

Neuroprotective Effects of Conessine in Beta-Amyloid-Induced SH-SY5Y Cells *in vitro*

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ABSTRACT

Objectives: Alzheimer's Disease (AD) is a brain disorder that affects the memory and thinking ability of the patient, characterised by beta-amyloid (A β) plaque accumulation, oxidative stress, impaired autophagy, and neuronal death, leading to cognitive decline. Current treatment for AD is limited to symptomatic relief, which highlights the demand for the discovery of newer drugs. Conessine, a steroidal alkaloid derived from *Holarrhena antidysenterica*, has demonstrated potential neuroprotective activity; however, its mechanism of action against beta-amyloid-induced toxicity remains unclear. **Materials and Methods:** In this study, we examined the protective effect of conessine on neurons in SH-SY5Y human neuroblastoma cells. MTT assay was used to study the general toxicity profile and the neuroprotective function of conessine. The level of autophagy was determined through flow cytometry analysis. The level of ROS generated in cells during beta-amyloid treatment was quantified using a fluorescence-based assay. Oxidative stress gene expression was analysed using real-time PCR experiments. **Results:** The results suggest that conessine at low doses (≤ 12.5 μ g/mL) protects cells from beta-amyloid-induced cytotoxicity and restores their viability and shape. Flow cytometry showed that conessine (12.5 μ g/mL) increased autophagy. In addition, DCFDA staining revealed that conessine reduced the production of Reactive Oxygen Species (ROS) induced by amyloid beta. Real-time PCR showed that conessine increased the expression of the antioxidant genes NRF2 and HMOX1 in A β -treated cells by 1.5 and 1.10 times, respectively, and even more in cells that were only treated with conessine (1.78 and 1.4 times). **Conclusion:** Collectively, these results suggest that conessine has potential as a therapeutic candidate for AD. More research is thus warranted to elucidate its molecular interactions and clinical applicability.

Keywords: Alzheimer's Disease, Conessine, Beta-amyloid, Neuroprotection, Autophagy, Oxidative Stress, NRF2, HMOX1.

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INTRODUCTION

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder affecting millions of individuals worldwide.¹ It is one of the causes of dementia, characterized by cognitive decline, memory loss and impairment in daily functions.² AD is expected to increase globally due to the aging population, and it is estimated to reach 150 million cases by 2050.³ This global increase in incidence could affect the worldwide healthcare and economic well-being. The primary signs of Alzheimer's disease in the brain

are the accumulation of beta-amyloid plaques outside cells and neurofibrillary tangles composed of hyperphosphorylated tau protein within cells.⁴ These pathological changes delay neurons' ability to communicate with each other, ultimately leading to oxidative stress, impaired autophagy, and the initiation of inflammatory responses.⁵ Later, all of these factors lead to neuron death and cognitive decline. Even though a lot of research has been done, there are still only a few effective treatments for AD, but unfortunately, they are less effective in treating.^{6,7} This highlights the importance of finding new ways to slow its progression.

The pathophysiology of AD is well-studied but complex. The reported histopathological studies show the extracellular aggregation of amyloid beta plaques, particularly A β 1-42, and intracellular aggregation of neurofibrillary tangles.^{8,9} In the case of amyloid beta plaque accumulation, it causes microglial activation and an inflammatory response in the affected area, which contributes to neurotoxicity. Amyloid beta causes toxicity in



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several ways, such as oxidative stress, mitochondrial dysfunction, and impaired autophagy, all of which lead to the death of neurons.¹⁰ Excessive ROS-mediated production of oxidative stress harms cell components like lipids, DNA and protein, which aggravates neuronal vulnerability. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates the expression of antioxidant genes, such as Heme Oxygenase-1 (HMOX1). It is essential for reducing oxidative stress.^{11,12} However, in AD, NRF2 signaling is often compromised, making it difficult for cells to fight against oxidative stress. Likewise, AD disrupts autophagy, a cellular process that enables the removal of damaged organelles and misfolded proteins. This causes toxic aggregates to build up. Thus, restoring autophagy and boosting antioxidant defences are considered effective ways to manage AD.

Natural products, particularly phytochemicals derived from various plants, have demonstrated promising activities against multiple diseases, including neurodegenerative disorders.^{13,14} They possess a high amount of antioxidant and anti-inflammatory activity, which makes them a very attractive choice for drug discovery. Moreover, many of the isolated compound target signaling pathways lead to relief from disease progression. Recently, many natural compounds with anti-neurodegenerative disease properties have been studied. Among these, conessine, a steroidal alkaloid derived from the plant *Holarrhena antidysenterica*, has emerged as a promising candidate.¹⁵ Preliminary studies have shown that conessine can induce antioxidant effects by modulating oxidative stress and neuroprotection.¹⁶ Its ability to cross the blood-brain barrier, a critical requirement for central nervous system therapeutics, further enhances its therapeutic potential. However, the specific mechanisms by which conessine exerts neuroprotection against A β -induced toxicity in AD models are not well explored.

The current treatment for AD is focused on symptomatic relief rather than a cure. For instance, drugs such as cholinesterase inhibitors and NMDA receptor antagonists provide only symptomatic relief and do not halt disease progression.¹⁷ Since AD is a multi-target disease condition, such as autophagy, oxidative stress, neurotoxicity, etc., the effective treatment must be able to act on all these aspects for relief to patients. Therefore, the objective of this investigation was to examine the effect of conessine in SH-SY5Y cells exposed to A β -induced toxicity, which is a well-studied *in vitro* model for AD.¹⁸ Previous studies have highlighted the potential of such alkaloids in various models. Additionally, conessine itself has shown some initial effect in neurodegenerative diseases such as cerebral ischemia and oxidative stress models by Bandaru *et al.*, 2020.¹⁶ However, they focused on behavioural outcomes and broad antioxidant enzyme modulation in a whole-animal model; on the contrary, our study approach enabled us to uncover novel, A β -specific protective effects of conessine that were not explored in the prior work.

Moreover, since it originates from natural sources, we expect the activity to be accompanied by the lowest level of toxicity.

MATERIALS AND METHODS

Chemicals and cell culture materials

Conessine has been purchased from a commercial supplier, Sigma. Human neuroblastoma cells (SHSY-5Y) were purchased from ATCC. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, USA) with 10% Fetal Bovine Serum (FBS), L-glutamine, sodium bicarbonate (Merck, Germany), and an antibiotic solution that had penicillin (100 U/mL), streptomycin (100 μ g/mL), and amphotericin B (2.5 μ g/mL). Cells were kept in 25 cm² tissue culture flasks at 37°C in an incubator with 5% CO₂ and humidity.

Neurotoxicity assay

For the MTT assay, confluent cell monolayers that were two days old were trypsinized, put in 10% growth medium, and seeded at 10000 cells/well in a 96-well tissue culture plate. Then, the plate was placed in a 5% CO₂ incubator at 37°C. To make the test compounds, 1 mg of conessine was dissolved in 1 mL of 0.1% DMSO, filtered through a 0.22 μ m Millipore syringe filter to make sure it was sterile, and then diluted in DMEM to make 100 μ g/mL, 50 μ g/mL, 25 μ g/mL, 12.5 μ g/mL, and 6.25 μ g/mL. After 24 hr, the growth medium was changed to 100 μ L of each compound concentration in three wells, along with untreated control cells. The cells were then incubated for another 24 hr. We observed neurotoxicity directly under an inverted phase contrast microscope and noted changes in cell shape, such as rounding, shrinking, granulation, or vacuolization, as signs of cytotoxicity. For the MTT test, MTT dye (5 mg/mL of PBS) was added (100 μ L) to each well after the test compounds were removed. After 4 hr at 37°C, the supernatant was discarded, and 100 μ L of DMSO was added to dissolve the formazan crystals. A microplate reader measured absorbance at 540 nm. We used the formula (Mean OD of samples/Mean OD of control group) $\times$ 100 to figure out the percentage of growth that was inhibited.

Neuroprotection assay

Human neuroblastoma cells (SHSY-5Y) were plated into a 96-well plate with 10,000 cells per well, as previously discussed. We prepared the test compounds by dissolving 1 mg conessine in 1 mL of 0.1% DMSO and then filtering the mixture through a 0.22 μ m Millipore syringe filter. The attached cells were treated with 10 μ M beta-amyloid to induce toxicity and then incubated for an hr. Next, test compounds were added to DMEM at concentrations of 25 μ g/mL, 12.5 μ g/mL, 6.25 μ g/mL, 3.1 μ g/mL, and 1.5 μ g/mL, in three wells each, and the mixture was left to sit for 24 hr together with the beta-amyloid added earlier at 37°C in a 5% CO₂ incubator. We kept the untreated control cells and the wells that only had beta-amyloid. We observed morphological changes

under a microscope, as we had done before, and used the MTT assay to assess cytotoxicity.

Autophagy assay

SHSY-5Y cells were grown in the usual way and then seeded as described above. 10 μ M beta-amyloid was added to the cells and incubated for an hour to induce toxicity. After that, conessine was added to DMEM at a concentration of 12.5 μ g/mL, and the cells were kept at 37°C in a humidified 5% CO₂ incubator for 24 hr. There were control groups made up of untreated cells, cells treated with only beta-amyloid, and cells treated with only the sample. At the end of the assay, cells were transferred into a 12×75-mm polystyrene tube to assess autophagy, with at least 1×10⁶ cells per sample. The samples were spun at 1000 rpm for 5 min, and the supernatant was carefully removed to keep the cell pellet, which looked like a white film or a visible pellet. We washed the pellet by resuspending it in cell culture medium or 1X Assay Buffer, then spinning it. Next, each cell sample was returned to 250 μ L of 1X Assay Buffer or cell culture medium that lacked indicators and contained 5% FBS. The Cyto-ID® Autophagy Detection commercial Kit was used to check for autophagy. To make the Cyto-ID® Green Detection Reagent, 1 μ L was mixed with 1 mL of 1X Assay Buffer or cell culture medium without indicators and 5% FBS. We added 250 μ L of this diluted stain solution to each sample, mixed it well, and let it sit in the dark at room temperature or 37°C for 30 min. After incubation, the cells were gathered by centrifugation, washed with 1X Assay Buffer, and then put back into 500 μ L of fresh 1X Assay Buffer. After that, a flow cytometer was used to measure the extent of autophagy.

ROS analysis

SHSY-5Y cells were cultured in T25 flasks at a density of 100,000 cells per flask. Separate flasks were maintained for control groups, including untreated cells, cells treated with beta-amyloid alone, and cells treated with the sample alone. The cells were allowed to attach overnight. Subsequently, one flask was treated with 12.5 μ g/mL of conessine, another with a combination of beta-amyloid and conessine, and an additional flask was maintained as a control. The treatment was continued for 24 hr. Afterwards, the flasks were removed from the incubator, the medium was discarded, and the cells were washed twice with PBS. Then, 50 μ L of DCFDA was added, and the cells were incubated in the dark for 30 min. The flasks were subsequently observed under a fluorescence microscope, and the fluorescence intensity was measured at an excitation wavelength of 470 nm and an emission wavelength of 635 nm.

Real-Time PCR

Standard lab methods were used to grow SHSY-5Y cells. The cells were placed in a culture flask and left overnight to attach. Separate flasks were maintained for control groups, including untreated cells, cells treated with beta-amyloid alone, and cells treated with

the sample alone. The flasks were treated accordingly and kept in the incubator overnight. After 24 hr, the flasks were removed from the incubator, and the RNA was extracted. Briefly, the RNA extraction was done using a commercial kit (Invitrogen's RNA isolation reagent). The process involved adding 1 mL of TRIzol reagent to the flask, setting it aside for 5 min, and then transferring the lysate to a clean Eppendorf tube. Then, 200 μ L of chloroform was added, and the mixture was spun in a centrifuge at 14,000 rpm and 40°C for 15 min. The upper aqueous phase was transferred into a new tube, mixed with 500 μ L of 100% isopropanol, and incubated at room temperature to promote growth. After that, it was spun in a centrifuge at 14,000 rpm at 40°C for 15 min. We removed the supernatant and washed the RNA pellet with 200 μ L of ethanol. Then we put it back in the centrifuge and spun it again at 14,000 rpm for 15 min at 40°C. The remaining RNA pellet was dried in air and then dissolved in TE buffer to facilitate cDNA synthesis later.

The obtained RNA was checked for purity and integrity. The cDNA was synthesised by adding 2 μ L of RNA with 5 μ L of RT easy mix and 0.5 μ L of oligo dT in an RNase-free PCR tube. The final volume of the mixture was made up to 20 μ L using sterile water. The entire content was mixed gently and cDNA was prepared in an Eppendorf Master Cycler by running it through 5 min at 25°C; 20 min at 46°C, and RT inactivation for 1 min at 95°C. We used real-time qRT-PCR with SYBR Green Master Mix on a Roche LightCycler 96 to look at the levels of the HMOX and Nrf2 genes in both control and treated cells. All reactions were carried out in triplicate and used the $\Delta\Delta$ Ct method to measure gene expression. The PCR protocol started with an activation step at 95°C for 2 min. Then, there were 40 cycles of denaturation at 95°C for 10 sec, annealing at 56°C for 1 min, and extension at 72°C for 1 min per kb. After cycling, the reaction stayed at 40°C. The primers that were used were: HMOX: sense 5'-CTCAAACCTCCAAAAGCC-3', antisense 5'-TCAAAAACCAACCCCAACCC-3'; Nrf2: sense 5'-CACATCCAGTCAGAAACCAGTGG-3', antisense 5'-GGAATGTCTGCGCCAAAAGCTG-3' and GAPDH: sense 5'-ACTCAGAAGACTGTGGATGG-3', antisense 5'-GTCATCATACTTGGCAGGTT-3'.

Statistical analysis

All data were presented as the means of triplicate data $\pm$ SD. ANOVA with *post hoc* tests was used to measure significant differences between multiple groups when appropriate. *p* values less than 0.05 were considered statistically significant.

RESULTS

Neurotoxicity Evaluation of Conessine in SH-SY5Y Cells

In order to know the general cytotoxicity of conessine, we have conducted an MTT assay in SH-SY5Y Cells. The assay revealed a dose-dependent cytotoxicity in the selected doses. The assay

was carried out for 24 hr, during which no cytotoxicity was observed in the control. However, the doses used, especially the higher doses, showed signs of toxicity, including cell detachment, rounding, and blebbing, as shown in Figure 1. Compared to lower doses, toxicity signs were visible at concentrations higher than 25 $\mu\text{g/mL}$; hence, all other studies were conducted using a lower and sub cytotoxic dose, i.e., 12.5 $\mu\text{g/mL}$.

Neuroprotection Effect of Conessine in beta Amyloid-Induced Toxicity in SH-SY5Y Cells

To assess conessine's ability to protect against neurotoxicity, we evaluated its toxicity in SH-SY5Y Cells using beta-amyloid. The results shown in Figure 2 indicate that beta-amyloid at a dose of 10 μM effectively induced toxicity. Later, we used a series of doses of conessine, ranging from 0.75 to 12.5 $\mu\text{g/mL}$. All selected doses significantly reduced beta-amyloid-induced toxicity. Figure 2A shows the normal morphology of SH-SY5Y cells. In contrast, the beta-amyloid-treated cells exhibited morphological changes, with reduced cell numbers indicating a level of toxicity that was restored, as shown in Figure 2G. The quantitative data showed a significant level of reactivation of cells, as shown in Figure 2H.

Effect of Conessine on the Autophagy Induced by β -Amyloid

A flow cytometry-based assay was carried out to determine the effect of β -amyloid on autophagy induction and the impact of conessine on it (Figure 3). Our results showed that natural autophagy was observed in control cells, which, upon treatment with β -amyloid, halted autophagy. To study the effect of conessine on this, 12.5 $\mu\text{g/mL}$ of the compound was tested, and it was found to enhance autophagy significantly ($p \leq 0.05$). In addition, it has been observed that conessine alone had a higher level of autophagy compared to the autophagy observed in the beta amyloid group.

Conessine-induced antioxidant effect in SH-SY5Y Cells via protecting against ROS generation

To study the level of ROS produced by β -Amyloid (10 μM), and to measure the protective effect of conessine, the DCFDA assay was used. As shown in Figure 4, β -Amyloid (10 μM) produced additional ROS, indicated by high fluorescence, which was well protected by conessine. The qualitative data in Figure 4 showed that the protective effect induced by conessine was statistically significant.

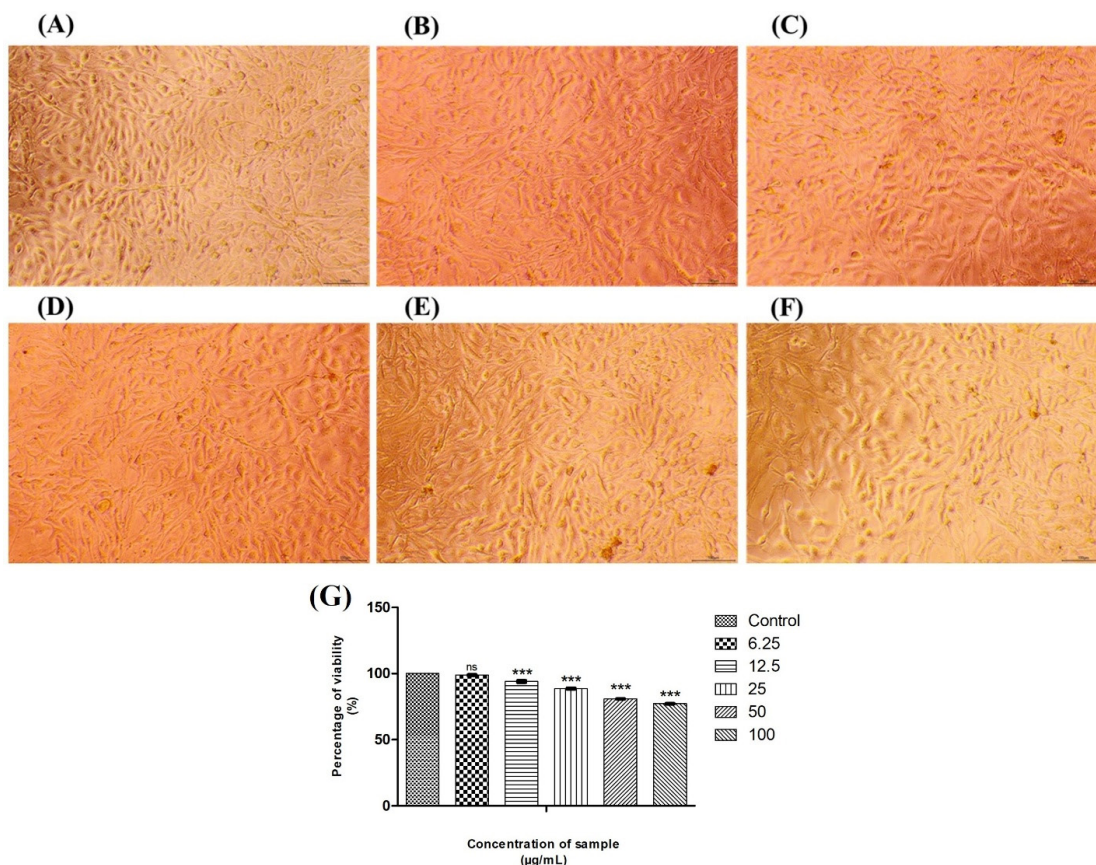


Figure 1: Cytotoxic effect of Conessine in SH-SY5Y Cells measured by MTT assay, and cytological changes were photographed. (A) Control, B-F is treated with Conessine 6.25, 12.5, 25, 50 and 100 $\mu\text{g/mL}$, respectively. (G) represents the quantified data obtained from neurotoxicity evaluation. All assays were carried out in triplicate. *** represent $p \leq 0.05$. ns means not significant.

Effect of conessin in the regulation of NRF2 and HMOX genes

Gene expression analyses were performed to assess the levels of oxidative stress- related genes NRF2 and HMOX1 using RT-PCR (Figure 5). β -Amyloid exposure reduced NRF2 expression below baseline (control set at 1.0), with levels decreasing to approximately 0.85-fold (-0.15 relative change). Treatment with conessine resulted in a modest increase in NRF2 expression, reaching approximately 1.5-fold in β -amyloid- treated cells, while conessine alone showed a slightly higher increase of around 1.78-fold.

A similar pattern was observed for HMOX1 expression. From the 1.0-fold control baseline, conessine treatment in the presence of β -amyloid produced a mild elevation to 1.10-fold, and conessine alone increased expression to about 1.4-fold. Although these fold changes are relatively small, they suggest a trend toward activation of the NRF2- HMOX1 antioxidant pathway following conessine treatment.

DISCUSSION

The present study investigated the effect of conessine in SH-SY5Y human neuroblastoma cells exposed to β -amyloid-induced toxicity, a well-established *in vitro* model of Alzheimer's Disease (AD). The current results showed that conessine has a significant neuroprotective effect in the selected cell lines against β -amyloid-induced cytotoxicity. Instead of only describing general antioxidant benefits, we now provide evidence that conessine protects neurons by restoring autophagy flux, suppressing β -amyloid-induced ROS, and activating the NRF2-HMOX1 pathway, indicating a direct molecular effect on cellular stress-response signaling. These findings, together with prior literature, suggest that conessine has promising therapeutic potential for AD.

The neurotoxicity assay performed revealed that conessine exhibits cytotoxicity, but only at concentrations higher than 25 $\mu\text{g/mL}$. This finding is in agreement with prior studies on similar alkaloids and conessine itself, which have shown changes in morphological structure and disturbances in cellular homeostasis.¹⁹⁻²¹ This cytotoxicity may be due to the effect of conessine in the cellular off-targets or signalling pathways, as seen with other steroidal alkaloids. However, at subcytotoxic

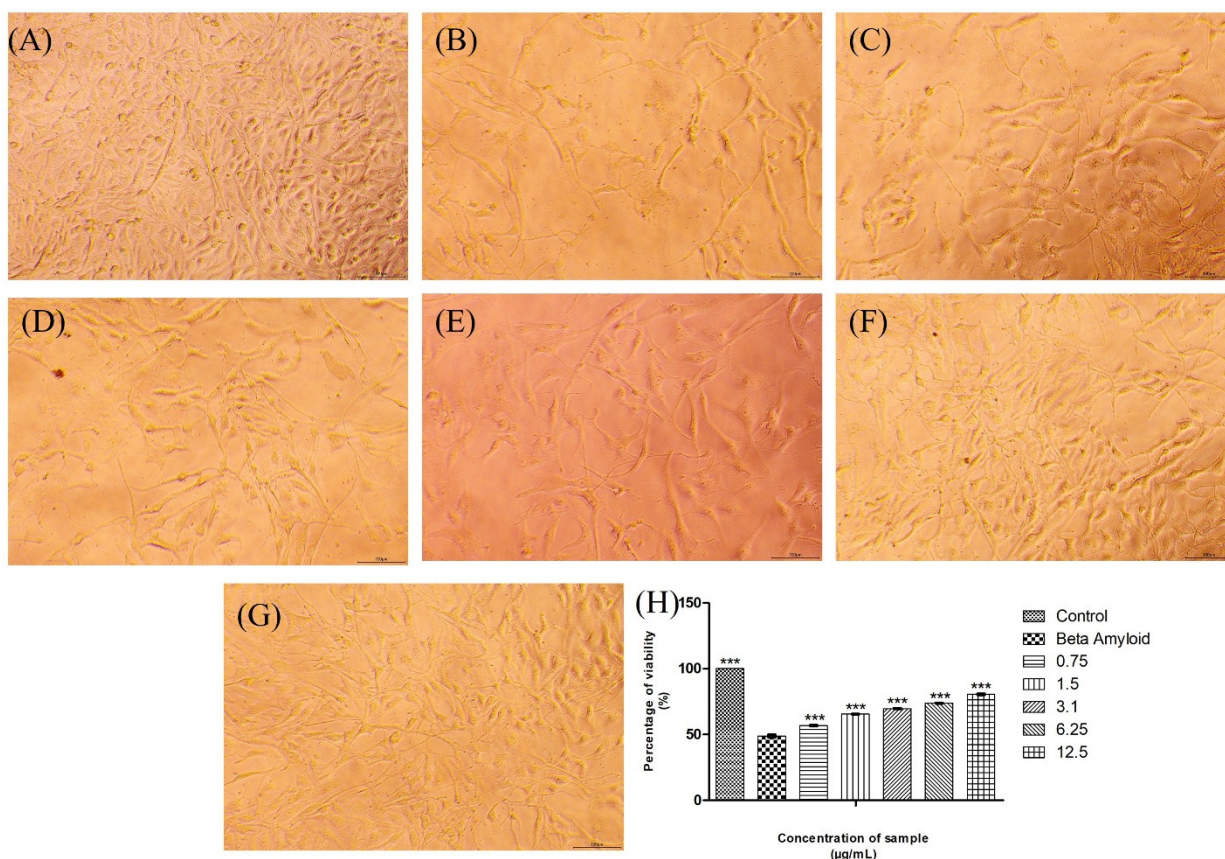


Figure 2: Neuroprotective effect of conessine in SH-SY5Y Cells induced with β -Amyloid (10 μM), and cytological changes were photographed. (A) Control, (B) β -Amyloid (10 μM) alone treated, (C-G) represent the cells pre-treated with β -Amyloid (10 μM) and exposed to conessine at 0.75, 1.5, 3.1, 6.25 and 12.5 $\mu\text{g/mL}$, respectively. (H) represents the quantified data obtained from the MTT assay. All assays were carried out in triplicate. *** represent $p \leq 0.05$.

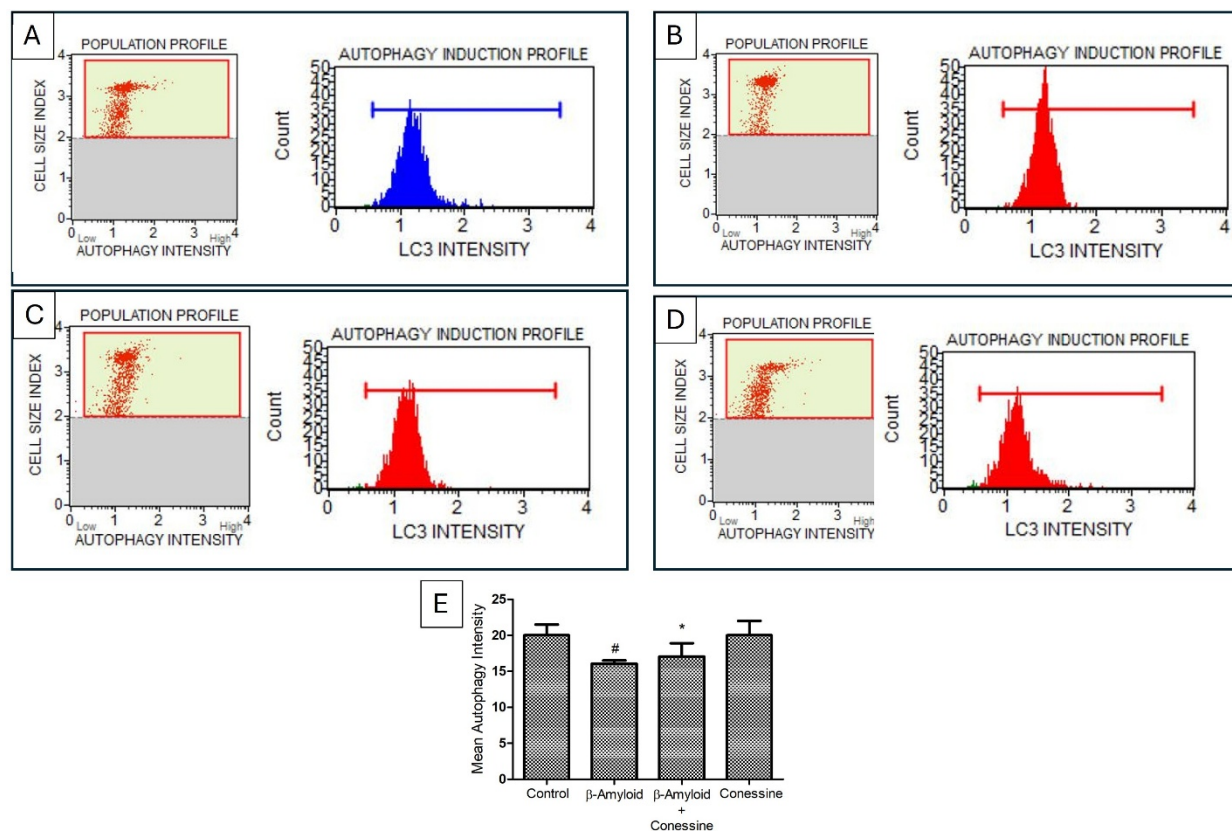


Figure 3: Effect of Conessine on the Autophagy Induced by β -Amyloid. Figure A-D shows the autophagy induction flow cytometry profile for Control, β -Amyloid (10 μ M) alone treated, β -Amyloid (10 μ M) and exposed to conessine at 12.5 μ g/mL and conessine alone, respectively. Figure E represents the quantitative data, where * represents $p \leq 0.05$ β -Amyloid Vs β -Amyloid + Conessine; # represents $p \leq 0.05$ Control vs β -Amyloid. All values were averaged from triplicate values $\pm$ S.D.

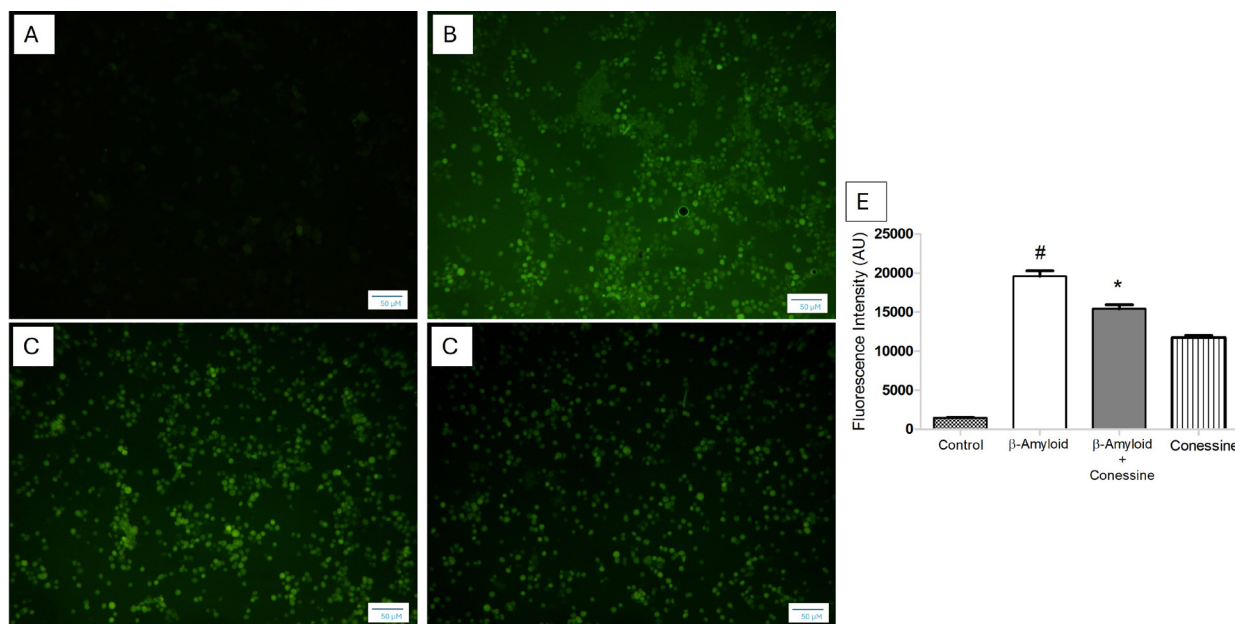


Figure 4: Measurement of ROS generation obtained by DCFDA staining. Control cells showed no fluorescence (A), β -Amyloid (10 μ M) showed the highest DCFDA staining (B), cells pretreated with β -Amyloid (10 μ M) and Conessine 12.5 μ g/mL showed less fluorescence than β -Amyloid (10 μ M) alone (C), and conessine alone reduced the fluorescence level less than β -Amyloid (10 μ M) and Conessine 12.5 μ g/mL (D). The level of fluorescence was quantified as shown in Figure 4E. All assays were carried out in triplicate. * represent $p \leq 0.05$ β -Amyloid Vs β -Amyloid + Conessine; # represent $p \leq 0.05$ Control Vs β -Amyloid.

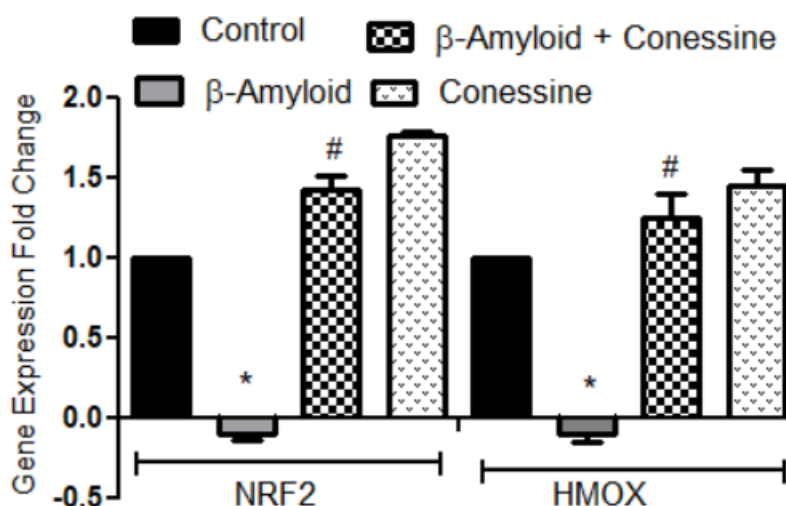


Figure 5: Gene expression of NRF2 and HMOX genes. All assays were carried out in triplicate. * represent $p \leq 0.05$ β -Amyloid Vs β -Amyloid + Conessine; # represent $p \leq 0.05$ Control Vs β -Amyloid.

doses (less than 12.5 $\mu\text{g/mL}$), conessine exhibited significant neuroprotection against β -amyloid-induced toxicity. The MTT assay quantitative data and the morphological figures explicitly showed that 12.5 $\mu\text{g/mL}$ of conessine restored cell viability and morphology, which were disturbed by β -amyloid, indicating a protective effect against neuronal damage. This dose-dependent pattern supports that conessine exerts regulated, pathway-specific actions at lower doses while causing non-specific toxicity only at higher concentrations. This result aligns well with other natural compounds, such as resveratrol and quercetin, which restore normal neuronal function by stabilizing mitochondrial function and inhibiting apoptotic cascades.^{22,23}

Autophagy is a cellular process by which the cell breaks down and removes the dysfunctional parts of the cell, and uses them for the repair of cells and as building blocks.²⁴ In AD, basal autophagy is essential for neuronal survival.²⁵ In our experiment, we have detected that β -amyloid significantly impaired the autophagy flux in SH-SY5Y cells, which is consistent with earlier studies showing that β -amyloid can disrupt lysosomal function and autophagosome clearance, leading to toxic accumulation.²⁶ As shown in Figure 3, conessine significantly enhances autophagy at 12.5 $\mu\text{g/mL}$, which was previously disturbed by β -amyloid. This enhancement suggests that conessine may act upstream on autophagy regulators, possibly by modulating AMPK/mTOR signaling, which are common targets for natural autophagy inducers; however, this requires further validation.²⁷ Restoration of autophagy by conessine in our studies aligns well with earlier reports on other natural compounds, such as rapamycin and resveratrol, which promote autophagic clearance of β -amyloid aggregates through the activation of pathways, including AMP-Activated Protein Kinase (AMPK), or the inhibition of the mechanistic Target of Rapamycin (mTOR).²⁸⁻³¹ Thus, conessine's

neuroprotective effect may partly originate from improving impaired proteostasis in β -amyloid-challenged neurons.

ROS plays a pivotal role in neurodegenerative diseases.³² In the case of AD, ROS has been identified as a key detrimental factor as per new and emerging research studies. Consistent with the oxidative stress hypothesis of AD, in our research, it has been found that β -amyloid significantly increased the ROS production in SH-SY5Y cells. This indicates that β -amyloid can trigger mitochondrial dysfunction and oxidative damage. β -amyloid has been shown to mediate its harmful effects in Alzheimer's patients' neurodegeneration via oxidative stress.³³ The effect of conessine in reducing the generation of ROS indicates that it has a potent antioxidant effect. Earlier, numerous studies have demonstrated that conessine has a significant impact in mitigating oxidative stress in various disease models.^{19,34} In our study, the reduction in ROS is linked not only to general antioxidant action but also to the activation of intrinsic cellular antioxidant pathways, as supported by the upregulated NRF2- HMOX1 expression. The statistically significant decrease in fluorescence intensity in cells treated with conessine suggests that it may either directly remove ROS or indirectly enhance antioxidant pathways. Our gene expression data, which we will discuss below, support the second possibility by demonstrating that the Nrf2-HMOX1 pathway is activated.

The gene expression study we conducted revealed modest increases of NRF2 and HMOX1 gene expression by conessine, suggesting a potential involvement of the NRF2- HMOX1 pathway in its antioxidant and neuroprotective effects. NRF2 is a transcription factor that regulates the defensive mechanisms against stress, leading to cellular damage.³⁵ This transcription factor regulates many cytoprotective genes, particularly those related to antioxidants, which aid in the synthesis of amino acid

substrates such as glutathione. One such gene regulated through NRF2 is HMOX1, which encodes heme oxygenase-1, an enzyme that produces antioxidant molecules such as biliverdin and carbon monoxide.³⁶ In our study, β -amyloid exposure slightly reduced NRF2 expression, which is consistent with previous reports showing that β -amyloid accumulation can diminish NRF2 signaling and weaken antioxidant capacity.³⁷ Conessine treatment moderately restored Nrf2 expression to 1.5 times its normal level and HMOX1 expression back to 1.10 times its normal level. Cells that were only treated with conessine had slightly higher levels of expression (1.78 fold for NRF2 and 1.54-fold for HMOX1). These findings indicate that conessine directly activates NRF2-dependent transcription and may facilitate NRF2 nuclear translocation, counteracting β -amyloid-induced suppression of this pathway. The higher expression of Nrf2 and HMOX1 in cells treated with conessine alone compared to those co-treated with β -amyloid suggests that β -amyloid may partially antagonize conessine's effects on gene expression, possibly due to its interference with the nuclear translocation of Nrf2.

CONCLUSION

In conclusion, our study demonstrated that conessine possesses significant neuroprotective properties in β -amyloid-induced SH-SY5Y cells, as evidenced by its ability to reduce cytotoxicity, enhance autophagy, mitigate ROS production, and upregulate Nrf2 and HMOX1 expression. These findings underline the potential of conessine and similar compounds as a target for AD and other neurodegenerative conditions. While our results provide a strong foundation, further mechanistic and preclinical studies are needed to overcome the limitations of this study. For instance, our *in vitro* model may not fully recapitulate the complex microenvironment of the AD brain, including glial interactions and chronic inflammation. Furthermore, confirmation of autophagic flux and the precise molecular interactions between conessine and β -amyloid remain unclear and could be addressed in future studies. These factors must be studied to fully elucidate conessine's therapeutic potential and facilitate its translation to clinical applications.

ACKNOWLEDGEMENT

None

ABBREVIATIONS

AD: Alzheimer's Disease; **AMPK:** AMP-Activated Protein Kinase; **ANOVA:** Analysis of Variance; **A β :** Beta-Amyloid; **DCFDA:** 2',7'-Dichlorofluorescein Diacetate (used for ROS analysis); **DMEM:** Dulbecco's Modified Eagle's Medium; **DMSO:** Dimethyl Sulfoxide; **FBS:** Fetal Bovine Serum; **HMOX1:** Heme Oxygenase-1; **mTOR:** mechanistic Target of Rapamycin; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (used in the MTT assay); **NF- κ B:** Nuclear Factor Kappa B;

NRF2: Nuclear Factor Erythroid 2-Related Factor 2; **PBS:** Phosphate-Buffered Saline; **qRT-PCR:** quantitative Real-Time Polymerase Chain Reaction; **ROS:** Reactive Oxygen Species; **SD:** Standard Deviation; **SYBR:** SYBR Green (fluorescent dye used in qPCR).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

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SUMMARY

This study investigates the neuroprotective effects of conessine, a steroidal alkaloid from *Holarrhena antidysenterica*, in beta-amyloid (A β)-induced SH-SY5Y human neuroblastoma cells, an *in vitro* model of Alzheimer's Disease (AD). Conessine at low doses (≤ 12.5 μ g/mL) significantly protected cells from A β -induced cytotoxicity, restoring cell viability and morphology, as shown by MTT assays. It enhanced autophagy, counteracting A β -induced autophagy impairment, as confirmed by flow cytometry. Additionally, conessine reduced Reactive Oxygen Species (ROS) production, as measured by DCFDA staining, indicating potent antioxidant effects. Real-time PCR revealed that conessine upregulated antioxidant genes NRF2 (1.5-fold) and HMOX1 (1.10-fold) in A β -treated cells, with higher expression (1.78- and 1.4-fold) in conessine-only treated cells. These findings suggest conessine's potential as a therapeutic candidate for AD by mitigating cytotoxicity, enhancing autophagy, and boosting antioxidant defences. Further mechanistic and preclinical studies are needed to explore its clinical applicability.

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