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IN VITRO NEUROPROTECTIVE POTENTIAL OF *CRESCENTIA CUJETE* LEAVES AGAINST MPTP- INDUCED TOXICITY IN SH-SY5Y NEUROBLASTOMA CELLS

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ABSTRACT

Objective: *Crescentia Cujete* are used traditionally to treat neurological disorders. The aim of this study was to evaluate the *invitro* neuroprotective potential of the plant, after induction of toxicity using MPTP, in SH-SY5Y neuroblastoma cells. **Methods:** In the present study, it is to investigate the effect of ethanolic extract of *Crescentia cujete* leaves on MPP_+ induced cell viability by MTT assay, SRB assay, Trypan blue exclusion assay, Lactate Dehydrogenase (LDH) and generation of reactive oxygen species (ROS) were measured in SH-SY5Y neuroblastoma cells. **Results:** The ethanolic extract of *Crescentia cujete* showed an IC50 of 159.29 µg/mL with R^2 value of 0.947 in MTT assay, SRB assay showed an IC50 was 162.5µg/ml with R^2 value of 0.884 and Trypan blue exclusion assay form a level of inhibition produced by different concentration increased with increasing concentration. Pre-treatment with the concentration of *Crescentia cujete* significantly diminished the release of LDH induced by MPP+ as LDH increases proportionally to the number of lysed cells and the ROS levels generated in response to ethanol extract of *Crescentia cujete* were significantly reduced in MPP+ treated SH-SY5Y cells than the standard **Conclusions:** The cell viability assays clearly show that the ethanol extract of *Crescentia cujete* leaves have the ability to protect SH-SY5Y cells against damage induced by MPTP and offer neuroprotection *in vitro*.

KEYWORDS: *Crescentia cujete*, Parkinson's disease, SH-SY5Y Neuroblastoma cells, MPTP.

INTRODUCTION

Parkinson's disease (PD) is a progressive neurological disorder, characterized by partial loss of dopaminergic neurons in the ventral midbrain area's substantia nigra (SN), resulting in a corresponding decrease in striatum levels of dopamine. Loss of dopaminergic supply to striatum results in imbalance with neurotransmitters such as acetylcholine and dopamine, leading to symptoms of PD.[1] PD is one of the most extensively studied neurodegenerative disorders, where the parkinsonian neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+)-mediated SH-SY5Y cellular toxicity the active metabolite of 1-methyl-4-phenyl-2,3,6-tetrahydropyridine (MPTP), is used as an in vitro model of the disease. MPP^+ is actively transported into dopaminergic neurons through the plasma membrane upon accumulation by DA transporter.[2] The molecule is sequestered into mitochondria where it shows selective inhibition of complex I of the electron transport chain (ETC). This inhibition of ETC complex I interfere with ATP production, disrupts mitochondrial membrane potential, and leads to the formation of reactive oxygen species (ROS).[3]

Currently, there are no specific or effective satisfying agents to limit neuronal damage and neurological dysfunction without disagreeable side-effects. Thus, there is a need to develop new protective representatives that can stop the advance of such neuronal apoptosis. Medicinal plants have long been known throughout the world for their unique and valuable benefits. Antioxidant activity dependent on disease cure or safe lifestyle maintenance may be the scientific basis of conventional herbal medicines such as those used in Ayurveda.[4] These plants are used in many countries for centuries due to their security, productivity, adequacy and comparably fewer side effects than chemical drugs.[5]

In the present study, the effect of *Crescentia Cujete* Leaves on neuroinflammation and intracellular ROS generation in MPP+‑induced neurotoxicity in human neuroblastoma SH‑SY5Y cells were investigated.

METHODS

Preparation of ethanolic extract of *Crescentia cujete* leaves

Fresh leaves of *Crescentia cujete* was collected from Anaikatti, Coimbatore. The samples were authenticated by Botanical Survey of India, Coimbatore. Voucher sample was prepared

and deposited in the Herbarium for reference. For preparation of extract, the dried leaves were pulverized and mixed with 50% ethanol in the ratio of 1:4 (w/v) and kept at 150 rpm in an orbital shaker for 72h and filtered through Whatmann No.1 filter paper. The filtrate was condensed in rotary evaporator and lyophilised. This lyophilised sample was used for *in vitro* assays.

Cell lines and culture medium

All the cell lines were procured from ATCC, stock cells were cultured in DMEM/ F12 supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO_2 at 37^oC until confluent. The cell was dissociated with a cell dissociating solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells is checked and centrifuged. Further, 50,000 cells well of Jurkat was seeded in a 96 well plate and incubated for 24 hrs at 37^oC, 5% CO_2 incubator.

MTT assay for cell viability

Cellular viability was measured in a 96- well plate by quantitative colorimetric assay with MTT, which represents a suitable indicator for mitochondrial activity of living cells (12). After the treatment schedule, medium was removed and the cells were incubated with 0.2.5 mg/ml MTT for 4 h at 37˚C. The reaction was stopped by adding DMSO. The amount of MTT formazan product was determined by measuring absorbance in a microplate reader at a test wavelength of 570 nm and a reference wavelength of 630 nm.[6] The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for each cell line.

% Inhibition = 100 – (Mean OD of individual test group / Mean OD of Control group) x 100.

Morphological evaluation of SH-SY5Y cells

Cells were cultured and grown at a density of 2.2x104 cells/well into 6‑well plates. SH‑SY5Y cells were treated with 0.1 mg/ml ethanol extract of *Crescentia cujete* leaves, after 24 h, cells were removed from the wells and centrifuged at 500 x g for 3 min. The supernatant was withdrawn from the tubes and phosphate‑buffered saline (PBS) was added to the cells. Cell volume was adjusted to 105‑106 cells/ml. A total of 95 µl cell suspension was

added to the microtube, and 5 µl acridine orange (AO), was added to the same tube. Cells were incubated at 37˚C for 15‑30 min in the dark. Subsequently, 10 µl cell staining solution was added to a glass slide and covered with a cover glass.[7] Cells were observed with a fluorescence microscope (Axiovert 2000; Carl Zeiss AG, Oberkochen, Germany).

SRB assay for cell viability

After the 24 h exposure period, 100 µL of the supernatant was aspirated from the wells and replaced with 100 µL of cold trichloroacetic acid (TCA) solution (30% w/v). Each plate was then incubated at 4°C for 1 h to fix cells to the plate. After incubation, the plate was gently washed with water (four times) to remove excess TCA. The plate was dried in a low-temperature oven, after which 100 µL of 0.057% (w/v) SRB solution was added to wells to stain the cellular protein contents. The plate was incubated for 30 min at 4°C and washed twice with 200 µL of a 1% acetic acid solution (v/v) to remove excess unbound dye. The plate was rinsed four times with 1% acetic acid to remove unbound dye. Plate was allowed to air-dry at room temperature. 50µl of 10mM Tris base solution (pH 10.5) was added to each well and the plate was shaken on an orbital shaker for 10 min to solubilize the protein-bound dye. Absorbance was measured at 560 nm in a plate reader.[8]

Measurement of cell viability by Trypan blue exclusion assay

SH‑SY5Y cells were incubated with different concentrations of EECC for 24 h, then added 1 mM MPP+ and incubated for 2 h. Following overnight incubation of fresh media with EECC, the number of viable cells was determined using trypan blue exclusion assay. Cells were collected and rinsed with PBS. After which they were immediately stained with 0.4% trypan blue solution, and the number of viable cells were counted using a haemocytometer under a light microscope.[9]

Lactate dehydrogenase (LDH) assay for cell membrane damage

The amount of LDH released into the medium is an index of cell membrane damage because of the enzyme's high intra cellular localization. The plasma membrane damage was evaluated by measuring extracellular LDH activity in the medium. SH-SY5Y cells were pretreated with concentrations of the ethanolic extract of *Crescentia cujete* leaves and then exposed to 1mM MPP+ for 24 h. After the incubation, 50 µl of culture supernatants were collected from each well. The LDH activity was determined with a colorimetric LDH assay kit. Total cellular LDH activity was determined by solubilizing the cell with 0.2. % Triton X-100.[10] The

release of intracellular LDH to the extracellular medium was expressed as a percentage of total cellular LDH activity.

Determination of reactive oxygen species production

ROS was determined using a fluorescent probe, 2',7'- dichlorofluorescin diacetate. SH-SY5Y cells were seeded in 96-well plates in growth medium at a density of 4,000 cells/well under standard conditions. Cells were treated with ethanolic extract of *Crescentia cujete* leaves (0.05 and 0.1 mg/ml). After 24 and 48 h, the medium was removed, and the cells were incubated with 5 µM DCFH-DA in the growth medium for 30 min at 37˚C and 5% CO2.[11] Fluorescence was measured at 24 and 48 h using a fluorescent plate reader at excitation emission (490 and 525 nm respectively).

Statistical evaluation

IC50 Value

The IC50 of a drug can be determined by constructing a dose-response curve and examining the effect of different concentrations of antagonist on reversing agonist activity. IC50 values can be calculated for a given antagonist by determining the concentration needed to inhibit half of the maximum biological response of the agonist.

IC_{50} values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoid dose response curve (variable) and computed using Graph Pad Prism 6 (Graph pad, San Diego, CA, USA).

RESULTS AND DISCUSSION

MTT assay for cell viability

The MTT is a yellow substratum for mitochondrial dehydrogenases in a surviving cell that generates a blue product (formazan) measurable by photometric techniques.[12] In order to assess the effects of MPP_+ and EECC leaves (10, 20, 40, 80,160 and 320 µg/mL) on cell viability, MTT assay was carried out. The percentage of cell viability was measured by comparing the optical density (OD) against the control. As the concentration increased the cell proliferation percentage increased, these results indicated that MPP+ had pronounced roles in inhibiting cell survive. Based on the findings, 24 h cell treatment with various doses of the extract (10, 20, 40, 80,160 and 320 µg/mL) exerted no major effects on the viability of the cells (Figure. 1). Incubation of the extract at 160 and 320 µg/mL could majorly inhibit

MPP+ mediated toxicity; however, it did not inhibit cellular damage at doses of 40 and 80 µg/Ml.

The *in vitro* percentage viability, IC50 and the regression analysis is reported in Table for the ethanolic extract of *Crescentia cujete*. The IC50 for EECC was arrived from the dose response curve and was found to be 159.29 µg/mL with R^2 value of 0.947 which suggests that *Crescentia cujete* exhibit toxic effects only at higher concentrations.

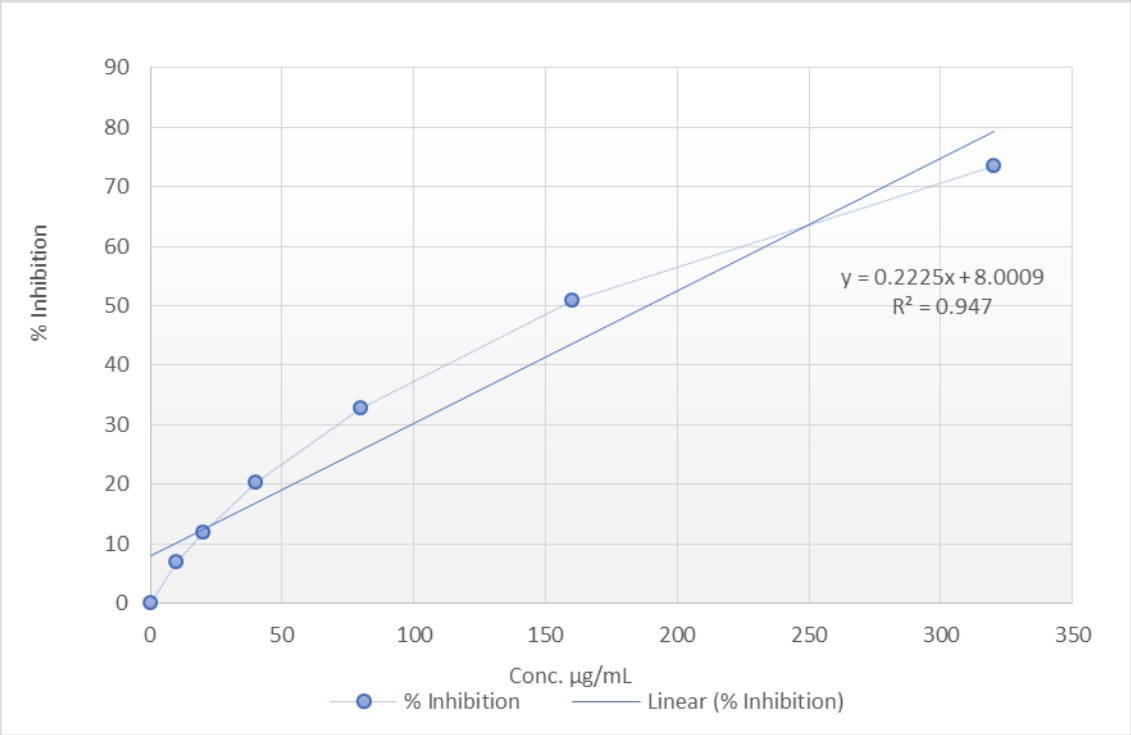
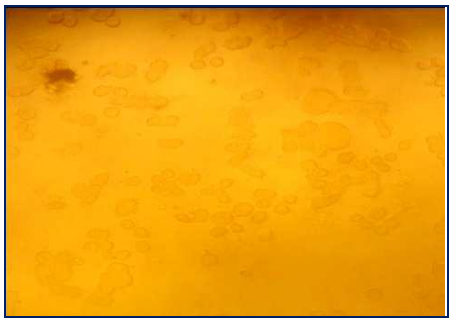


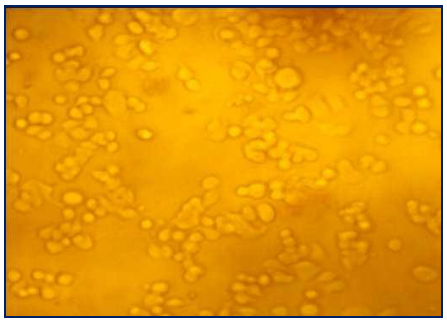
Figure 1: MTT assay % Cell Inhibition and IC50 of ethanolic extract of Crescentia *cujete* leaves.

Morphology of SH-SY5Y cells following ethanol extract of *Crescentia cujete* leaves pretreatment

Morphological evaluation of the SH-SY5Y cells treated with MPP+ alone showed shrinkage and rounding up of cell bodies while ethanol extract of *Crescentia cujete* leaves pre-treatment appeared to have prevented this MPP+-induced morphological alterations. On the other hand, the morphology of cells exposed to ECC alone appeared similar to that of the untreated cells as shown in plate 1

(A) NORMAL (B) SH-SY5Y cells treated with MPP+ alone





(C) SH-SY5Y cells pre-treated with ECC (D) SH-SY5Y cells treated with ECC only and then treated with MPP+

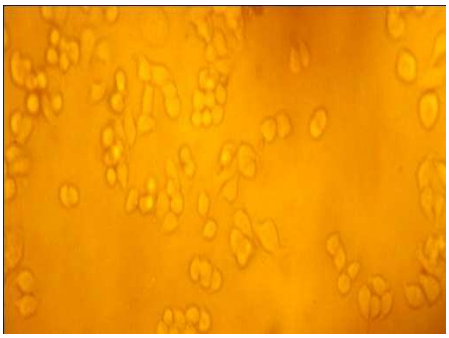


PLATE 1: Effects of treatment with ECC extract on MPP+ induced morphological alterations in SH-SY5Y cells.

SRB (Sulpho Rhodamine B) assay on SH-SY5Y neuroblastoma cell line

SRB assay is one of the major techniques used to assess the cell growth. The cell response to plant extract was by dose dependent manner (10, 20, 40, 80,160 and 320 µg/mL). Great cytotoxic effect was seen with increase in concentration (Figure 2). At concentrations from 10µg/ml to 320 µg/ml, EECC pre-treatments showed protective effects against cytotoxicity. When cells were pre-treated with 10–40 µg/ml cc, the cell viability was significantly higher than that of the control cells. The IC50 was 162.5µg/ml µg/mL with R^2 value of 0.884.

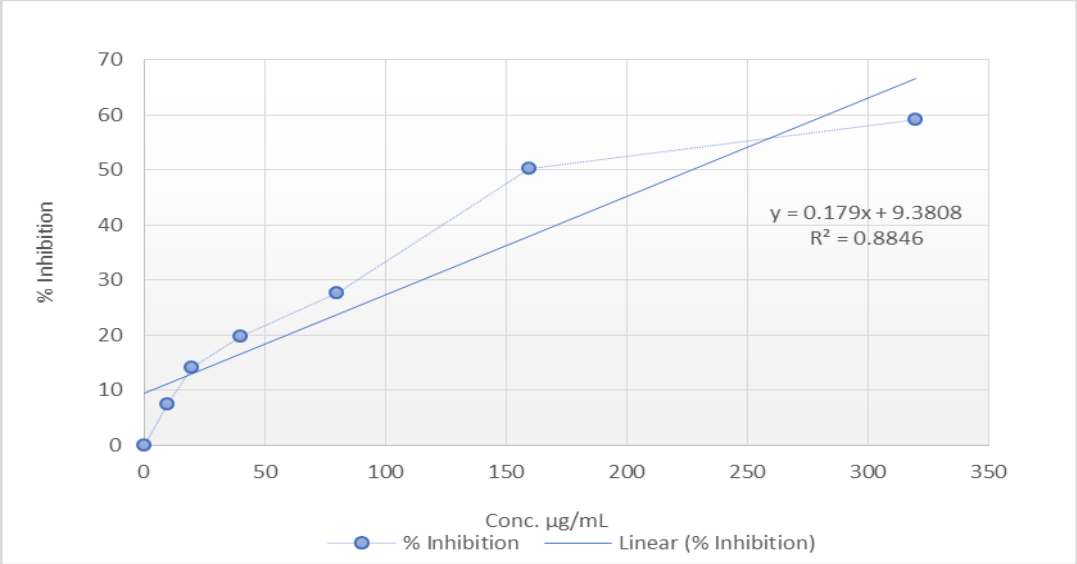


Figure 2: Cytotoxic effect of ethanol extract of *Crescentia cujete* leaves on SH-SY5Y cell line by SRB Assay.

Trypan blue exclusion assay on SH-SY5Y neuroblastoma cell line

The trypan blue test was performed to determine changes in the viability of each cell line after being exposed to the EECC with respect to the time. EECC at increasing concentrations (10, 20, 40, 80,160 and 320 µg/mL) reduced the cell viability in a concentration dependent manner when compared with control (Figure 3) The level of inhibition produced by different concentration increased with increasing concentration.

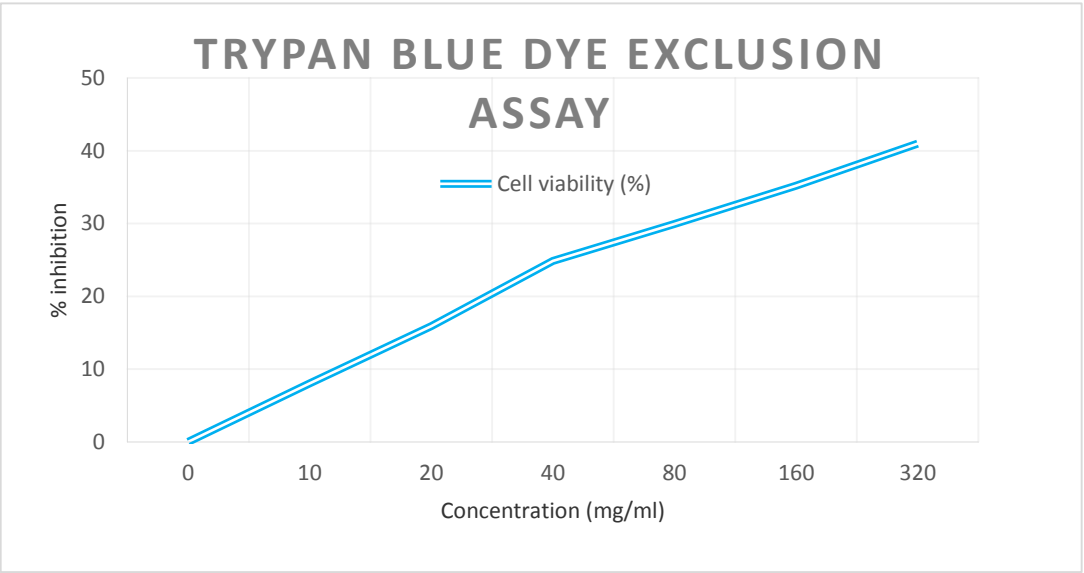


Figure 3: Effect of ethanol extract of *Crescentia cujete* leaves on trypan blue dye exclusion assay.

Lactate dehydrogenase (LDH) assay for cell membrane damage

The release of LDH is more pronounced during necrotic cell death compared to apoptotic cell death. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage. This assay is based on the principle where, LDH oxidizes lactate to pyruvate which then reacts with tetrazolium salt to form formazan the amount of which directly relates to the increase in the number of lysed cells.[13] In high concentration (160 and 320 µg/ml of EECC alone treatment to SH-SY5Y cells induced exhibited slight cytotoxicity, EECC protected against cell death induced by 1mM MPP+ pre-treatment with the concentration of 10 and 20 µg significantly diminished the release of LDH induced by MPP+ as shown in figure 4.

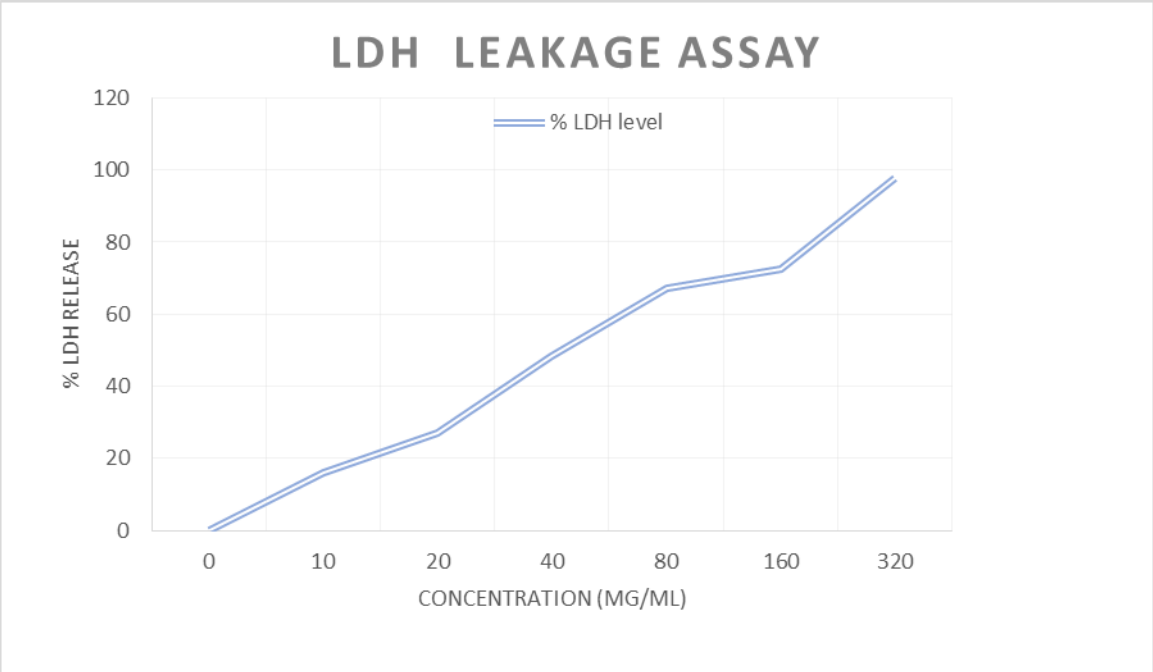


Figure 4: Effect of ethanol extract of *Crescentia cujete* leaves on SH-SY5Y neuroblastoma cell line by LDH Leakage Assay.

Generation of free Radicals

The principal aim of MPP_+ is the mitochondria, where it inhibits Complex I in the mitochondrial respiratory chain, with the consequential termination of oxidative phosphorylation. It is known to bind to complex I of the electron transport chain, preventing the transfer of electrons from iron sulphur clusters to ubiquinone [14], reducing oxidative phosphorylation, and generating more and more reactive oxygen species. Several studies have shown the involvement of radical oxygen species (ROS) in MPP+ -induced neurotoxicity. MPP+ treatment increased the levels of ROS. Oxidative stress, characterized

by increased levels of reactive oxygen species and diminished levels and activities of antioxidants, also take a place in the death of dopaminergic cells.[15]

ROS have an essential role in facilitating signal transduction processes within the intracellular region. Intracellular ROS generation was determined using a DCFH-DA fluorescent probe. Fluorescence analysis indicated that the green fluorescence intensity of DCF was significantly reduced in the treated cells, as compared with the control cells (Figure 5). Furthermore, fluorescence analysis indicated the apparent action of *Crescentia cujete* leaves extract on intracellular ROS generation in a dose dependent manner.

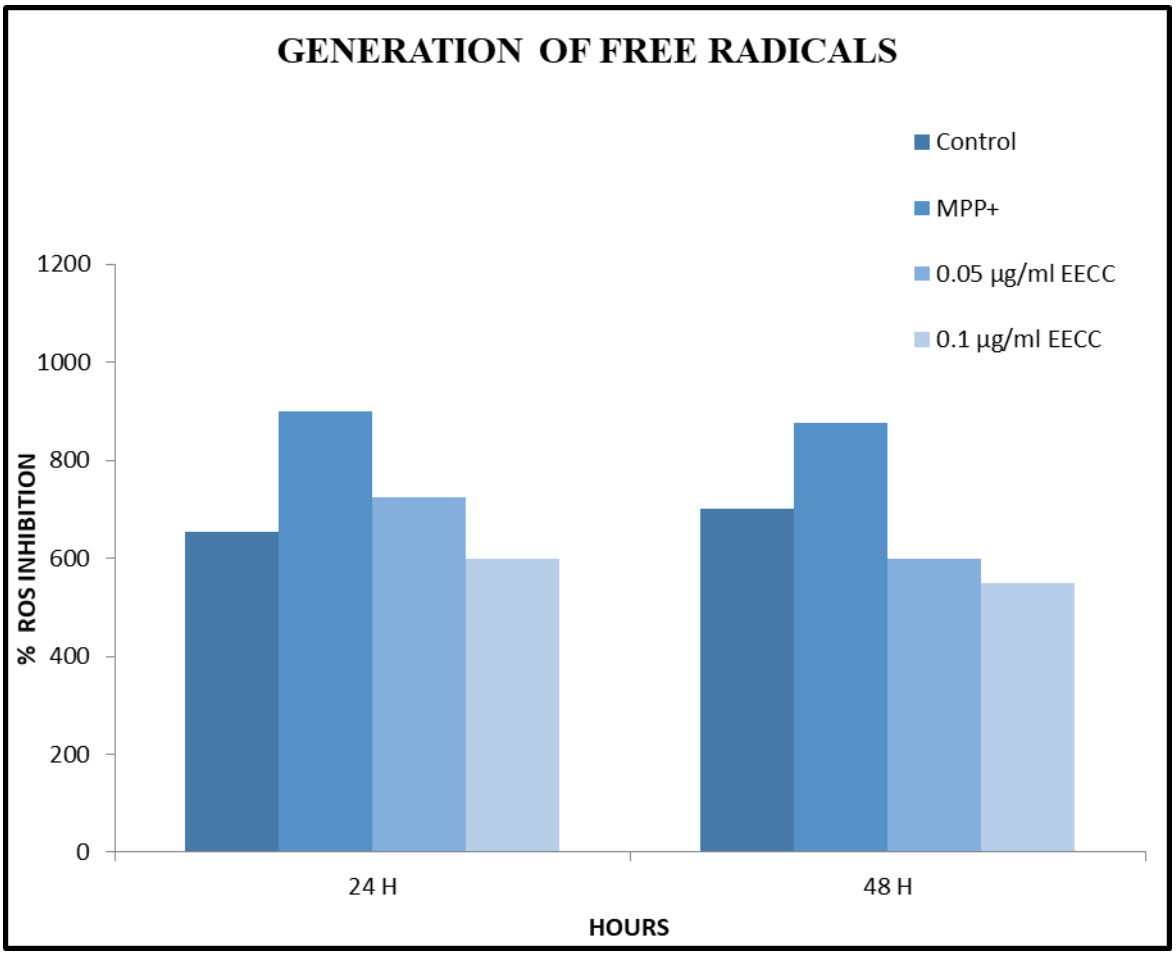


Figure 5: Effect of ethanolic extract of *Crescentia cujete* leaves on generation of free radicals (ROS Levels).

CONCLUSION

The cell viability assays clearly show that the ethanol extract of Crescentia cujete leaves have the ability to protect SH-SY5Y cells against damage induced by MPTP and offer neuroprotection in vitro. This protective effect of Crescentia cujete leaves might be mediated

by its potent antioxidant properties. The observed effect of this medicinal plant can be further analysed by *in vivo* models of Parkinson's diseases and the plant can be used to develop new drugs that can treat neurodegenerative diseases.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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