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RESEARCH ARTICLE

In Vitro Pharmacological Evaluation of Fulvic Acid and Humic Acid Novel Combination in Amyloid Beta Intoxicated SH-SY5Y Cell Lines Model of Alzheimer's Disease

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Abstract

Alzheimer's disease is the most widespread advancing neurodegenerative disorder, and it is estimated that more than 50 million people have been affected worldwide. It is characterized by dementia, loss of sensory and motor function and difficulty in performing work. NMDA antagonists and acetylcholinesterase inhibitors are currently being used as treatment options for AD, but the treatment would not decline the disease progression as well as neurodegeneration. We used a unique humic acid and Fulvic acid mixture in the current investigation for the treatment of amyloid-β intoxicated Alzheimer's *in vitro* model in SH-SY5Y cell line. Humic acid and fulvic acids are organic substances which will be produced by microorganism from the biomass such as dead matter of animal as well as plants. Both the components were used in the 1:1 ratio against AD induced cell line with optimum concentration 50µM/ml of each compound. The inflammatory mediators like Reactive Oxygen Species (ROS) were estimated using flow cytometry - H2DCFDA staining assay and cytokines such as TNF-α and IL-1β levels were estimated using ELISA. The results obtained from present study implicating that the humic acid and fulvic acids and their novel combinations lessen the ROS, cytokines level and decreased the apoptosis levels there by it exhibiting the neuro-protective mechanism via inhibiting neuro-inflammatory pathway in AD.

Keywords: Alzheimer's Disease, Cytokines, Dementia, Fulvic Acid, Humic Acid, Neurodegeneration

1. Introduction

Alzheimer's Disease (AD) is a classic neurodegenerative disease that primarily affects the ageing brain and is characterised by a variety of cognitive deficits, including difficulties with working, emotional, and sensory-motor gating. An estimated 50 million people worldwide have AD, according to estimates[1], there would be 82 million cases of AD worldwide in 2030 and 152 million cases in 2050[2]. It is also linked to neuropathological alterations due to the deposition of β-amyloid peptide (Aβ) fragments in extracellular space around the neurons. Amyloid Precursor Protein, a transmembrane protein, is processed by the proteases β-secretase and γ-secretase to create Aβ[3].

Aβ induces oxidative stress resultantly it enhances the level of β-secretase and γ-secretase, which further up regulate the production of Aβ in AD affected brain[4]. As tau proteins are formed into hyper phosphorylated, insoluble aggregates known as neurofibrillary tangles by Aβ fragments, which are more toxic to neurons, cholinergic neurons in the hippocampus and cortical regions of the brain are more susceptible to degeneration, which lowers the level of acetylcholine. One of the causes of altered neurophysiology, including the cholinergic pathway's signal transmission and AD, is a depleted acetylcholine level[5]. Thus, reducing or suppressing oxidative stress and Aβ-mediated cytotoxicity may be a promising preventative or therapeutic intervention in AD.

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Aβ (25-35) has been used frequently in studies of Aβ characteristics because it is a simpler alternative to the native full-length peptide, Aβ (1-42), which is known to be implicated in the pathophysiology of AD. These experiments assume that the mechanisms of action of Aβ (25-35) and Aβ (1-42) are similar. In comparison to the original Aβ (1-42), the shorter peptide is more toxic to cultured neurons, exhibits toxicity early, oxidises membrane proteins more, and aggregates more quickly. In fact, although these traits are enhanced, Aβ (25-35) matches the toxicological and aggregation properties of the full-length peptide[6]. Tumour necrosis factor (TNF-α), which increases apoptosis and necrosis events in the brain's sensitive regions like the hippocampus, is produced by the Aβ (25-35), which also causes oxidative damage, free radical production, microglial activation via cytokines, and microglial activation in the rat brain[7].

Glutamate antagonists and cholinesterase inhibitors are two of the available treatments for AD. These symptomatic treatments have side effects and neither slows down the disease's course nor stop neuronal deterioration. As a result, many medications are being developed that aim at a variety of targets and are anticipated to treat AD more effectively than single-targeting drugs[8-10].

Fulvic Acid (FA) was previously tested for cardio protective, anti-inflammatory, anti-diabetic, antioxidant activity, and in an *in vitro* model, it was shown to prevent the aggregation and disintegration of tau fibrils linked to AD. Humic acid was previously tested for neuroprotective and hepatoprotective activity in both *in vitro* and *in vivo* studies[11-16]. Therefore, in the present study, SH-SY5Y *in vitro* cell line model administration with Aβ (25-35) was suggested as a logical strategy to create a sporadic AD model for assessing the effects of a novel 1:1 ratio of FA and HA.

2. Materials and Methods

2.1 Materials

Amyloid beta (Aβ (25-35), ≥95 %) and Minimum Essential Medium (MEM) was purchased from AllianzBio (Maharastra, India), dimethylsulfoxide (DMSO,≥99.9%),ELISAkitand3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT, ≥99.9 %) were obtained from Sigma-Aldrich (St. Louis, MO). 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, ≥99.9 %) was purchased from Thermo Fisher Scientific. All other reagents were from commercial suppliers and of standard biochemical quality.

2.2 *In Vitro* Study (Aβ 25-35 Intoxicated SH-SY5Y Cell Lines Model)

2.2.1 Maintenance of Cells

The human neuroblastoma cell line SH-SY5Y was purchased from NCCS, Pune, India. The 1% antibiotic-antimycotic solution and 10% FBS were added to MEM media to sustain SH-SY5Y cells, which were then subcultured every two to three days at a temperature of 37°C in a CO_2 incubator[17].

2.2.2 Cell Viability Assessment – MTT Assay

The MTT [(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide)] assay, which is based on the theory of conversion of MTT to formazan, was used to assess the neuronal viability in terms of mitochondrial metabolic function. The SH-SY5Y cells were pre-treated with Aβ 25-35 (10μM) for 2 hours to determine the protective effect and then post-treated with Fulvic Acid (FA), Humic Acid (HA), and FA+HA (1:1) using a non-toxic concentration of 50 μM/ml. A microplate reader was used to measure the absorbance at 570 nm after additional cells were treated with MTT (0.5 mg/ml in medium) for 3 hours at 37 °C in 5 % CO_2. The formazan crystals that formed as a result were then dissolved in DMSO. The conventional formula was used to determine the percentage of SH-SY5Y cells that were viable[18-20].

2.2.3 Aβ(25-35) Intoxication and FA, HA and FA+HA (1:1) Treatment

In Minimum Essential Medium, the SH-SY5Y cell lines were cultured in accordance with the prescribed protocol. 20,000 cells per well were sown in 96-well plates after a haemocytometer was used to count the cells and 99% viability was established. The cells were next treated post-induction with FA, HA, and FA+HA (1:1) at a concentration of 50 μM/ml and incubated for 24 hours. The cells had previously been exposed with Aβ 25-35 (10 μM) for 2 hours to induce neurotoxicity[21]. Following therapy, the following assessments were carried out.

2.2.4 Cell Morphological Observation

The morphological alterations in the SH-SY5Y cell lines were observed and documented with the help of inverted phase contrast microscopy (CKX-41, Olympus, Tokyo, Japan) at a 20x magnification.

2.2.5 Measurement of IL-1β and TNF-α (Pro-inflammatory Cytokines) by ELISA

After treating Aβ-induced SH-SY5Y cells with FA, HA, and combination of FA and HA in a 1:1 ratio, the levels of IL-1β and TNF-α were determined in the cell culture supernatants using commercially available ELISA kits (Ray Biotech, Norcross, GA), in accordance with the manufacturer's instructions. A 96-well plate covered with an antibody specific for human IL-1β (mouse IL-6 antibody) or TNF-α (mouse TNF-α antibody) is used in the experiment. Standards and samples were pipetted into the wells, and the immobilised antibody was employed to bind any IL-1β and TNF-α present in the sample to the wells. After washing the wells, an anti-human IL-1β /TNF-α biotinylated antibody was added. HRP-conjugated streptavidin was pipetted into the wells after unbound biotinylated antibody was removed with washing. The wells were once again rinsed, and then a TMB substrate solution was added. The amount of IL-1β or TNF-α bound determines how much colour appears in the wells. The stop solution (0.16M sulfuric acid) turns the colour from blue to yellow, and the microplate reader (ELX-800, BioTek, USA) was used to measure the colour's intensity at 450 nm [22].

2.2.6 Assessment of Reactive Oxygen Species (ROS) Level in Aβ Induced SH-SY5Y Cells by Flow Cytometry

The ROS level in SH-SY5Y cells was assessed using a flow cytometer from BD Biosciences in San Jose, California. H2DCFDA, a cell-permeable non-fluorescent dye that was used to identify ROS in treated cells and causes them to produce DCF. Oxidative stress and the elevated DCF intensity were inversely related. In brief, the cells were cultivated in a 6-well plate at a density of 0.5 x 10^6 cells per millilitre and then incubate at 37 °C in a CO_2 incubator overnight. Aspirate the used medium, incubate the cells for two hours with Aβ protein (10 µM/mL) to cause neuro-inflammation or neurotoxicity, and then added 50 uM/ml of experimental chemicals (HA, FA, and HA+FA) to one millilitre of culture medium. To serve as a negative control, one well has been left untreated[23].

The cells were collected and used medium at the end of the treatment cycle, placed in 5 ml polystyrene tubes, and centrifuged the tubes for five minutes at 300 x g at 25 °C. After carefully decanting the supernatant, it was washed twice with PBS and finally, PBS was decanted. The cells were re-suspended in an H2DCFDA working solution, generated in culture media at 1 x 10^6 cells/ml density, and incubated at 37 °C for 30 min while being shaded from the light. The tubes were centrifuged at 150 x g for 5 minutes. The supernatant was gently removed and then the cells were re-suspended in 400 ml of pre-warmed DPBS. A flow cytometry (BD FACS Calibur) analysis was carried out using a 488 nm and a 535 nm (FL1) laser for excitation and detection, respectively. Software developed by BD Cell Quest pro was used to assess the percentage of ROS level under every circumstance[24].

2.2.7 Live/Dead Assay by Confocal Fluorescence Microscopy

The cells were cultured overnight at 37 °C in a CO_2 incubator in a 6-well glass bottom plate with a cell density of 0.5 × 10^6 cells/2 ml. The cells were exposed to Aβ protein (10 µM/mL) for 2 hours, followed by fulvic acid, humic acid, and FA+HA (1:1) at a concentration of 50 µM/ml in 1000 µl of culture media, and the cells were then incubated for 24 hours. As a control, cells that weren't treated at all were used. Each well's medium was taken out and put into 12 × 75 mm polystyrene tubes. The tubes were then washed with 500 µl of PBS. PBS was taken out, 250 µl of trypsin-EDTA solution was added, and the mixture was incubated at 37 °C for 3 to 4 minutes. The culture liquid was added back to each well, and the cells were then immediately harvested into 12 × 75 mm polystyrene tubes. The cells were stained for 10 minutes using 200 µL staining solution with 100 µg/ml of EtBr and acridine orange. The edges of the cover slip and a DPX mount were used to secure a 100µl cell suspension. A filter cube and a fluorescence microscope were used to observe under conditions where EtBr is excited at 560/40 nm and emitted at 645/75 nm, and acridine orange is excited at 470/40

nm and emitted at 525/50 nm. Using Image J Software version 1.48, images were superimposed[25].

3. Results

3.1. Cell Viability Assessment – MTT Assay

Effect of HA, FA, and HA+FA on Aβ 25-35-impaired SH-SY5Y cell lines' cell viability levels.

The viability of Vehicle control cells was defined as 99.66 %. The results are the mean ± SD, n = 3. Superscript '***' denotes P< 0.001 between treatment versus amyloid beta, ### denotes P< 0.001 between vehicle control versus amyloid beta (Figures 1 and 2).

Table 1. The percentage cell viability values of Aβ induced SH-SY5Y cells upon treatment of FA, HA and FA+HA with 50µM/ml concentration after the 24 hours treatment

Culture condition	% Cell viability ± SD
Untreated	99.66±0.6
$A\beta_{25-35}$ (10µM)	42.00±1.2###
$A\beta_{25-35}$(10µM) +FA (50µM)	78.33±1.15***
$A\beta_{25-35}$(10µM) + HA (50µM)	66.66±1.52***
$A\beta_{25-35}$(10µM) +FA+ HA (1:1) (50µM)	87.66±1.52***

3.2 Statistical Analysis

GraphPad Prism Version 6.0, one-way ANOVA and Tukey's multiple comparison tests were used to establish the statistical significance (Table 1).

3.3 Evaluation of FA, HA and FA+HA on Pro-inflammatory Cytokines Expression in Aβ Exposed SH-SY5Y Cells

The effects of HA, FA, and HA+FA on the levels of cytokines like TNF-α and IL-1β in SH-SY5Y cell lines exposed to Aβ 25-35 were observed (Table 2).

Table 2. Effect of FA, HA and FA+HA on pro-inflammatory cytokines concentration in Aβ exposed SH-SY5Y cells

Treatment Conc. -(µM/ml)	TNF-alpha (pg/ml) ± SD	IL-1 beta (pg/ml) ± SD
Vehicle Control	217.33 ± 13.97	3.33 ± 0.52
($A\beta_{25-35}$)	1619.17 ± 19.03###	27.66 ± 0.81###
$A\beta_{25-35}$ + FA 50 µM	442.66 ± 15.91 ***	6.33 ± 0.52 ***
$A\beta_{25-35}$ + HA 50 µM	622.16 ± 18.26 ***	8.5 ± 0.55 ***
$A\beta_{25-35}$ + (FA+ HA) 50 µM	337.83 ± 14.61 ***	4.16 ± 0.75 ***

Results are the mean ± SD, n = 3. Superscript '***' denotes P< 0.001 between treatment versus amyloid

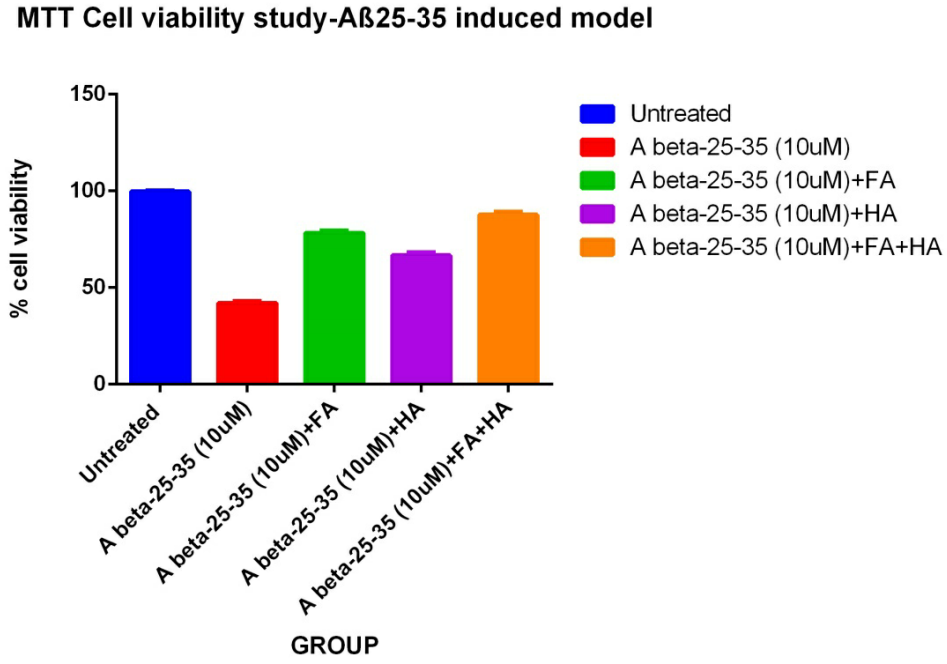

Figure 1. Overlaid bar graph depicted the percentage cell viability values of FA, HA and FA+HA with 50µM/ml concentrations treated on Aβ stimulated SH-SY5Y cells in comparison to Aβ alone and vehicle control. Aβ – Amyloid beta, FA – Fulvic acid, HA – Humic acid.

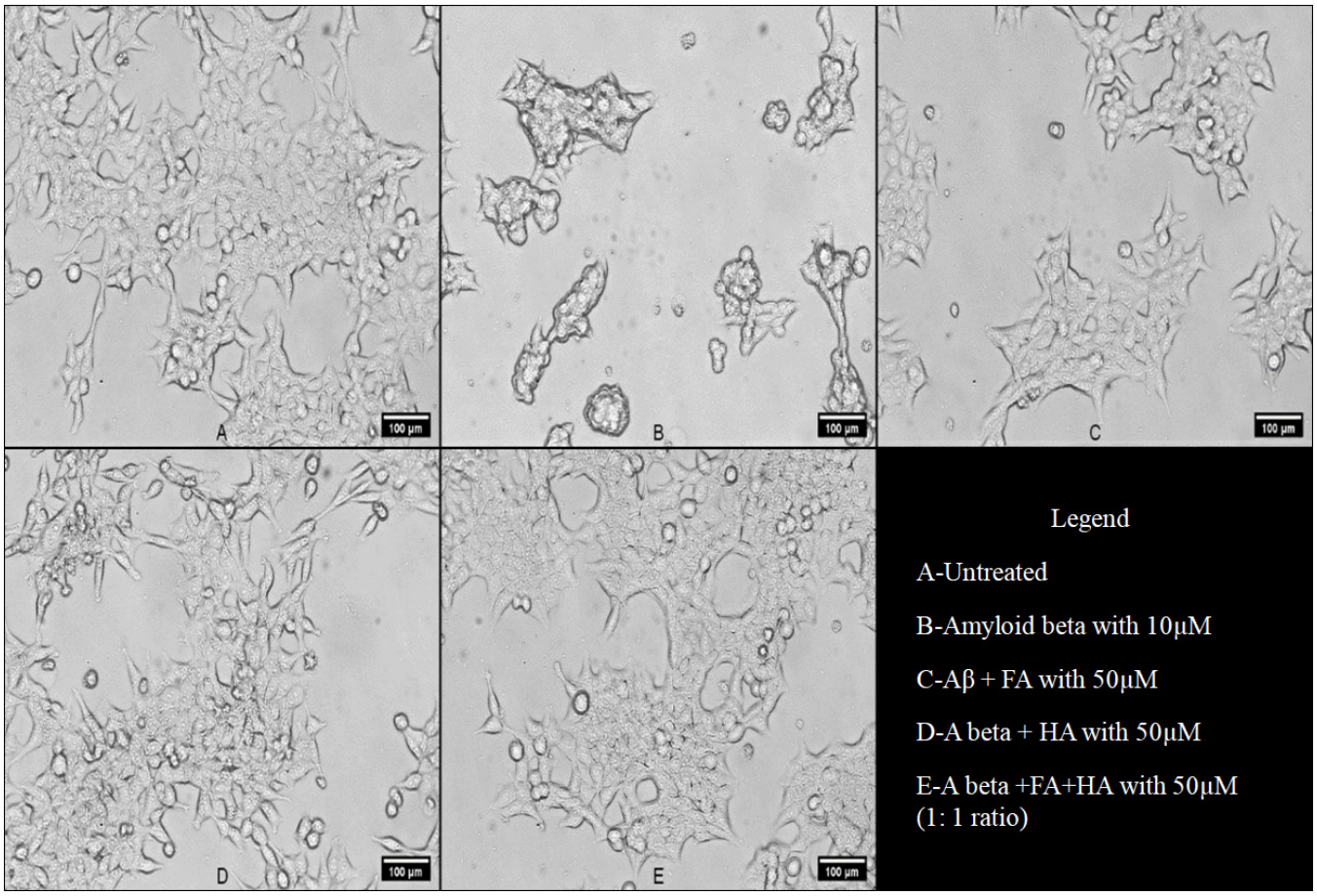

Figure 2. Microscopic observations of Fulvic acid, Humic acid and a combination of Fulvic acid and Humic acid in 1:1 ratio treated on β-amyloid stimulated SH-SY5Y cells with 50µM/ml concentration after the incubation period of 24 hours. All the images were captured at 20x magnification. Aβ – Amyloid Beta, Scale bar - 100µm, FA – Fulvic acid, HA – Humic acid.

beta, $^{###}$ denotes P< 0.001 between vehicle control versus amyloid beta (Figures 3 and 4).

3.3 Evaluation of Impact of HA, FA and HA+FA on ROS level in Aβ Exposed SH-SY5Y Cells by Flow Cytometry

The impact of HA, FA and HA+FA on ROS level was determined in SH-SY5Y cells by incubating with and without 10 uM/ml Aβ for a period of 2 hours and treat the cells with 50 µM/ml HA, 50 µM/ml FA and 50 µM/ml of HA+FA independently for a period of 24 hours, later the cells were stained using H2DCFDA dye and ROS in the cells were quantified by flow cytometer. By labelling the cells with H2DCFDA dye, which following reaction with cells subjected to oxidative stress transforms into DCF, the amount of ROS intensity in the cells was determined. Oxidative stress (ROS) and DCF intensity expression were directly proportional.

It was discovered that untreated SH-SY5Y cells have reduced DCF expression. After exposing SH-SY5Y cells to Aβ 25-35 for 24 hours, the DCF level was increased. As determined by H2DCFDA labelling using a flow cytometer, HA and FA treatment for a period of 24 hours decreased the ROS levels in Aβ-exposed SH-SY5Y cells, as did a combination of HA and FA for a period of 24 hours.

The ROS level and oxidative stress were decreased in Aβ-exposed SH-SY5Y cells treated with HA, FA, and HA+FA. Therefore, the findings of the H2DCFDA investigation suggest that all of the substances had a protective effect against the ROS level and oxidative stress caused by Aβ in SH-SY5Y cells. The results of the current study suggested that combining FA and HA in a 1:1 ratio may have a greater impact on neuroprotective capacity than performing individually.

Results are presented as the mean ± SD for n = 3. The superscripts *** indicate that the comparison between treatment and amyloid beta is significant (P<0.001),

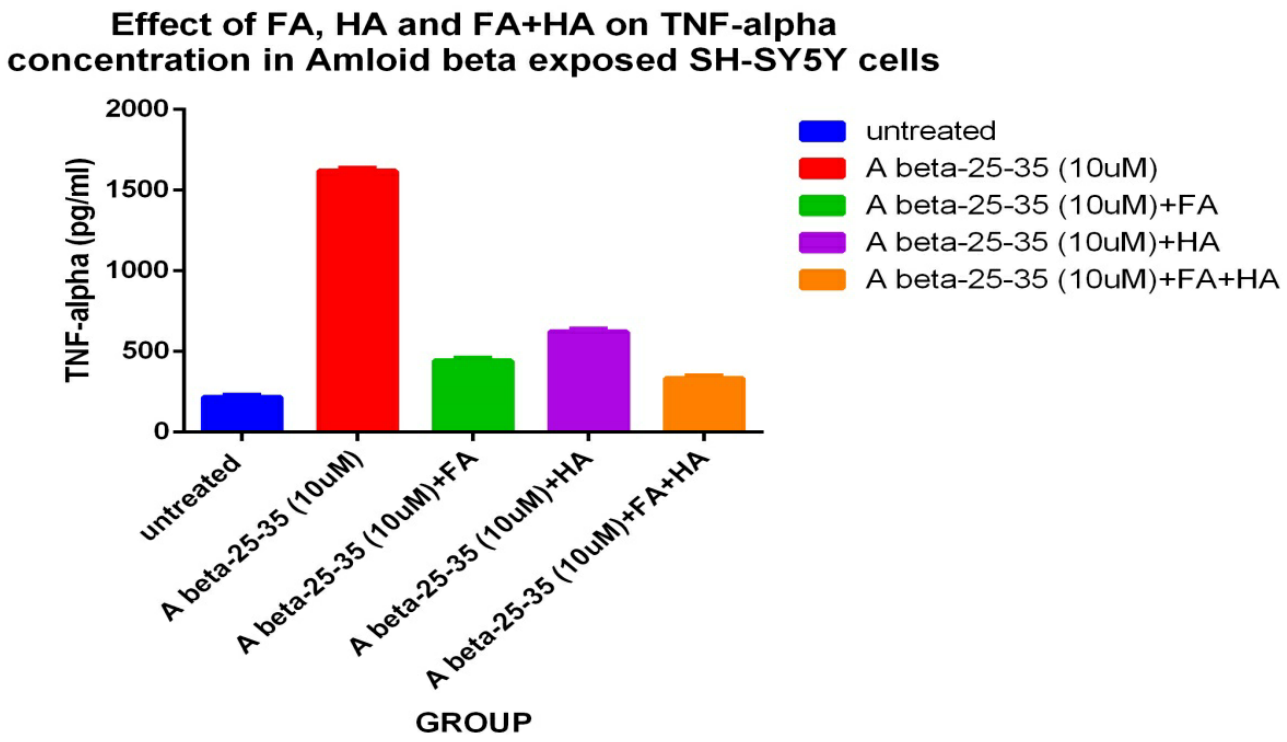

Figure 3. Overlaid bar graph depicted the TNF-α concentration observed in FA, HA and FA+HA with 50µM/ml concentrations treated on Aβ stimulated SH-SY5Y cells in comparison to the Aβ alone and vehicle control cells. TNF-α concentration was expressed in terms of pg/ml. Aβ - Amyloid beta, FA – Fulvic acid, HA – Humic acid.

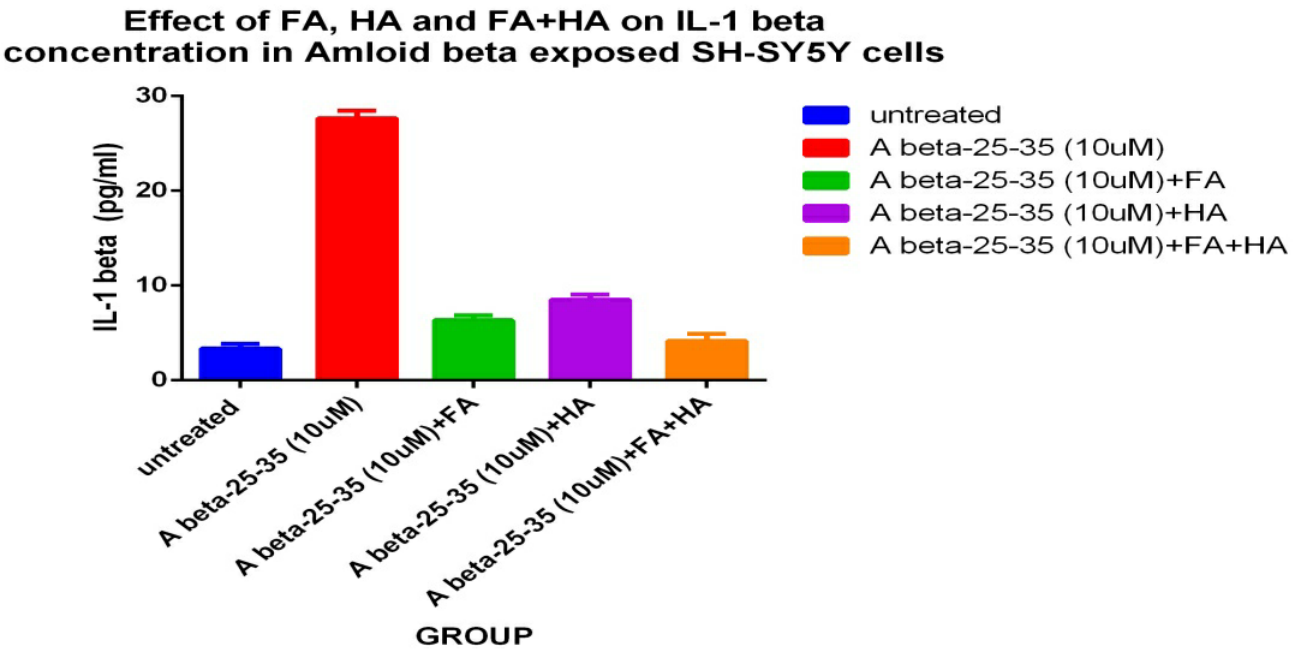

Figure 4. Overlaid bar graph depicted the IL-1β concentrations observed in FA, HA and FA+HA with 50µM/ml concentrations treated on Aβ stimulated SH-SY5Y cells in comparison to the Aβ alone and Vehicle control cells. IL-1β concentration was expressed in terms of pg/ml. Aβ – Amyloid beta, FA – Fulvic acid, HA – Humic acid.

Table 3. H2DCFDA expression study of the FA, HA and FA+HA against SH-SY5Y cell line induced by Aβ

Culture condition	Percentage cells expressed DCF intensity ± SD
Untreated	0.36 ± 0.14
Aβ-10 µM alone	72.29 ± 2.13###
Aβ+ FA	32.81 ± 2.26***
Aβ+ HA	44.11 ± 2.27***

similarly ### indicates P < 0.001 between untreated and amyloid beta (Table 3).

The intensity of Dichlorodihydrofluorescein (DCF) was inhibited in FA, HA and FA+HA combinations in Aβ intoxicated cells. % Cells expressed DCF intensity in different culture conditions of FA, HA and FA+HA with 50µM concentrations exhibited 32.81 ± 2.26 %, 44.11 ± 2.27 % and 23.35 ± 3.29 % cells whereas Aβ with 10µM alone showed 72.29 ± 2.13 % of ROS expression respectively. Cells without treatment were served as a control group (Figure 5).

The intensity of ROS was drastically depleted with the novel combination FA+HA 50µM (1:1) concentration, neuronal cell death and apoptosis significantly inhibited in amyloid beta intoxicated SH-SY5Y neuronal cells and statistically, it was significant and the p-value found that <0.001.

3.4 Live/Dead Assay by AO/EtBr Staining

Legend: 1. Untreated, 2. Aβ, 3. Aβ + FA, 4. Aβ + HA and 5. Aβ + (FA + HA) [Aβ - Amyloid beta]

Untreated and Aβ-induced SH-SY5Y cells were post-treated with 50µM concentrations of FA and HA separately and in combination at a 1:1 ratio in a study

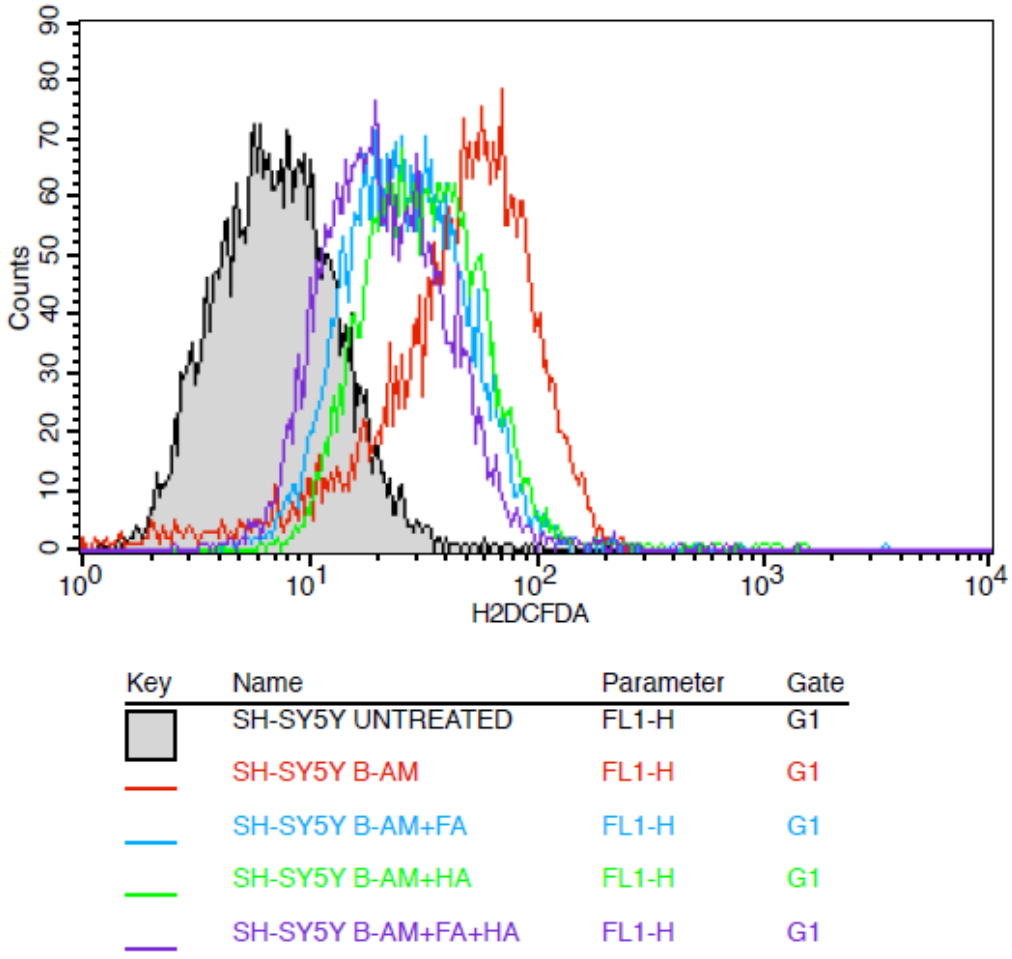

Figure 5. Overlaid histograms depicted the H2DCFDA expression in Untreated, Aβ alone with 10uM/ml, Aβ +FA-50µM/ml, Aβ+HA-50µM/ml and Aβ+HA+FA-50µM/ml concentration against the SH-SY5Y cells using BD FACS Calibur and obtained data was analysed by BD CellQuest pro (ver.6.0). B-AM– Amyloid beta, FA – Fulvic acid, HA – Humic acid.

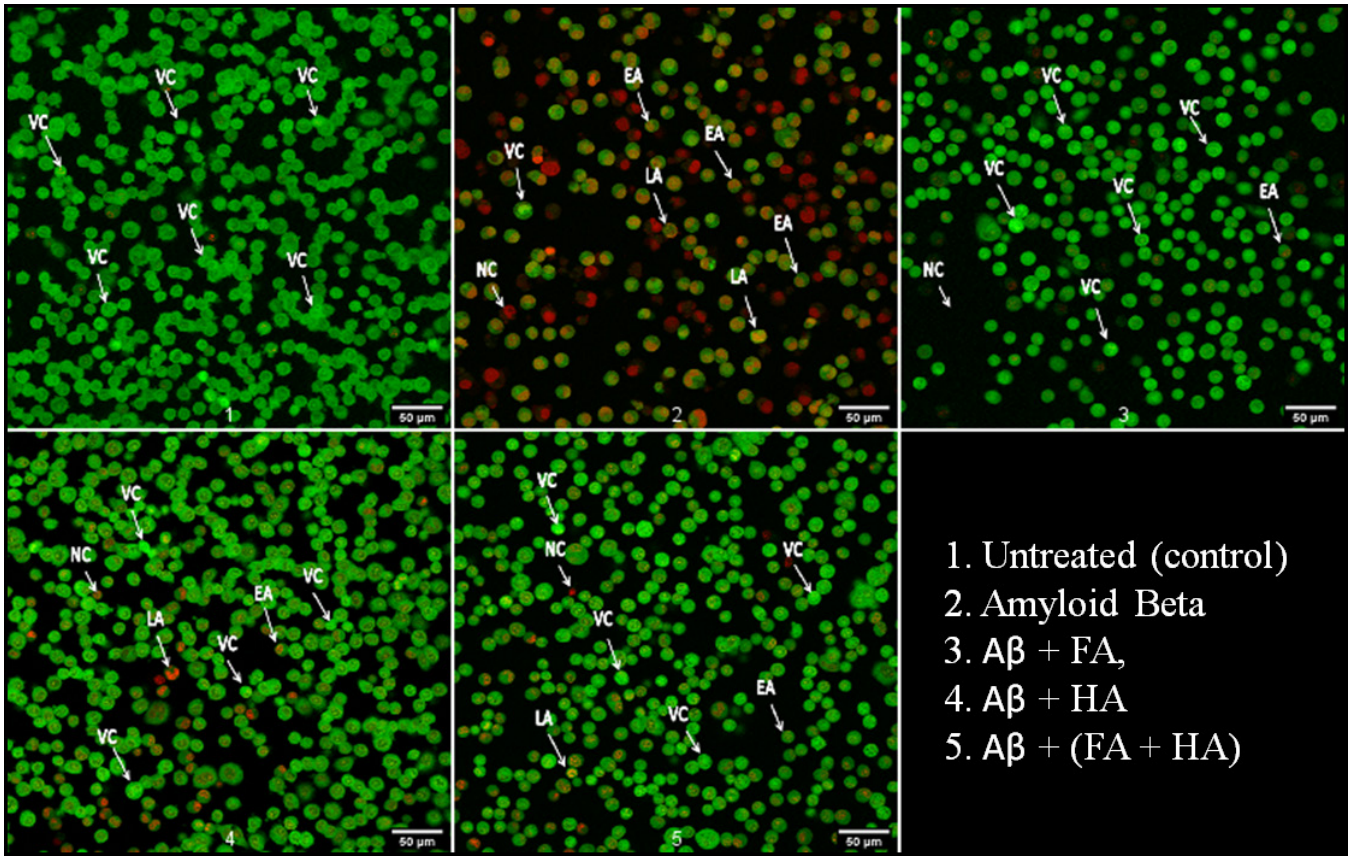

Figure 6. Live/dead assay by AO/EtBr staining.

using AO and EB dual-labeling on SH-SY5Y cells. 40 x magnifications were used to take the pictures. Images show viable cells (VC), early (EA), late (LA), and necrotic (NC), respectively (Figure 6).

4. Discussion

The neuroprotective impact of HA, FA, and their combined form (HA+FA) was investigated through *in vitro* analysis using neurodegenerative cells induced by Aβ. In the present study, we have used five incremental doses ranging from 6.25 to 100 µM/ml concentrations. HA, FA and HA+FA (1:1) compounds showed non-toxic potency till 50µM/ml. 100µM/ml concentration caused moderately alters cell viability on SH-SY5Y cells. In the combinational treatment of these compounds in Aβ induced model, 50µM/ml caused effective recovery from Aβ induced neurotoxicity. We have then compared the individual compounds; combination of both compounds enhanced high cell proliferation and confirmed the neuroprotective ability of both molecules. Cell viability significantly increased up to 88.82 ± 1.6 % (50 µM/ml), 79.64 ± 1.49 % (50 µM/ml) and 90.04 ± 1.1 % (50 µM/ml) for FA, HA and HA+FA (1:1) respectively when compared to Aβ alone group. Therefore, the present study result reveals that optimum dose (50 µM/ml) may play an important factor for the pharmacological efficacy of these compounds. The percentage of cell viability was assessed after appropriate treatment with designed combination, the results were significantly indicating the potent anti-oxidative effect in Aβ intoxicate SH-SY5Y cells, the efficacy novel combination (HA+FA) was significant against vehicle control, FA, HA alone and the statistical significance was found to be $p<0.001$.

Live/dead assay by Acridine orange/Ethdium bromide staining results depicted similar trend to ROS study by inhibiting the amyloid induced neurotoxicity[26]. In AD induced model, Apoptotic cells were highly observed and combination of FA+HA combination effectively inhibited the rate of apoptosis caused by

amyloid beta whereas in individual treat of FA and HA groups, FA showed better recovery of amyloid toxicity than the HA group and confirmed that FA have better neuroprotective effect than the HA.

The estimation of TNF-α and IL-1β, pro-inflammatory cytokines levels have been done using commercially available ELISA kits[27]. SH-SY5Y cells were treated with Aβ, Aβ +FA, Aβ + HA and Aβ+ FA+HA respectively. FA, HA and FA+HA effectively inhibited the levels of TNF-α and IL-1β compared to Aβ alone treated SH-SY5Y cells. Observed results suggested that FA, HA and FA+ HA by depleting the cytokines level and proved to be potent agents in order to protect the neuroblastoma cells against Aβ induced neuro-inflammation. Overall, the observed cytokines expression studies by ELISA method suggested that the combination of the non-toxic concentrations of both FA and HA showed better inhibitory effect than the individual compounds and proved that combination of FA and HA in 1:1 ratio may have better neuro-protective effect[28].

Humic acid and fulvic acid are naturally available bio-active compounds which have been used in the traditional use of medicine to cure various diseases. Due to its immune regulatory properties of fulvic acid, it has found utilization as a traditional Chinese medicine. FA constitutes a blend of polyphenolic acid compounds sourced from humus, peat, lignite, and aquatic surroundings. Its application in traditional medicine extends to the treatment of disorders related to the digestive tract[29]. Humic and fulvic acids are abundant sources of minerals that are readily absorbable by our bodies, prompting a range of specific and nonspecific biological activities. In the realm of natural medicine's extensive database, humic substances emerge as therapeutic agents with diverse bioactive capabilities. These substances are being advocated for use as dietary supplements and are even finding application in cosmetic products[30].

Fulvic acid has already been reported for its anti-oxidant as well as neuroprotective properties by inhibiting the neuro-inflammatory mechanism on the other hand humic acid has also been reported as a strong anti-oxidant[31,32]. In the present study, we have used the combination of HA+FA (1:1) against Aβ intoxicated neuroblastoma cells with various concentrations of treatment and then treated and untreated combinations were examined for various anti-inflammatory cytokines., TNF-α and IL-1 β and DCF expression. The level of ROS and cytokines was significantly depleted in treatment groups. FA (50 µM/ml), HA (50 µM/ml) and FA+HA (50 µM/ml) treated SH-SY5Y cells which were pre-stimulated with 10µM/ml of Aβ significantly suppressed the expression of DCF in Aβ induced model as compared to Aβ alone treated SH-SY5Y cells[33].

In summary, based on cell viability studies in β amyloid-induced neuronal cells, it was observed that neuronal cell death caused by β amyloid (42.00 ± 1.2 %) whereas the treatment of FA, HA, and the combination of FA and HA showed 78.33 ± 1.15, 66.66 ± 1.52, and 87.66 ± 1.52 % respectively. These results revealed the fact that both agents have the neuroprotective ability by recovering the cell death caused by β amyloid agent. Surprisingly both compounds showed a similar trend of cell recovery in terms of viability of cells. Further combinational treatment of both FA and HA in 1:1 ratio with 50µM concentration exhibited the 90.04 ± 1.1 % in terms of % cell viability and proved to be a better formulation to recover the oxidative stress or apoptosis caused by β amyloid[34].

Further to prove the neuroprotective ability of molecules, pro-inflammatory cytokines were measured in the supernatants of all treatment groups including controls[35]. In the current study, the levels of interleukin-1 beta and tumour necrosis factor-alpha (TNF-α) were measured. In the β amyloid treatment group, both cytokines effectively enhanced due to oxidative stress and gradually decreased in FA, and HA individual groups and significantly reduced in combinational treatment of FA+HA. The obtained results of quantitative measurements of cytokines expression double confirmed the neuroprotective ability of molecules similar to the cell viability studies[36].

Oxidative stress is a key factor in causing neuronal cell death or neurotoxicity. Neurotoxicity caused by β amyloid agents resembles Alzheimer's disease AD in humans. To study the oxidative stress in all the treated and non-treated groups of neuroblastoma cells, ROS study was employed by H2DCFDA staining by Flow Cytometry method. Percentage cells expressed DCF intensity in all the different conditions of untreated, β amyloid alone and β amyloid with fulvic acid;

humic acid and a combination of both in 1:1 ratio were screened by flow cytometry. In all the groups, 10,000 cells were counted to measure the DCF expression[37,38]. Combinational treatment of FA and HA with 50µM showed more efficient protective ability than the individual groups in Neuroblastoma cells. β-amyloid alone enhanced DCF expression in SH-SY5Y cells.

5. Conclusion

In conclusion, the study concludes that the innovative combination of FA and HA (fulvic acid and humic acid), represented as FA+HA, exerts more robust effects when compared to the separate administration of FA and HA. This synergistic combination has demonstrated a remarkable capacity for safeguarding human neuronal cells (specifically, SH-SY5Y cells). The substantiation of this protective efficacy stems from a multi-faceted analysis involving the quantification of cytokine expression levels using Enzyme-Linked Immunosorbent Assay (ELISA), the reduction of apoptosis as observed under Confocal Microscopy, and the attenuation of DCF (2',7'-dichlorofluorescin) intensity via Flow Cytometry within a model environment where β amyloid induction is implemented.

This comprehensive study provides compelling evidence for the neuroprotective potential of the FA+HA combination. However, to establish a more profound understanding of the underlying molecular mechanisms responsible for the observed neuroprotective attributes of FA and HA, and to pave the way for their potential application in the therapeutic landscape of Alzheimer's disease, further investigation in an *in vivo* context is imperative. By delving into the intricacies of these compounds' interactions within a living organism, we can garner insights that will augment the translational significance of these findings, potentially bringing us closer to viable treatments for Alzheimer's disease.

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7. References

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