RESEARCH PAPER

Neuroprotective Role of Proline Isolated from *Cassia alata Linn*. in Hydrogen Peroxide Induced Oxidative Stress and Cellular Dysfunction

Shanmugam V M[1,2]., Manjula. K. R[1]*. and Jolitha A. B.[3]

[1]*Department of Biotechnology, School of Applied Sciences, REVA University, Bengaluru – 560064, Karnataka, India*
[2]*Department of Biotechnology, Jyoti Nivas College Autonomous, Bengaluru- 560095, Karnataka, India*
[3]*Department of Biotechnology, Maharani Lakshmi Ammanni College for Women, Autonomous Bengaluru –560012, Karnataka, India*

Corresponding Author:
Dr. Manjula K.R
School of Applied Sciences, REVA University, Rukmini Knowledge Park, Kattigenahalli, Yelahanka, Bengaluru – 560064, Karnataka, India. E-mail address: manjula.kr@reva.edu.in

ABSTRACT
Oxidative stress mediated neuronal loss due to misfolded protein is the main etiological hallmark for various progressive degenerative disorders of neurons. The phytocompounds and osmolytes from medicinal plants are increasingly explored as multifunctional antioxidant, anti-inflammatory, and neuroprotective abilities. Current research study is to investigate proline, as osmolyte extracted from the *Cassia alata Linn*. leaves, for its ability to protect SH-SY5Y neuroblastoma cells from oxidative stress induced by hydrogen peroxide. Proline was extracted and its purity was confirmed by RP-HPLC, showed a single peak (99.99% area coverage), retention period of 3.0369 minutes, indicating high abundance and purity of proline. Neuroprotective efficacy was evaluated using MTT assay and phase contrast microscopy. Proline treated cells exhibited significantly improved viability and preserved morphology compared to hydrogen peroxide treated cells. Mitochondrial function measured by JC-1 staining, demonstrated that proline pretreatment restored mitochondrial membrane potential observation ($\Delta\Psi m$) and reduced H_2O_2 induced depolarization, indicating the restored mitochondrial structural integrity. Flow cytometric analysis further showed that proline at 125 µg/mL reduced *COX-2* expression to a mean fluorescence intensity (MFI) of 1756.33 ± 296.49 compared with 2818.33 ± 780.13 in cells treated with 300 µM H_2O_2 alone, highlighting attenuation of oxidative stress associated inflammatory signalling, aligned with other antioxidant interventions in SH-SY5Y cells. Collectively, these findings indicate the proline from *Cassia alata Linn*. functions as a potent antioxidant osmolyte that limits the oxidative damage, maintains mitochondrial membrane integrity along with its functions and suppress *COX-2* linked inflammatory responses in neuronal cells, supporting its potential as plant derived neuroprotective candidate

Keywords: Inflammatory Biomarker, *COX-2*, Hydrogen peroxide, mitochondrial integrity, oxidative stress.
How to cite this article*:* Shanmugam VM, Manjula KR, Jolitha AB. Neuroprotective Role of Proline Isolated from Cassia alata Linn. in Hydrogen Peroxide Induced Oxidative Stress and Cellular Dysfunction. Int J Drug Deliv Technol. 2026;16(56s): 694-702. DOI: 10.25258/ijddt.16.56s.73
Source of support: Nil.
Conflict of interest: Nil.

INTRODUCTION

Progressive neuronal dysfunction accompanied by oxidative stress, misfolded proteins, loss of synaptic transmission, mitochondrial dysfunction and chronic neuroinflammation characterizes degenerative of neurons in age related disorders such as Parkinson's disease (PD), Alzheimer's disease (AD)[1]. Numerous research documentation on clinical and experimental evidences suggest that oxidative stress is a key etiological and pathogenic mechanism for these disorders, causing neuronal damage through excessive ROS production, lipid peroxidation, depolarization across membrane of mitochondria led to activated proinflammatory & proapoptotic signaling pathways[2-4]. To address these issues and to develop neuroprotective treatments for amyloid toxicity, AD and other related disorders, it has become crucial to identify bioactive compounds that can reduce oxidative stress, stabilize mitochondrial functions and suppress various neuroinflammatory cascades metabolic pathways[5,6].

Neuroprotection can be achieved by utilizing the rich source of bioactive phytocompounds isolated and characterized from medicinal plants, which have been shown to reduce inflammatory actions, protection of cells against oxidative stress and its cytotoxic effects[7]. *Cassia alata Linn*., a widely used ethnomedical plant, has been traditionally employed for the treatment for various dermatophytes infections, inflammatory conditions, fungal and other microbial infections and metabolic pathway disturbances[8].

**Author for Correspondence: Dr. Manjula K.R*

According to the recent research studies, *Cassia alata Linn.* extracts have immunomodulatory effects by reducing proinflammatory mediators such as tumor necrosis factor-α (TNF-α)[9]. However, there is a limited work documented on the potential contribution of small amino acids such as proline, a natural osmolyte towards protection of neuronal cells. Proline normally expressed during stress conditions in plants to protect the cells from various environmental factors and establishing osmotic balance between cell and extracellular system. It helps in osmotic adjustment, ROS scavenging, protein and membrane stabilization[10-12].

Proline has been demonstrated to assist in redox buffering, quench singlet oxygen and superoxide and promote the cellular antioxidant defenses in addition to its traditional role in plant stress physiology[13]. According to recent research indicates, proline metabolism affects redox homeostasis, mitochondrial integrity and apoptosis in mammalian cells. Proline mediated stabilization of mitochondrial function and mitigation of ROS induced cellular injury may provide major therapeutic benefits in neuronal protection, since oxidative damage and mitochondrial dysfunction are important aspects of the etiology of neurodegenerative diseases[14-16].

To establish an effective in vitro paradigm for investigation causes of neuronal damage and its loss and screening potential neuroprotective drugs the effective SH-SY5Y human neuroblastoma cell lines are widely used as model cell line systems. Exposure of SH-SY5Y cells to hydrogen peroxide mimics the oxidative driven neuronal degeneration by causing excessive ROS production, loss of potential across mitochondrial membrane, decreased cell viability, activation of inflammatory mediators such as cyclooxygenase – 2 (*COX-2*) and initiation of apoptotic cell death pathways[17]. Hence, the compounds that attenuate H_2O_2 induced depolarization across mitochondrial membranes, damage due to oxidative stress and activation of inflammatory cascade mechanism in this model are therefore considered promising route for the development of promising neuroprotective drug.

By consideration of the potential gaps as mentioned, the current research study aimed to characterize the isolated proline using Reverse Phase – High Performance Liquid Chromatography (RP-HPLC) analysis and to investigate its neuroprotective efficacy through MTT assay, mitochondrial membrane potential (MMP) assay and flow cytometric analysis of *COX-2* inflammatory biomarker expression in SH-SY5Y neuronal cell lines. The findings of this study may contribute toward the development of plant-derived osmolyte-based neuroprotective therapeutics for oxidative stress-mediated neurodegenerative disorders.

2. MATERIALS AND METHODS

2.1 Procurement of plant sample

Cassia alata Linn. plant saplings collected from FRLHT, Bengaluru and authenticated by a botanist at Botany Department, Mysuru University, A voucher specimen (Voucher number UOMBOT24CA019) was deposited in the department herbarium. Fresh leaves of *Cassia alata Linn*. were collected from the treatment plants at 12 hours' time interval upon water stress and thoroughly washed with distilled water to remove contaminant and dust particles present over the surface of the plant.

2.2 Extraction and estimation of proline

The finely chopped leaf material served as a primary source for the extraction and isolation of proline. 500 mg of finely chopped leaf sample was subjected for homogenization by adding 3% sulfosalicylic acid (10 mL) under chilled conditions. Sulfosalicylic acid was used for its efficiently precipitating ability of proteins while releasing free amino acids. The resultant homogenate mixture was centrifuged for 10 minutes at 4^0C and 10000xg to obtain a clear supernatant containing soluble osmolytes and amino acids. The collected supernatant was used for further estimation and isolation procedures.

Proline content was estimated as natural osmolytes and biochemical marker for oxidative stress using the slight modification with acid ninhydrin method[18]. To 1.0mL of the cell extract, 1.0mL of acid ninhydrin reagent and 1.0mL of glacial acetic acid were added. The resultant reaction mixture was then incubated at 100^0C for 60 minutes in a thermoregulated water bath, leading to the formation of chromophore complex. The reaction was stopped by placing the tubes immediately on ice. 4 mL of toluene mixed vigorously with the resultant chromophore sample for 30 seconds, the chromophore containing toluene layer was separated and its absorbance was measured using a UV-visible spectrophotometer, calibrated at 520 nm. Proline concentration was quantified by preparing a standard curve using L-proline (ranging from 10 - 100µg/mL). The results thus obtained were expressed as µmol proline/g of fresh weight of plant extract.

$$\text{Proline (µmol/g FW)} = \frac{(C \times V_t)}{W \times V_s}$$

Where: C = Concentration of proline obtained from the standard curve (µg/mL)

V_t = Total volume of the extract (mL)

V_s = Volume of the extract used in assay (mL)

W = Fresh weight of leaf sample (g)

2.3 RP-HPLC analysis of Proline

The HPLC analysis of proline isolated from *Cassia alata Linn.* leaf extracts were carried out using RP-HPLC for characterization both qualitatively and quantitatively for the isolated osmolyte. The extracted proline fraction obtained after acid ninhydrin extraction was separated by 0.22 µm membrane filter filtration prior to chromatographic analysis. Chromatographic separation was performed using a C18 reverse-phase column (specification: Symmetry C18, 4.6 mm × 250 mm with 5 µm particle size). 70% methanol in distilled water (v/v), which was filtered and degassed before use and used as mobile phase. The analysis was carried out at the constant flow rate (1.0 mL/min) with an isocratic mode.

Standard L-proline solution (0.4 mg/mL) was prepared using the mobile phase solvent system. The plant extract sample containing isolated proline was dissolved in the same mobile phase at a concentration of 10 mg/mL. 20 µL

of both standard and proline solutions were subjected for separation by injecting into the HPLC instrument using an auto-sampler. The purity was detected by using UV–Visible detector maintained at 254 nm and the chromatographic run time was maintained for 30 min. The chromatograms obtained were analysed based on retention time (RT), peak area, height and percentage area normalization. Identification of proline in the sample was achieved by carrying out a comparison study of the retention time of the peak of sample and the standard L-proline chromatogram.

2.4 *In vitro* cell culture study

2.4.1 Cell line procurement and revival of cell culture

The neuronal cell line model SH-SY5Y cells were sourced from NCCS, Pune, and restoration of culture and preservation of cell cultures by using Dulbecco's Modified Eagle Medium (DMEM) with 10% foetal bovine serum (FBS) + 1% Penicillin-Streptomycin + % L-glutamine at 37°C in a humidified preset incubator containing 5% CO_2. Once the cell density achieved around 80–90% confluence, they were passaged using 0.25% trypsin EDTA solution. The trypan blue exclusion method was conducted to observe the cellular viability prior to conducting experiments.

2.4.2 Cytotoxicity and Neuroprotective Activity of *Cassia alata Linn.* against H_2O_2 induced toxicity

The MTT assay was performed to assess the cytotoxic and neuroprotective effects of proline. SH-SY5Y neuronal cell lines were seeded in 96 well plates at a density of 1 x 10^4 cells per well and treated with different concentration of proline ranging from 10 to 200 µg/mL) for one day (24 hours). Induction of oxidative stress was carried out by taking the and subjected to 150 to 300 µM H_2O_2 for 3 hours. Following treatment, 20 µL of MTT at the concentration of 5 mg/mL was added to each well and the mixture was incubated for four hours. The formazan crystals were dissolved in 150 µL of Dimethyl sulphoxide (DMSO) after the medium was decanted and the absorbance was measured at 570 nm using ELISA plate reader. The IC_{50} values were calculated using non-linear regression analysis and the cell viability was calculated in percentages relative to untreated control cells.

2.5 Mitochondrial membrane permeability assay

The neuroprotective effect of proline on mitochondrial membrane function and its integrity was carried out by taking the cells and pretreated with different concentrations of isolated proline obtained from *Cassia alata Linn.* leaf extracts for 24 hours. Oxidative stress induced mitochondrial dysfunction was generated by exposing the cells to hydrogen peroxide under optimized experimental conditions. The untreated cells served as normal control while hydrogen peroxide treated cells served as negative control for the comparison of the effect with the proline as treatment groups.

Following treatment, trypsinization method was employed for the isolation of cells and washed with 0.1M PBS (phosphate buffer saline, pH 7.4) for two times. the cellular pellet was resuspended with fresh serum free medium containing JC1 dye (final concentration of 2 - 5µM) and incubated in dark conditions for 30 minutes at 37^0C. Following the incubation, treatment of washing by using phosphate buffer saline (PBS) will minimize the background fluorescence by washing all the unbound stain, facilitate good experimental results by flow cytometric analysis. The cells that are stained were analysed by flow cytometer under 488 nm excitation laser, JC1fluroresence channel (PE; ~ 590 nm). In contrast to the cells with depolarized or damaged mitochondria, JC1 monomeric form will be formed and produced increased green fluorescence, healthy cells with intact mitochondria with membrane potential forms JC-1 Aggregates within mitochondria, creating strong red fluorescence. At least 10000 events were recorded for each sample, and changes in the polarization and membrane potential of mitochondria ($\Delta\Psi m$) were determined by computing the ratio of red to green intensity.

2.6 Flow Cytometric Analysis of *COX-2* expression

In a humidified CO_2 incubator, SH-SY5Y cell lines were sown in six well culture plates with a cell density of 3×10^5 cells/2 mL and cultured by incubating for the entire night at 37°C. Following a 24-hour period, the cells incubated further for a period of 4 hours at 37°C and treated with the required concentrations of proline prepared in 2 mL of complete culture media. Subsequently, all groups except the untreated control were stimulated with hydrogen peroxide (H_2O_2; 300 µM) to induce oxidative stress. To facilitate intracellular accumulation of expressed cytokines, 2 µL of protein transport inhibitor (BD GolgiStop™ containing monensin) was added to each well, to promote intracellular accumulation of cytokines. The cells were collected into a sterile 5 mL centrifuge tubes after the completion of treatment period, and centrifuged at 300 x g for 5 minutes at 25^0C. PBS was used twice to wash the cell pellet after the supernatant was carefully decanted. Following washing, the cells were fixed for 20 minutes at room temperature using a 0.5 mL paraformaldehyde solution and then they were washed with 0.5 % BSA prepared in 1X PBS. The cells were treated with 0.1% Triton X 100 prepared in 0.5 % BSA solution, incubated for 10 minutes and then rinsed once again with 0.5 % BSA in PBS to permeabilize them. The addition of diluted primary antibody (Final volume of 100 µL cell suspension), the cells were then incubated in the dark for 30 minutes at room temperature. Following incubation, the cells were thoroughly mixed, resuspended in 0.5 mL PBS, cleaned with 0.5% BSA and then subjected for flow cytometric analysis.

2.7 Data analysis using statistics

The results thus obtained from the various experiments were run independently in triplicates. The results were produced as mean ± SD. Statistical comparative analysis was carried out using GraphPad Prism software (version 10.0, GraphPad Software Inc., CA, USA). Differences across experimental groups were evaluated using one way

ANOVA followed by Tukey's post hoc multiple comparison tests to determine intergroup significance. The student's t test was used for pairwise comparison as needed. Statistical significance was defined as a p value of less than 0.05 ($p<0.05$).

3. RESULTS

3.1 Proline determination

Proline concentration showed a slight increase in response to the stress from 81.33 ± 1.33 µg/ml at 12 hours to 88 ± 1.20 µg/ml at 24 hours, indicating a modest initial rise in response to the treatment. Proline level increased to 127.33 ± 0.16 µg/ml by 36 hours, indicating a sharper accumulation phase than the previous time periods. Proline levels marginally dropped to 122.66 ± 2.1 µg/ml after 48 hours, indicating that proline accumulation peaks about 36 hours and is not sustained at the maximal level at subsequent time interval points. This temporal pattern suggests that proline is actively produced by the treatment, but after peaking at 36 hours, it may be partially digested, redistributed or downregulated to prevent the cellular damage due to stress. Proline serves more as a transitory osmolyte and signalling molecular during the mid phase of stress than as a continually accumulating solute, as seen by the slight decrease from 127.33 ± 0.16 µg/ml at 36 hours to 122.66 ± 2.1 µg/ml at 48 hours (Figure 1).

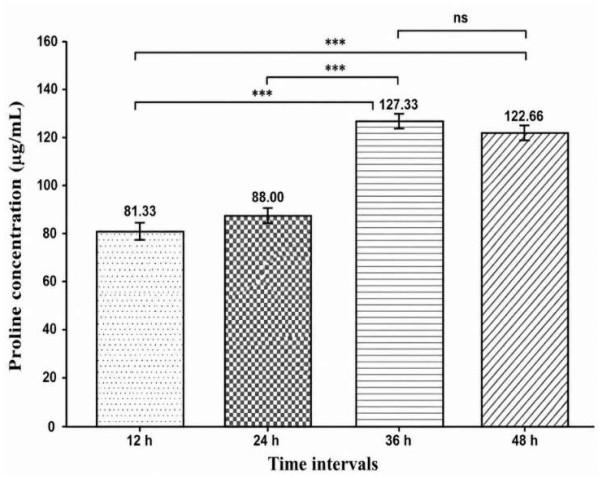

Figure 1: Estimation of proline concentration at different time interval. Data represented as mean ± SD (n =3); one way ANOVA followed by Tukey's multiple comparison test (ns indicates non-significant, *** $p < 0.001$).

3.2 HPLC analysis of proline sample

The chromatographic profile exhibited a sharp and well resolved major peak corresponding to the proline, indicating the successful isolation and purification from *Cassia alata Linn.* leaf extracts. The peak normalization and comparison with standard calibration data was performed. The HPLC chromatogram of proline showed a prominent and sharp peak at a retention time (RT) of 3.039 min. this peak accounted for 99.9% of the total area, indicating that the isolated fraction was predominantly proline with very high purity. The minor peak was observed at 2.483 min contributing 0.01% of the total area, suggesting the presence of negligible impurities. The high area and height of the major peak confirm the abundance of proline in the isolated sample (Figure 2).

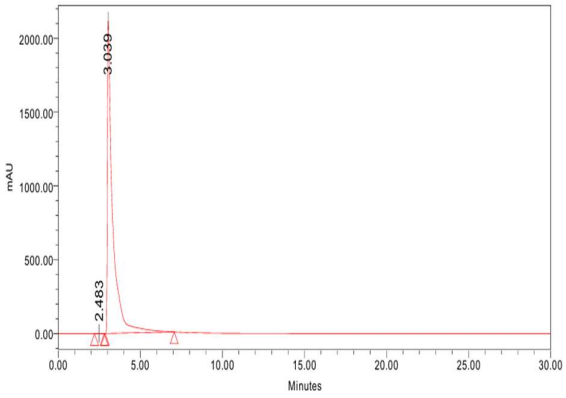

Figure 2: RP-HPLC chromatogram for proline extract from *Cassia alata Linn.* leaf sample

3.3 Cell culture cytotoxic study

The phase-contrast microscopic images showed a clear difference in SH-SY5Y cell morphology among the groups. A healthy, closed spaced monolayer of cells with typical shape and strong cell adhesion was seen in the untreated control. In contrast, the H_2O_2 treated group showed marked cellular damage, including cell shrinkage, rounding, detachment, and reduced density, indicating oxidative stress-induced injury. With improved cell adhesion and survivability, the quercetin-treated group maintained a morphology more comparable to the control. Furthermore, the proline-treated group excelled the H_2O_2 group in terms of cell density and maintaining proper cellular morphology, indicating a preventing effect against stress caused by hydrogen peroxide (as shown in figure 3).

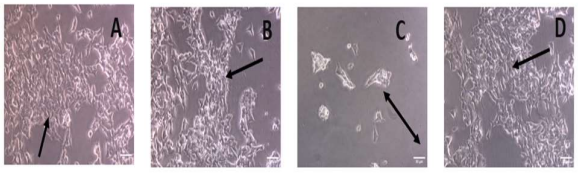

Figure 3: Phase contrast microscopic images of neuroprotective effect of proline in neuroblastoma (SH-SY5Y cell lines) compared with untreated (A), quercetin as positive control (B), negative control with hydrogen peroxide (C) and 300 µM hydrogen peroxide (H_2O_2) + proline composite treated cells (D). The → indicates the cellular density with cellular aggregates showing cellular communications and their viability. ↔ indicates the cellular morphological changes and lesser adhesion and damaged cell. The concentration for the Quercin, and proline was maintained at 250 µg/mL, hydrogen peroxide used for the induction of oxidative stress is at 300 µM concentration.

3.4 Mitochondrial membrane potential (MMP) analysis

Mitochondrial membrane potential (MMP) analysis was performed by conducting *in vitro* cell culture assay in SH-SY5Y neuronal cells using JC-1 fluorescent staining to evaluate mitochondrial integrity under oxidative stress conditions. JC-1 dye produces aggregates that produce red fluorescence in healthy mitochondria with intact membrane potential, but JC-1 monomers cause green fluorescence in damaged or injured neuronal cell with depolarized mitochondria. Healty mitochondrial activity and intact mitochondrial membrane potential were indicated by the untreated/protected cells with strong red fluorescence and weak green fluorescence. On the other hand, after being exposed to H_2O_2, oxidatively stressed cells exhibited strong green fluorescence and slightly decreased red fluorescence, indicating severe mitochondrial depolarization and breakdown of mitochondrial integrity.

Compared to the H_2O_2 treated group, proline treated cells led to a significant decrease in green fluorescence and a restoration of red fluorescence intensity. Cells exposed to proline showed relatively higher red fluorescence among the treated groups, suggesting better mitochondrial stability and successful retention of mitochondrial membrane stability and potential. The observed fluorescence pattern suggested reduced mitochondrial damage and enhanced cellular survival in treated neuronal cells. Overall, the JC-1 fluorescence images confirmed that oxidative stress induced significant mitochondrial dysfunction, whereas pretreatment with the test compounds effectively protected mitochondrial integrity and reduced mitochondrial depolarization in SH-SY5Y cells (figure 4)

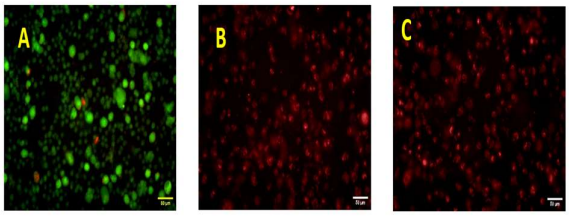

Figure 4: Flow cytometric images for mitochondrial membrane potential (ΔΨm) calculation. Green fluorescence indicates the JC1 aggregates as mitochondrial membrane depolarized state. Whereas red fluorescence indicates the mitochondrial polarized state with membrane integrity (A: Hydrogen peroxide treated, B: Quercetin as positive control and C: proline treated cells).

The 10000 events were collected for each sample and the changes in potential across mitochondrial membrane ((ΔΨm) were computed using the ratio of red to green fluorescence intensity. While the restoration of red fluorescence in proline treated groups indicated mitochondrial stability and cytoprotective activity, a decline in the red/green fluorescence ratio indicated mitochondrial membrane depolarization and increased membrane permeability.

The obtained flow cytometric data were analysed using FlowJo software platform. Results were expressed as percentage of mitochondrial membrane potential restoration relative to control groups. The assay enabled assessment of the neuroprotective efficacy of proline against oxidative stress-mediated mitochondrial dysfunction in SH-SY5Y neuronal cells.

3.5 *COX-2* biomarker analysis

Flow cytometric analysis of *COX-2* expression in SH-SY5Y cells (PE-conjugated anti-*COX-2* antibody, FL2 channel) demonstrated a strong upregulation of *COX-2* upon exposure to H_2O_2 (300 µM), indicating oxidative-stress–induced inflammatory activation. Pre-treatment with Quercetin and proline-rich fraction (CAP) at 125 µg/mL for 4 h prior to H_2O_2 exposure significantly reduced *COX-2* mean fluorescence intensity (MFI) in correlation comparison to H_2O_2 treated cells. The geometric mean MFI values (average of 3 replicates) for the relevant groups are briefed in the table 1 and figure 5.

Table 1: Flow cytometric analysis for *COX-2* expression in neuroblastoma cell lines (SH-SY5Y) showing the Mean fluorescence intensity (MFI).

Sl. No.	Sample Group	MFI (Mean ± SD)
1	Control (Untreated)	1381.00 ± 63.01
2	H_2O_2 (300 µM)	2818.33 ± 80.13
3	Quercetin – 125 µg/mL + H_2O_2	897.67 ± 195.24
4	CAP – 125 µg/mL + H_2O_2	1756.33 ± 96.49

Data represented as mean ± SD (n =3); [Abbreviations: H_2O_2– hydrogen peroxide, CAP – Proline extract from *Cassia alata Linn*. SD – standard deviation; MFI – Mean Fluorescent intensity].

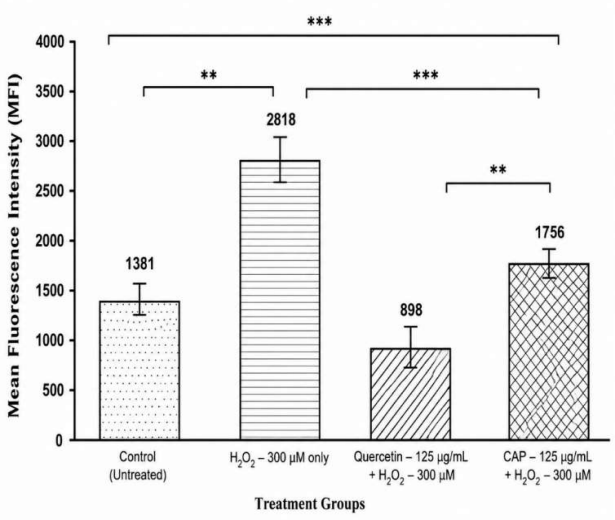

Figure 5: Flow cytometric analysis of *COX2* expression in SH-SY5Y cell lines. Data represented as mean ± SD (n =3); one way ANOVA followed by Tukey's multiple comparison test ANOVA ** p< 0.01 *** p < 0.001 compared with H_2O_2 treated group. [Abbreviations used: *COX - 2* – Cyclooxygenase 2; H_2O_2 = hydrogen peroxide; CAP – proline extracted from *Cassia alata Linn*].

4. DISCUSSION

Proline is a non-essential amino acid that functions as one of the most compatible naturally occurring osmolyte across various forms of species including plants, aquatic life and bacteria[19]. Proline serves a crucial role in stress tolerance, membrane stabilization and protection against oxidative damage. The elevated levels of proline in *Cassia alata* Linn. provides a natural defence mechanism against environmental stress through mechanisms involving osmotic adjustment and antioxidant defence[20]. The elevated levels of proline might possibly be a factor in the pharmacological effects reported in the study. The enrichment of compatible osmolytes such as proline and glycine betaine is regarded as an important adaptive response in plants exposed to environmental and oxidative stress conditions[21]. These low molecular weight compounds function as osmoprotectants by stabilizing proteins, cellular membranes, enzymes, and mitochondrial structures while preserving intracellular osmotic stability and redox homeostasis[22, 23].

In the present study, the results reported that proline accumulation increased progressively with longer incubation periods, which indicates the activation of cellular defence mechanisms[24]. Proline accumulation increased substantially up to 36 hours, highlighting its active involvement in osmotic adjustment and antioxidant defence. Proline has been recognized as a versatile stress-responsive metabolite capable of scavenging free radicals, stabilizing proteins and lipid bilayers, and regulating cellular redox homeostasis[25]. A slight decline in the proline concentration was observed at 48 hours, potentially an indication of metabolic utilization during sustained adaptation to stress conditions or conversion into other metabolic intermediates linked with cellular repair and restoration mechanisms. Several other stress-induced plant metabolic studies have reported a similar circumstance where proline accumulation gradually increases at initial stages of stress conditions followed by stabilization or reduction during extended stress exposure[26].

High content of proline accumulation suggests that it could serve as predominant osmoprotective compound in *Cassia alata Linn*. under the tested conditions[27]. However, proline aids in protecting cellular structures from oxidative stress mediated damage. The enhanced accumulation of proline is particularly significant in context of neuroprotective research, due to its predominant antioxidant and cytoprotective properties that may contribute to stabilization of mitochondrial function and reduction of neuronal oxidative injury. The observed osmolyte accumulation supports the hypothesis that *Cassia alata Linn*. exhibits substantial stress responsive metabolic potential and may serve as an important natural source of bioactive osmoprotectant with clinical relevance. The elevated levels of proline provide an additional validation of their potential contribution toward antioxidant, anti-inflammatory, and neuroprotective responses demonstrated in subsequent cellular and molecular investigations.

HPLC analysis was employed for isolation and estimation of proline content from *Cassia alata Linn*. leaf samples[28]. A dominant peak at RT 3.039 min with 99.99% of the total area was observed in the HPLC chromatogram of proline, which signifies the presence of proline with a greater abundance and purity form then isolated fraction. The negligible area percentage of the peak denotes the absence of significant interfering compounds, which provides additional evidence supporting the high purity of proline extracted from leaf samples.

The untreated control represents the normal healthy state of SH-SY5Y cells, these cells exhibit intact morphology and strong adherence, a typical character expressed by viable neuronal-like cells[29]. The positive reference group constitutes cells treated with quercetin, these cells exhibit a well-preserved cellular structure, substantiating its established antioxidant and cytoprotective properties. Cells treated with H_2O_2 presented pronounced cellular damages such as rounding, shrinkage, detachment, and reduced cell density, these cellular impairments are a plausible cause of damage caused to membranes, proteins, and other cellular components by reactive oxygen species (ROS) produced by hydrogen peroxide[30, 31]. The proline-treated group showed a marked improvement over the H_2O_2 only group. The proline treated exhibited better adhesion and comparatively fewer damaged cells were observed. These findings substantiate the role of proline in exerting protective effect against oxidative stress and prevent cellular damages by preserving cellular structures and minimizing ROS-mediated injury. However, given that the cell shape and morphology seem to differ slightly from that of the untreated or quercetin-treated groups, it suggests that the protection is only partial rather than complete. Overall, the image supports the conclusion that proline exerts neuroprotection to SH-SY5Y cells under oxidative stress conditions induced by H_2O_2.

Mitochondrial dysfunction is one of the most important pathogenic processes in neurodegenerative diseases especially Alzheimer's diseases[32]. The excess production of reactive oxygen species (ROS) produced by oxidative stress, which disrupts mitochondrial membrane function and its integrity, leading to impairment of mitochondrial membrane potential, impaired ATP synthesis, release of cytochrome C and activation of apoptotic signalling pathways[33, 34]. Protecting and stabilizing the potential across mitochondrial membrane is thought to be a crucial sign of neuronal survival and neuroprotective efficacy as a s result of these disruptions and cellular damages.

In the current study, it was observed that SH-SY5Y neuronal cells displayed marked mitochondrial depolarization under H_2O_2 induced oxidative stress conditions. This was evidenced by an increase in green fluorescence and reduced red fluorescence in JC-1-stained cells. The fluorescence signalling indicates profound mitochondrial injury, variations in cell membrane permeability, and the initiation of apoptotic cellular process The findings are consistent with previous reports demonstrating that oxidative stress-mediated ROS accumulation damages mitochondrial respiratory chain components and disrupts neuronal energy metabolism[34,35]. Pretreatment with proline from *Cassia alata Linn.* (CAP) and other phytocompounds significantly restored

mitochondrial membrane potential, as indicated by enhanced red fluorescence intensity. Among the tested compounds, proline exhibited superior mitochondrial protective activity, suggesting its strong osmoprotective and antioxidant potential. Proline is known to function as a compatible osmolyte and ROS scavenger capable of stabilizing proteins, lipid membranes, and mitochondrial structures under stress conditions[36]. The preservation of mitochondrial polarization in proline treated cells indicated inhibition of mitochondrial permeability transition and prevention of oxidative damage-induced apoptosis.

The mitochondrial membrane depolarization is an early indicator of apoptosis and neuronal degeneration, the JC-1 assay is regarded as a very specific, sensitive and highly reliable technique for assessing mitochondrial health[37]. Restoration of mitochondrial membrane potential by proline indicates its potential role as an antioxidant osmolyte capable of preserving mitochondrial integrity, reducing oxidative stress, and preventing neuronal apoptosis associated with neurodegenerative disorders such as Alzheimer's disease.

COX-2, encoded by PTGS2, is an inducible enzyme that is rapidly upregulated during inflammation, oxidative stress, and other cellular stress conditions, where it contributes to the synthesis of inflammatory prostaglandins[38, 39]. The marked increase in *COX-2* expression in SH-SY5Y cells following H_2O_2 exposure therefore confirms that oxidative stress activated a pro-inflammatory response in this neuronal cell model. The substantial suppression of *COX-2* expression by quercetin is consistent with previous evidence showing that quercetin attenuates *COX-2* induction under oxidative and inflammatory stress conditions[39]. In the present study, the mean MFI in the quercetin-pretreated group was lower than even the untreated control, suggesting that quercetin not only counteracted H_2O_2 induced inflammatory signalling but may also have reduced basal *COX-2* activity under the experimental conditions.

Proline also reduced H_2O_2-induced *COX-2* expression, although its effect was less pronounced than that of quercetin. The reduction in mean MFI from 2818.33 ± 780.13 in H_2O_2 treated cells to 1756.33 ± 296.49 after CAP pretreatment indicates a clear attenuation of oxidative stress-associated inflammatory signalling. This finding suggests that CAP possesses bioactive properties capable of limiting the pathways leading to *COX-2* induction, possibly through modulation of intracellular redox status or suppression of signalling cascades that regulate inflammatory mediator expression.

The biological implication of this study outcome lies in the central role of *COX-2* which facilitates production of inflammatory prostaglandins, and propagation of oxidative stress-associated cellular injury[40]. SH-SY5Y cells are widely used as a neuronal model in oxidative stress and neuroinflammation studies[41, 42]. A substantial reduction in *COX-2* after CAP pretreatment was observed, which asserts its potential anti-inflammatory effect in response to stress conditions. Although proline extracted from the selected plant did not match the inhibition achieved by quercetin, its moderate yet distinct reduction of *COX-2* expression denotes potential value for further assessment in oxidative stress-related neuroprotective studies.

5. CONCLUSION

Proline, an amino acid, produced during various abiotic stresses, maintains the protein integrity by protecting them from the misfolding. The underexplored concept of protein in neuroprotective action is a key research gap found by extensive review work. The present study signified that proline extracted from leaves of *Cassia alata Linn* exhibit marked neuroprotective potential against SH-SY5Y neuronal cells exposed to hydrogen peroxide induced oxidative stress. One of the most crucial osmolyte, proline was optimally extracted, quantified and characterized as demonstrated in the study. The methods employed such as acid-ninhydrin method and RP-HPLC analysis confirmed the presence of high concentration and purity of the compound present in the plant extract. In proline treated cells, JC-1 membrane potential study showed protection against mitochondrial depolarization and restoration of mitochondrial permeability, indicating successful stabilization of mitochondrial permutability under oxidative stress. Further it was found that proline treatment considerably decreased the expression of the inflammatory biomarker *COX-2*. These findings demonstrate the therapeutic value of proline, a natural osmolyte extracted from the plant as a viable neuroprotective alternative for the treatment of neurodegenerative disorders lead by protein misfolding linked to oxidative stress, including Alzheimer's disease. Further investigations of its therapeutic applications and development of proline as natural protective amino acid drug needs *in vivo* animal model and clinical validations.

6. ACKNOWLEDGEMENT

We would like to acknowledge the facilities given by REVA research Centre, REVA university, Bengaluru and constant support in carrying out the research work.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. AUTHOR'S CONTRIBUTION

All the authors have contributed equally to the conceptualization of the study, designing the research study, data collection for the experimental study and result interpretation along with manuscript reparation.

9. DATA AVAILABILITY STATEMENT

The data generated and analyzed for the interpretation of the experiments are included in the article.

10. ORCID IDs

Shanmugam Veeraswamy Manikyam - https://orcid.org/0000-0001-6602-3537

Manjula Kannasandra Ramaiah - https://orcid.org/0000-0002-2000-1321

Jolitha adiyara bosse - https://orcid.org/0000-0002-2504-737X

REFERENCE

1. Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature. 2006; 443(7113):787-95. DOI: https://doi.org/10.1038/nature05292
2. Sultana, R., Butterfield, D.A. Oxidatively modified, mitochondria-relevant brain proteins in subjects with Alzheimer disease and mild cognitive impairment. Journal of Bioenergy and Biomembranes, 2009; 41, 441–446. DOI: https://doi.org/10.1007/s10863-009-9241-7
3. Misrani A, Tabassum S, Yang L. Mitochondrial dysfunction and oxidative stress in Alzheimer's disease. Frontiers in aging neuroscience. 2021; 18(13):617588. DOI: https://doi.org/10.3389/fnagi.2021.617588
4. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacology. 2009; 7(1):65-74. DOI: https://doi.org/10.2174/157015909787602823
5. Onyango IG, Khan SM. Oxidative stress, mitochondrial dysfunction, and stress signaling in Alzheimer's disease. Current Alzheimer's Research. 2006; 3(4):339-49. DOI: 10.2174/156720506778249489.
6. Wang X, Wang W, Li L, Perry G, Lee HG, Zhu X. Oxidative stress and mitochondrial dysfunction in Alzheimer's disease. Biochimia Biophysica Acta. 2014; 1842(8):1240-7. DOI: https://doi.org/10.1016/j.bbadis.2013.10.015
7. Mittal P, Dhankhar S, Chauhan S, Garg N, Bhattacharya T, Ali M, Chaudhary AA, Rudayni HA, Al-Zharani M, Ahmad W, Khan SU. A review on natural antioxidants for their role in the treatment of Parkinson's disease. Pharmaceuticals. 2023; 16(7):908. DOI: https://doi.org/10.3390/ph16070908
8. Fatmawati S, Purnomo AS, Bakar MF. Chemical constituents, usage and pharmacological activity of Cassia alata. Heliyon. 2020; 6(7). e04396. DOI: https://doi.org/10.1016/j.heliyon.2020.e04396
9. Colin MN, Claudiana NS, Kaffah AU, Hasanah AN, Megantara S. Review on *Cassia alata* Bioactive Compounds: In silico, in vitro, and in vivo Studies. Drug design, development and therapy. 2024:4427-47. DOI: https://doi.org/10.2147/DDDT.S477679
10. Szabados, L. and Savoure, A. Proline: A Multifunctional Amino Acid. Trends in Plant Science, 2010; 15, 89-97. DOI: https://doi.org/10.1016/j.tplants.2009.11.009
11. Waseem M, Liu P, Aslam MM. Salinity and drought stress in plants: understanding physiological, biochemical and molecular responses. Frontiers in Plant Science. 2023; 14:1277859. DOI: https://doi.org/10.3389/fpls.2023.1277859
12. Ben Rejeb K, Abdelly C, Savouré A. How reactive oxygen species and proline face stress together. Plant Physiology & Biochemistry. 2014; 80:278-84. DOI: https://doi.org/10.1016/j.plaphy.2014.04.007
13. Rehman AU, Bashir F, Ayaydin F, Kóta Z, Páli T, Vass I. Proline is a quencher of singlet oxygen and superoxide both in *in vitro* systems and isolated thylakoids. Physiology Plant. 2021; 172(1):7-18. DOI: https://doi.org/10.1111/ppl.13265
14. Phang JM, Liu W, Hancock C, Christian KJ. The proline regulatory axis and cancer. Frontier in Oncology. 2012; 2:60. DOI: https://doi.org/10.3389/fonc.2012.00060
15. Donald SP, Sun XY, Hu CA, Yu J, Mei JM, Valle D, Phang JM. Proline oxidase, encoded by p53-induced gene-6, catalyses the generation of proline-dependent reactive oxygen species. Cancer research. 2001;61(5):1810-5. DOI: https://doi.org/
16. Rossman TC, Purohit G, Daudu OI, Becker DF. Proline Metabolism in Cancer: Emerging Roles in Redox Homeostasis and Therapeutic Opportunities. Cancers (Basel). 2025; 17(19):3156. DOI: https://doi.org/10.3390/cancers17193156
17. Zhong L, Zhou J, Chen X, Lou Y, Liu D, Zou X, Yang B, Yin Y, Pan Y. Quantitative proteomics study of the neuroprotective effects of B12 on hydrogen peroxide-induced apoptosis in SH-SY5Y cells. Scientific Reports. 2016; 6:22635. DOI: https://doi.org/10.1038/srep22635
18. Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. Plant and soil. 1973; 39(1):205-7. DOI: https://doi.org/10.1007/BF00018060
19. Yoshiba Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K. Regulation of levels of proline as an osmolyte in plants under water stress. Plant and cell physiology. 1997; 38(10):1095-102. DOI: https://doi.org/10.1093/oxfordjournals.pcp.a029093
20. Parveen S, Shahzad A. A review on in vitro culture of *Cassia alata Linn*. (Senna alata): Analysis of metabolites and biological activities. Journal of Functional and Environmental Botany. 2015; 5(2):78. DOI: https://doi.org/10.5958/2231-1750.2015.00016.5
21. Pal P, Singh AK. Chapter 9: Understanding the roles of glycine betaine for plant's tolerance and acclimatization under changing environmental situations. In Roles of Osmolytes in Changing Environment: Plant Biology, sustainability and climate change 2025; 179-198. Academic Press. DOI: https://doi.org/10.1016/B978-0-443-26549-5.00014-X
22. Wright MM, Rajewski BH, Gerrein TA, Xu Z, Smith LJ, Seth Horne W, Del Valle JR. Stabilization of a miniprotein fold by an unpuckered proline surrogate. Communications Chemistry. 2025 ;8(1):76. DOI: https://doi.org/10.1038/s42004-025-01474-6
23. Venkatesan A, Bernstein AM. Protein misfolding and mitochondrial dysfunction in glaucoma. Frontiers in cell and developmental biology. 2025; 13:1595121. DOI: https://doi.org/10.3389/fcell.2025.1595121
24. Mushtaq NU, Saleem S, Rasool A, Shah WH, Tahir I, Seth CS, Rehman RU. Proline tagging for stress tolerance in plants. International Journal of Genomics. 2025; 2025(1):9348557. DOI: https://doi.org/10.1155/ijog/9348557

25. Rao MJ, Duan M, Zhou C, Jiao J, Cheng P, Yang L, Wei W, Shen Q, Ji P, Yang Y, Conteh O. Antioxidant defence system in plants: reactive oxygen species production, signaling, and scavenging during abiotic stress-induced oxidative damage. Horticulturae. 2025; 11(5):477. DOI: https://doi.org/10.3390/horticulturae11050477
26. Khan N. Exploring plant resilience through secondary metabolite profiling: advances in stress response and crop improvement. Plant, Cell & Environment. 2025; 48(7):4823-37. DOI: https://doi.org/10.1111/pce.15473
27. Lai Z, Datir S, Weber J, Belford E, Regan S. Differential response of Senna occidentalis L. to arsenic and cadmium contaminated soil. Bulletin of Environmental Contamination and Toxicology. 2026; 116(4):89. DOI: https://doi.org/10.1007/s00128-026-04237-8
28. Mohammadrezakhani S, Rezanejad F. Improving the Antioxidant Defense System in Different Species of Citrus Fruits under Low-temperature Stress using Osmolytes. International Journal of Horticultural Science and Technology. 2025; 12(4):1105-16. DOI: https://doi.org/10.22059/ijhst.2025.357397.631
29. Aydemir M, Kurt MŞ, Daş AS, Arslan ME, Arslan E, Türkez H. Oxidative DNA damage and necrotic cell death in differentiated SH-SY5Y cells induced by continuous extremely low-frequency magnetic field exposure. Environmental Toxicology and Pharmacology.2026; 2026:105011. DOI: https://doi.org/10.1016/j.etap.2026.105011
30. Anikina VA, Kozlov DA, Popov AL, Zamyatina EA, Popova NR. Antioxidant activity and cellular response of novel cerium oxide nanoparticles stabilized with triethylene glycol under H_2O_2 stress in fibroblasts and keratinocytes. Biomaterials Translational. 2025. DOI: https://doi.org/10.12336/bmt.25.00047
31. Sagnia B, Fedeli D, Casetti R, Montesano C, Falcioni G, Colizzi V. Antioxidant and anti-inflammatory activities of extracts from *Cassia alata*, *Eleusine indica, Eremomastax speciosa, Carica papaya* and *Polyscias fulva* medicinal plants collected in Cameroon. PloS one. 2014;9(8):e103999. DOI: https://doi.org/10.1371/journal.pone.0103999
32. Devi SA, Chandrasekar BS, Manjula K, Ishii N. Grape seed proanthocyanidin lowers brain oxidative stress in adult and middle-aged rats. Experimental Gerontology. 2011; 46(11):958-64. DOI: https://doi.org/10.1016/j.exger.2011.08.006
33. Shubha Beaulah Angel P, Manjula K R, Phytochemical analysis, antioxidant potential and identification of bioactive compounds using GC–MS of the leaf extracts of *Clitoria ternatea*. Research Journal of Pharmacy and Technology. 2025; 18(10): 4803-4809. DOI: https://doi.org/10.52711/0974-360X.2025.00692
34. Devi SA, Manjula KR, Subramanyam MV. Protective role of vitamins E and C against oxidative stress caused by intermittent cold exposure in aging rat's frontoparietal cortex. Neuroscience letters. 2012; 529(2):155-60. DOI: https://doi.org/10.1016/j.neulet.2012.09.041
35. Kobayashi CI, Suda T, Regulation of reactive oxygen species in stem cells and cancer stem cells. Journal of Cell. Physiology. 2012; 227: 421-430. DOI: https://doi.org/10.1002/jcp.22764
36. Kheshin ME, Hmmam I. Polyethylene glycol and proline synergistically improve salinity tolerance via physiological and biochemical reprogramming in mango. BMC Plant Biology. 2025;25(1):1161. DOI: https://doi.org/10.1186/s12870-025-07211-4
37. Nadareidze V. Mitochondrial Health. Annals of Rejuvenation Science. 2026; 1(2). 1-14. DOI: https://doi.org/10.65649/za7k8128
38. Guardado MM, Lutz V, Hengstschläger M, Dolznig H. The role of prostaglandins as major inflammatory mediators in colorectal cancer. International Journal of Molecular Sciences. 2025; 26(24):12191. DOI: https://doi.org/10.3390/ijms262412191
39. Zhang E, Yan X, Shen H, Zhao M, Gao X, Huang Y. Intracranial aneurysm biomarkers: a convergence of genetics, inflammation, oxidative stress, and the extracellular matrix. International Journal of Molecular Sciences. 2025; 26(7):3316. DOI: https://doi.org/10.3390/ijms26073316
40. Sharma V, Sharma P, Singh TG. Therapeutic potential of *COX-2* inhibitors in neuropsychiatric disorders. Journal of neural transmission. 2025; 132(7):999-1011. DOI: https://doi.org/10.1007/s00702-025-02932-0
41. Pinheiro NR, Carneiro CF, Cancelliero GS, Nogueira GO, Almeida GM, Fernandes N, Wasilewska-Sampaio AP, Martins SM, Felix A, Amaral OB, Sebollela A. The human neuroblastoma SH-SY5Y cell line as a model to assess β-amyloid neurotoxicity: A systematic review and meta-analysis. bioRxiv. 2025; 2025-10. DOI: https://doi.org/10.1101/2025.10.20.683497
42. Prisacar M, Esser S, Hausherr M, Karacora B, Vyushkova Y, Eisenacher M, Grugel R, Marcus K, Eggers B. Systematic Analysis of SH-SY5Y Differentiation Protocols and Neuronal Subtype Abundance. Cellular and Molecular Neurobiology. 2025; 45(1):104. DOI: https://doi.org/10.1007/s10571-025-01627-0