of *C. paniculatus* (CPME) and provides avenue for identifying novel therapeutic lead molecules for neurodegenerative diseases. However, further preclinical studies are essential in order to validate the neuroprotective effects of CPME.

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