Assessment of neuroprotective effect of *Grewia tiliaefolia* and vitexin against Aβ$_{25-35}$ and glutamate induced toxicity in Neuro-2a cell lines
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5.5. Summary
Alzheimer’s disease (AD) is a severe progressive neurodegenerative disorder among the elderly population. World Alzheimer Report, 2015 has estimated that globally 46 million people were affected by dementia, of which 80% of the cases accounts for AD (Matrone and Brattico, 2013). The drugs currently licensed for the treatment of AD is the use of cholinesterase inhibitors, which increases the availability of the neurotransmitter acetylcholine (ACh) in the brain (Onor et al., 2007). However, this treatment strategy is not helpful in the long run, as AD is characterized by several pathological hallmarks (Bu et al., 2016). Secretion, aggregation and deposition of Aβ has been said to be a crucial process in the development of AD, and prevention of this process can be considered as an effective therapeutic approach for alleviating the progression of the disease (Stefani and Rigacci, 2014). The proteolytic processing of amyloid precursor protein (APP) by β and γ- secretases gives rise to Aβ peptides of varying length, which are made of 40-42 amino acid residues (Dong et al., 2012). Though the most abundant form of peptides produced in the brain are Aβ1-40 and Aβ1-42, a relatively smaller fragment Aβ25-35 consisting of 11 amino acid sequences which causes similar kind of aggregation pattern, oxidative and neurotoxicity as that of full length peptide has also been found in the brain of AD patients (Sato et al., 1995; Shanmugam and Jayakumar, 2004). This makes it worthy to use Aβ25-35 in AD related studies, to understand the pathogenesis caused by full length peptide.

Aggregated Aβ peptides in the brain apart from directly inducing neurodegeneration, interferes with several signaling pathways including glutamatergic system. This interference removes the blockade of N-methyl-D-aspartate receptor (NMDAR) leading to its continuous activation and glutamate excitotoxic condition. Glutamate is the most abundant and major endogenous excitatory neurotransmitter involved in learning and memory. It is synthesized through several metabolic pathways and its availability at appropriate levels for cellular signaling is strictly under the control of glutamate uptake and recycling mechanisms (Danbolt, 2001). A high concentration of extracellular glutamate exists in the synaptic cleft during AD (Lewerenz and Maher, 2015) and the chief mechanism through which glutamate evokes its toxicity in neuronal
cells is via the increased influx of Ca\textsuperscript{2+} ions through the continuous activation of NMDAR mediated by oxidative stress. Prevailing of such conditions for a longer time subsequently leads to the release of apoptotic factors and cell death (Danysz and Parsons, 2012).

Although the progression of AD is a complex process, several reports suggest that elevated oxidative stress by Aβ and glutamate excitotoxicity plays a crucial factor (Kamat et al., 2016). Enhanced production of reactive oxygen species/reactive nitrogen species, along with the deterioration of antioxidant defense system and altered signaling pathways, results in the oxidation of lipids, proteins and DNA leading to neurodegeneration (Markesbery and Camey, 1999). Based on this notion, supplementation of antioxidants has been considered as one of the effective treatment approaches against AD. With the preliminary studies pointing towards methanol extract of G. tiliaefolia and its active constituent vitexin to be promising against AD, they were employed for the identification of neuroprotective effect and possible mode of action against Aβ\textsubscript{25-35} and glutamate induced toxicity in Neuro-2a cells.

5.2. MATERIALS AND METHODS

5.2.1. Extract preparation

Methanol leaf extract of G. tiliaefolia (GT) was prepared as mentioned in Section 3.2.1.

5.2.2. Cell culture

Neuro-2a cells were purchased from National Centre for Cell Sciences (NCCS), India. Cells were cultured with Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), 1X Penicillin-Streptomycin antibiotic and maintained at 37 °C in a humidified incubator containing 5% CO\textsubscript{2}.

5.2.3. Evaluation of neuroprotective effect of GT/vitexin against Aβ\textsubscript{25-35} toxicity in Neuro2a cells

5.2.3.1. Cell viability assay

Neuro-2a cells (2 x 10\textsuperscript{5} cells) were treated with various doses of Aβ\textsubscript{25-35} (25 - 100 µM) in a 96-well plate and incubated for 24 h. After treatment, cells were washed with
PBS and 1 mg/ml MTT was added and incubated for 3 h at 37 °C. Finally after incubation, MTT was removed and dimethyl sulphoxide was added and the absorbance was measured at 540 nm using spectrophotometer.

To find the protective effect, the cells were pre-treated with GT (25 - 100 μg/ml)/vitexin (25 - 100 μM) for 2 h prior to Aβ25-35 (50 μM) treatment and incubated for 24 h and then subjected to MTT assay (Suganthy and Devi, 2016).

5.2.3.2. Neurite outgrowth assay

Neuro-2a cells (4 x 10⁴ cells) were seeded in 96-well plate with complete medium (DMEM, 10% FBS, 1X Antibiotic) and allowed to adhere to the surface. After the adherence of cells, complete medium was removed and cells were treated with GT (50 μg/ml)/Vitexin (50 μM) in treatment medium (DMEM, 2% FBS, 1X Antibiotic) and incubated for 48 h. After the incubation period, the cells were retrieved and microscopic examination of cells for neurite outgrowth and imaging was performed (Yeyeodu et al., 2010).

5.2.3.3. Measurement of intracellular ROS level

Reagents

3 mM DCFH-DA
(Working concentration- 10 μM)
- 1.45 mg/ml acetone

Lysis buffer (pH-8.0) (100 ml)
10 mM Tris
- 121.11 mg
20 mM EDTA
- 744.48 mg
0.25% Triton X-100
- 250 μl

Procedure

The accumulation of intracellular ROS was assessed using spectroflourimetric assay and confocal microscopy (Suganthy and Devi, 2016). For microscopic analysis 1 × 10⁴ cells were grown in glass cover slips. After the attachment of cells to the cover slips, the cells were pre-treated with GT (25, 50 μg/ml)/vitexin (25, 50 μM) for 2 h and then treated with 50 μM Aβ25-35. After the treatment period (24 h), the medium was removed, washed with PBS and were incubated with 10 μM DCFH-DA for 30 min at 37
C in darkness. Again the cells were washed with PBS to remove the extracellular DCFH–DA and were examined under confocal microscope (Zeiss LSM 710, Germany) equipped with an argon laser and the images were analysed using Zeiss LSM image examiner version 4.2.0.121. ROS produced by Neuro-2a cells was quantified in a spectrofluorimeter according to the method of Suganthy and Devi (2016). In brief, after incubation for 24 h, the cells were washed with PBS pH 7.4 and 10 μM DCFH-DA was added and incubated for 30 min at 37 °C in dark. After washing with PBS, the cells were lysed with lysis buffer pH 8.0 and the fluorescence was measured at 480 nm (Excitation) and 535 nm (Emission) using SpectraMax M3 Microplate Reader.

5.2.3.4. Determination of mitochondrial membrane potential

Reagent

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock Rh 123</td>
<td>1 mM (1 mg/250 μl DMSO)</td>
</tr>
<tr>
<td>Working</td>
<td>5 μM</td>
</tr>
</tbody>
</table>

Mitochondrial membrane potential (MMP) was monitored using the fluorescent dye Rhodamine 123 (Rh 123) (Suganthy and Devi, 2016). Treatment to Neuro-2a cells were done as explained earlier. After incubation, cells were incubated with Rh 123 (5 μM) for 30 min at 37 °C. The cells were removed from the 24-well plate and washed twice with PBS and the fluorescence intensity was measured with the excitation and emission wavelength of 480 and 530 nm respectively. For the visualization of change in MMP, cells grown in cover slip were washed with PBS after treatment and incubated with 5 μM Rh123 for 30 min and visualized under confocal microscope.

5.2.3.5. Preparation of cell lysate

Following incubation, cells from the experimental groups were washed with ice cold PBS (pH-7.4) and lysed using lysis buffer (pH-8.0). The lysates were centrifuged at 4000 g for 30 min at 4 °C and supernatant was stored at -80 °C until further use. Protein estimation was performed for all the samples by Bradford’s method.
5.2.3.6. Assessment of Cholinesterase inhibitory activity of GT and vitexin

Reagents

- 3 mM DTNB - 11.90 mg in 10 ml of Tris-Cl (pH 8.0/7.4)
- 15 mM ATCI - 20 mg in 5 ml of d.H2O
- 15 mM BTCI - 24 mg in 5 ml of d.H2O

Procedure

AChE and BuChE (Butyrylcholinesterase) inhibitory activity was evaluated by the method developed by Ingkaninan et al., (2000). Cell lysate (100 µg of protein) from each experimental group was taken and allowed to react with 3 mM DTNB. The substrates ATCI or BTCI (1 mM) was added to initiate the enzyme activity. The formation of 5-thio-2-nitrobenzoate anion was detected by yellow coloration and the absorbance was detected in the wavelength of 405 nm. The specific activity of the enzyme was determined for all the control and test samples.

Calculation

\[
\text{Specific activity} = \frac{\text{Change in OD/ min} \times \text{Reaction volume} \times 1000}{\text{Sample volume} \times \epsilon \lambda \times \text{Protein concentration}}
\]

The molar extinction coefficient for DTNB is \( \epsilon_\lambda = 13,600 \text{ M}^{-1} \text{ cm}^{-1} \) (1 Unit = nM of thiocholine idodide formed/min/mg of protein).

5.2.3.7. Measurement of reactive nitrogen species

Reagents

- 50 mM Phosphate buffer (pH 7.4)

  Solution A - K2HPO4 - 870 mg in 100 ml of d.H2O
  Solution B - KH2PO4 - 680.45 mg in 100 ml of d.H2O

- 81 ml of solution A was mixed with 19 ml of solution B and pH was adjusted to 7.4

Griess Reagent (5 ml)

- 0.1% N-naphthyl ethylene diamine - 5 mg/5 ml
- 1% Sulfanilamide - 50 mg in 5 ml of 2.5% phosphoric acid
10 mM Sodium nitrate - 6.9 mg in 10 ml of d.H₂O

For the measurement of RNS, cell lysate (100 µg of protein) obtained after treatment was incubated with Griess reagent for 30 min in dark at room temperature. The supernatant was collected and absorbance was measured at 548 nm in comparison with the standard sodium nitrite (5 - 25 µM). The results were represented as mmoles of nitrite/mg of protein (Jang and Surh, 2005).

5.2.3.8. Determination of lipid peroxidation

Reagents
15% Trichloroacetic acid (TCA) - 3.75 g of TCA in 25 ml of d.H₂O

0.37% Thiobarbituric acid (TBA) - 92 mg/25 ml 0.2 N HCl

TCA and TBA were mixed in the ratio of 1:1

100 µM Malondialdehyde (MDA)
(Working concentration- 10 µM)

For the determination of lipid peroxidation, an equal volume of ice-cold 15% TCA was added to 100 µg cell lysate and centrifuged at 3000 rpm for 15 min. To the supernatant, the reaction mixture containing 0.37% TBA and 15% TCA was added and the samples were incubated at 100 °C for 20 min. Samples were cooled and the absorbance was measured at 532 nm. Malondialdehyde (MDA) was used as the standard. The results were expressed as nmol of MDA/mg of protein (Yagi and Rastogi, 1979).

5.2.3.9. Determination of protein carbonyl content

Reagents
0.2% DNPH - 50 mg of DNPH in 25 ml of 2.5 N HCl
2M Guanidine HCl - 9.5 g in 50 ml of d.H₂O
10% TCA - 5 g of TCA in 50 ml of d.H₂O
Ethanol : Ethyl acetate - 1:1

For the quantification of protein oxidation, cell lysate (100 µg) was incubated with 0.2% Di-nitro phenyl hydrazine (in 2.5 N HCl) for 1 h in dark followed by precipitation with 10% TCA. The precipitates were washed three times with ethanol:
ethyl acetate (1:1) and the final pellet was resuspended in 2 ml of 2 M guanidine HCl and measured at 360 nm. The results are expressed as mmoles of free carbonyl content/mg of protein (Levine et al., 1990).

**Calculation**

\[
\text{Free protein carbonyl content} = \frac{\text{Absorbance} \times \text{Dilution factor}}{\text{Extinction coefficient} \ (\varepsilon_\lambda)}
\]

where \(\varepsilon_\lambda = 22000 \text{M}^{-1} \text{cm}^{-1}\).

**5.2.3.10. Measurement of glutathione content**

**Reagents**

- Glutathione - 1 mM (3.73 mg/10 ml)
- DTNB
  - Stock - 10 mM (39.6 mg/10 ml)
  - Working conc. - 0.6 mM
- Phosphate buffered saline - pH 7.4

The level of non-enzymatic antioxidant glutathione was measured according to the method of Sedlak and Lindsay (1968) with slight modification. Cell lysate containing 100 µg of protein was precipitated with equal volume of 5% TCA and the precipitate was removed after centrifugation at 4200 rpm for 15 min. The pellet containing protein was suspended in PBS and 0.6 mM DTNB. The reaction mixture was incubated for 5 min at room temperature. The absorbance was measured at 412 nm with glutathione (10 – 50 µM) as standard and the results were expressed as µmoles of GSH/mg of protein.

**5.2.3.11. Acridine orange/Ethidium bromide dual staining**

**Reagents**

- Phosphate buffered saline pH 7.4 (100 ml)
- Acridine orange
  - Stock - 100 µg/ml (50 µl from 0.2% stock)
  - Working conc. - 10 µg/ml H₂O
Ethidium bromide

Stock - 10 mg/ml H₂O

Working conc. - 10 μg/ml H₂O

Cells were grown and treatment was done as explained earlier. After 24 h, cells were washed with PBS and stained with AO/EtBr solution (1:1 v/v) at a concentration of 10 μg/ml for 10 min and were visualized under confocal microscope (Suganthy and Devi, 2016).

5.2.3.12. Caspase-3 colorimetric assay

Reagents

Lysis buffer pH 7.4 (100 ml)

N-(2-Hydroxyethyl) piperazine-N'-(2ethane sulfonic acid) (HEPES) - 50 mM (1.19 gm)

Dithiothreitol (DTT) - 5 mM (77.12 mg)

EDTA - 0.1 M (3.74 gm)

Triton X-100 - 0.2%

Assay buffer pH 7.4 (100 ml)

Tris-HCl - 50 mM (605 mg)

NaCl - 100 mM (584 mg)

EDTA - 1 mM (37.4 mg)

Glycerol - 10%

DTT - 10 mM (154 mg)

Ac-DEVD-pNA - 1 mM (1.27 mg/2 ml assay buffer)

Stock p-nitroaniline (pNA) - 1 mM (1.38 mg/10 ml)

Caspase-3 activity was measured based on the ability of caspase-3 in the cell lysate to hydrolyze the substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide to release p-nitroaniline. Briefly, reaction mixture consisting of 100 μg cell lysate, 200 μM Ac-DEVD-pNA and caspase-3 assay buffer was incubated at 37 °C for 1 h under dark. The
cleavage of the substrate was measured at 405 nm with p-Nitroaniline as the standard. The results were expressed as μmol of pNA/min/mg of protein (Ochu et al., 1998).

**Calculation**

Caspase 3 activity was calculated as

\[
\text{Specific activity} = \frac{\mu\text{mol pNA} \times \text{dilution factor}}{\text{Time} \times \text{volume of sample} \times \text{Protein concentration}}
\]

5.2.3.13. RNA isolation and qPCR analysis

Total RNA was isolated from cell extracts using the standard Trizol procedure (RNA X Press reagent, Sigma), according to the manufacturer’s recommendations. RNA concentration was quantified by Nanodrop spectrophotometer and reverse-transcribed using oligodT primer and MultiScribe™ Reverse Transcriptase (Applied Biosystems) enzyme. FastStart Universal SYBR Green Master mix (Roche Life Science) was used for performing real-time PCR (qPCR) analysis and amplified using gene specific primers (Table-5.1). The amplified genes were normalized with GAPDH gene as endogenous control. qPCR analyses were performed with two independent experimental samples in duplicates and average of gene expression levels were plotted with standard deviation. Fold changes were quantified with the standard formula by calculating $2^{-\Delta\Delta Ct}$.

**Table-5.1: Sequences of Primers Used for Quantitative Real-Time PCR**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Gene Name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAPDH</td>
<td>TCAACGGCACAGTCAAGG</td>
<td>ACTCCACGACATACTCAGC</td>
</tr>
<tr>
<td>2</td>
<td>LXR-α</td>
<td>GCAGGACCAGCTCAGTAG</td>
<td>GGTCACCACCTCCATTAGC</td>
</tr>
<tr>
<td>3</td>
<td>APOE</td>
<td>AACAGACCCAGCAAATACGC</td>
<td>GGTGATGATGGGTTGGTAG</td>
</tr>
<tr>
<td>4</td>
<td>ABCA-1</td>
<td>AGCCAGAGGGAGTGTCAGA</td>
<td>CATGCCATCTCGGTAACCT</td>
</tr>
<tr>
<td>5</td>
<td>Seladin-1</td>
<td>TGTTGCTGAGCTTGATGAC</td>
<td>AGCTCGTAGGCAGTGCAAAT</td>
</tr>
<tr>
<td>6</td>
<td>Pgc-1α</td>
<td>AATGCAGGCTCTTAGCAGCT</td>
<td>ACCTTTTGTGCTTCTTCCT</td>
</tr>
<tr>
<td>7</td>
<td>Nrf-2</td>
<td>GTGCTCTATGCTGAATCC</td>
<td>GCGGCTTGAATGTGTGTCTT</td>
</tr>
<tr>
<td>8</td>
<td>HO-1</td>
<td>TGCTGTAGAACACTCTGG</td>
<td>TCCTGCTGACATCGCCTG</td>
</tr>
<tr>
<td>9</td>
<td>Cyp-D</td>
<td>CTGTATGCCGGGAAGCATATG</td>
<td>CCAGTCATCCCTCCTTCTCA</td>
</tr>
<tr>
<td>10</td>
<td>Grp-78</td>
<td>TGCAGCACGACATCGTTC</td>
<td>TTTCTTTGTGGCGAAATGTC</td>
</tr>
<tr>
<td>11</td>
<td>Gadd153</td>
<td>CGGCGAGGAGAGACGAC</td>
<td>CGGTCTGGGGATGAGATA</td>
</tr>
</tbody>
</table>
5.2.3.14. Molecular docking

Molecular docking was performed with Discovery studio package, version 2.5 (Accelrys, Inc., San Diego, CA). The 3D structure of LXR-α (PDB ID – 5AVI) was downloaded from Protein Data Bank and the structure of vitexin was retrieved from Pubchem database (Pubchem ID: 5280441). Analysis was carried out as per the protocol explained in the section 3.2.7.

5.2.3.15. Western blot

Reagents

RIPA buffer pH 8.0 (100 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris (pH 8.0)</td>
<td>50 mM (605 mg)</td>
</tr>
<tr>
<td>NaCl</td>
<td>150 mM (876 mg)</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1%</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>1% (1 g)</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1% (100 mg)</td>
</tr>
</tbody>
</table>

Following the treatment period, the cells were collected, washed with PBS and proteins were obtained by lysis with RIPA buffer. Cell lysates (100 µg of protein) were electrophoresed on 12% SDS gels followed by transferring to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked in 5% skim milk for overnight followed by 6 h incubation with corresponding primary antibodies (β-actin (sc-130657), Bcl-2 (sc-783), Bax (sc-493); Santa Cruz; 1:1000 dilution) and then for 3 h incubation with anti-rabbit IgG- alkaline phosphatase conjugated secondary antibody (Sigma; A3687; 1:2000 dilution). The bands were developed with the substrates nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3′-indolyphosphate (BCIP) and quantified with ImageJ software.

5.2.3.16. STRING analysis

The interaction network of differentially expressed genes studied by qPCR analysis and proteins through in silico analysis was created by STRING analysis (http://string-db.org/) using the parameters; species- Homo sapiens; active interaction sources- all; medium confidence- 0.400; maximum number of interactors- 25.
5.2.4. Evaluation of neuroprotective effect of GT/vitexin against glutamate excitotoxicity in Neuro-2a cells

5.2.4.1. Cell viability assay

Neuro-2a cells (2 x 10^5 cells) were incubated with various doses of glutamate (1 – 10 mM) in a 96-well plate and incubated for 24 h. Cell viability was measured according to the protocol described in section 5.2.3.1. To find the protective effect, the cells were pre-treated with GT (25 - 100 μg/ml)/vitexin (25 - 100 μM) for 2 h prior to glutamate (5 mM) treatment and incubated for 24 h and then subjected to MTT assay (Suganthy and Devi, 2016).

5.2.4.2. Measurement of intracellular ROS level

The level of intracellular ROS was assessed with DCFH-DA using spectrofluorimetric assay. Neuro-2a cells were pre-treated with GT (25, 50 μg/ml) /vitexin (25, 50 μM) for 2 h and then treated with 5 mM glutamate for 24 h. Quantification of ROS was done according to the protocol described in section 5.2.3.2.

5.2.4.3. Determination of mitochondrial membrane potential

Estimation of mitochondrial membrane potential was done as described in the section 5.2.3.3.

5.2.4.4. Cell lysate preparation

After treatment, cells were washed with PBS (pH 7.4), and were collected and lysed with lysis buffer (10 mM Tris, 20 mM EDTA, 0.25% TritonX-100; pH 8.0). The cell lysates were centrifuged at 4000 g for 30 min at 4 °C; protein estimation was done by Bradford method and the samples were stored at -80 °C until further use.

5.2.4.5. Measurement of RNS

Cell lysates (100 μg of protein) from each group were treated with Griess reagent (0.1% NED and 1% sulphanilamide), and incubated at dark for 30 min in a 96-well plate. After incubation, the measurements were taken at 548 nm using Multilabel reader with sodium nitrite (5 - 25 μM) as the standard and the results were represented as mmoles of nitrite/mg of protein (Jang and Surh, 2005).
5.2.4.6. Antioxidant assays

The level of lipid peroxidation and protein carbonyl content was measured according to the protocols described in the section 5.2.3.7 and 5.2.3.8 respectively. The amount of glutathione in cells was quantified according to the protocol described in section 5.2.3.9.

5.2.4.7. Caspase-3 colorimetric assay

Caspase-3 activity was estimated as per the protocol described in section 5.2.3.11.

5.2.4.8. Molecular docking

Molecular docking was performed with Discovery studio package, version 2.5 (Accelrys, Inc., San Diego, CA). The 3D structure of targets were downloaded from Protein Data Bank (NMDAR (PDB ID: 3QEL) and GSK-3β (PDB ID: 1Q5K)) and the structure of vitexin was retrieved from Pubchem database (Pubchem ID: 5280441). Analysis was carried out as per the protocol explained in the section 3.2.7.

5.2.4.9. RNA isolation and qPCR analysis

RNA isolation and qPCR analysis was performed as explained in the section 5.2.3.12. The list of genes which were analyzed are tabulated in Table-5.2.

Table-5.2: Sequences of Primers Used for Quantitative Real-Time PCR

<table>
<thead>
<tr>
<th>S.No</th>
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<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAPDH</td>
<td>TCAACGGCACAGTCAAGG</td>
<td>ACTCCACGACATACTCAGC</td>
</tr>
<tr>
<td>2</td>
<td>Cyp-D</td>
<td>CTCATCTGGACGGGAAACAT</td>
<td>CCACTCATCCCCCTCTTCA</td>
</tr>
<tr>
<td>3</td>
<td>Nrf-2</td>
<td>GTGCTCTATTCGTAATCC</td>
<td>GCGTCTTAATGTTGTCTT</td>
</tr>
<tr>
<td>4</td>
<td>HO-1</td>
<td>TGCTGAAATGACACTCTGG</td>
<td>TCCTCTGTCACGACACCT</td>
</tr>
<tr>
<td>5</td>
<td>NQO-1</td>
<td>GTTTCTGTGGCTCCAGGTCA</td>
<td>CTCTTCTCCATCCTTCCAG</td>
</tr>
<tr>
<td>6</td>
<td>Gpr-78</td>
<td>TGCAGCAGCATCAAGTTC</td>
<td>TTTCTTGCTGGGCAATGTC</td>
</tr>
<tr>
<td>7</td>
<td>Gadd153</td>
<td>CTGCCTTTTACACCTGGAGAC</td>
<td>CGTTTCTCGGAGGATGAGA</td>
</tr>
<tr>
<td>8</td>
<td>NMDAR</td>
<td>CGGCCTTGGAGATACAGC</td>
<td>GTGAAGTGTCGGTGAGAGT</td>
</tr>
<tr>
<td>9</td>
<td>Calpain</td>
<td>CTGGAGGCTGCAGGAACTAC</td>
<td>CTCCCGGTTGTCATGTCGT</td>
</tr>
<tr>
<td>10</td>
<td>GLAST-1</td>
<td>TGCTCTTCCATGCTTTCG</td>
<td>ACATATCACCAGCCAAAT</td>
</tr>
<tr>
<td>11</td>
<td>GLT-1</td>
<td>TCTGAGGAGGCAATACCAC</td>
<td>AACTCCAGGCCCCCTTGT</td>
</tr>
</tbody>
</table>

5.2.4.10. Western blot

Western blot analysis was performed according to the protocol described in section 5.2.3.14 for evaluating the expression of the proteins β-actin, Bcl-2 and Bax.

5.2.4.11. STRING analysis

The interaction network of differentially expressed genes studied by qPCR analysis and proteins through in silico analysis was created by STRING analysis (http://string-db.org/) using the parameters; species- Homo sapiens; active interaction sources- all; medium confidence- 0.400; maximum number of interactors- 25.

5.2.5. Statistical analysis

All the experiments were done in triplicates and expressed as mean ± SD. A one-way anova followed by a Duncan’s post hoc test was performed to compare the groups for all the experiments. For the qPCR analysis Student’s t-test has been performed and p<0.05 was considered to be significant.

5.3. RESULTS AND DISCUSSION

5.3.1. Neuroprotective effect of G. tiliaefolia and vitexin against Aβ25-35 toxicity

Alzheimer’s disease, one of the most common forms of dementia is characterized mainly by the development and deposition of amyloid plaques formed by the proteolytic processing of β and γ secretases. Although the major component of the senile plaque is the full length Aβ peptides of 40-42 amino acid sequences, the much shorter fragment Aβ25-35 is extensively studied, since it mimics the characteristics of the full length peptide and hence proposed to be the biologically active region (Sato et al., 1995). These Aβ fragments, aggregates among themselves and get deposited in the brain as senile plaques which further causes oxidative stress and impair neuronal cells. The following section deals with the study of neuroprotective effect of GT/vitexin against Aβ25-35 induced toxicity in Neuro-2a cells.
5.3.1.1. Effect of GT/Vitexin on Aβ25-35 induced toxicity in Neuro-2a cells

Wide range of studies have indicated that Aβ25-35 induces death in neuronal cells through the activation of apoptotic pathways by altering the antioxidant status and interfering with several cellular signaling (Clementi et al., 2005; Ferreiro et al., 2007). The present study also demonstrated the induction of neuronal death upon Aβ25-35 treatment and its prevention upon pre-treatment with GT/vitexin. Initially, to check the optimum toxic dose, Neuro-2a cells were treated with different concentrations of Aβ25-35 (25 μM – 100 μM). Aβ25-35 treatment for 24 h affected the viability of cells in a dose dependent manner. At 50 μM concentration, Aβ25-35 significantly \((p<0.05)\) reduced (by 40%) the viability of cells (Fig-5.1.A). Hence, 50 μM Aβ25-35 was used for further experiments. GT (50 μg/ml), vitexin (50 μM) pre-treatment significantly \((p<0.05)\) protected the cells and restored the viability to 92.3 ± 2.37% and 92.86 ± 5.57% respectively (Fig-5.1.B). Induction of cell death upon Aβ25-35 treatment and the restoration of cell viability upon GT/vitexin treatment were shown in Fig-5.2.A-D. Cell shrinkage, loss of cell to cell contact and death was observed in Aβ25-35 treated group whereas, GT/vitexin treatment retained the normal morphology and established cell to cell contact, similar to that of control.

Fig-5.1: (A) Dose dependent cytotoxicity exerted by Aβ25-35 at 24 h. (B) Protective effect of GT and vitexin on Aβ25-35 induced neurotoxicity (significance at \(p<0.05\)).
Fig-5.2: Microscopic examination of the protective effect of GT and vitexin
(A) Control (B) Aβ25-35 (50 μM) (C) GT-50 μg/ml + Aβ25-35 (D) Vitexin-50 μM + Aβ25-35

5.3.1.2 Neurite outgrowth

One of the pathological features occurring during AD is the change in morphology of neurites, which leads to the cognitive impairment (Spires et al., 2005). Neuritogenic agents have shown promising efficacy in inducing the neurite outgrowth and restoring normal functions during AD (Li et al., 2002). Neuro-2a cells treated with GT and vitexin showed extensive neurite spread from the neuron and exhibited neurite networks. The results of neurite outgrowth assay indicates that GT and vitexin has the ability to induce neurite growth compared to the control and can act as potent neuritogenic agents (Fig-5.3).
Fig-5.3: Microscopic examination of the neurite outgrowth efficacy of (A) Control (0 h) (B) Control (48 h) (C) GT (50 μg/ml) and (D) Vitexin (50 μM)

5.3.1.3. Cholinesterase inhibition

Cholinesterase enzymes (AChE and BChE) play a major role in neurotransmission regulating the level of ACh in cholinergic neurons. Accumulating evidence indicate that apart from involving in cholinergic dysfunctions, they are also involved in the processing, deposition and aggregation of Aβ (Anand et al., 2011). In this context, cholinesterase inhibitory effect of GT and vitexin were evaluated in Neuro-2a cells. A significant ($p<0.05$) increase in the enzyme activity of both the enzymes were found in Aβ$_{25,35}$ treated group, whereas GT and vitexin exhibited significant dual cholinesterase inhibitory activity in a dose dependent manner (Fig-5.4). Result concludes that GT and vitexin protects the Neuro-2a cells from Aβ$_{25,35}$ induced alteration in the cholinergic neurotransmission.
Fig-5.4: Dose dependent dual cholinesterase inhibitory effect of GT and vitexin (significance at $p<0.05$; # Control vs Aβ$_{25-35}$; *Aβ$_{25-35}$ vs GT/Vitexin treated).

5.3.1.4. Effect of GT and Vitexin on Aβ$_{25-35}$ induced ROS formation

Though several hypotheses have been suggested, Aβ induced oxidative stress is one of the prime events contributing to the pathology of AD. Production of free radicals by Aβ makes the biomolecules susceptible to deleterious effects like DNA damage, oxidation of proteins, lipids and neuronal damage (Fang et al., 2002). Controlling the production of ROS or scavenging them is considered to be an important phenomenon in alleviating the effect of Aβ toxicity. Exposure of Neuro-2a cells with Aβ$_{25-35}$ for 24 h significantly increased the level of intracellular ROS (1931.78 ± 23.44 DCFDA fluorescence intensity) when compared to the control. However, 2 h pre-treatment with GT and vitexin significantly ($p<0.05$) attenuated the increase in intracellular ROS level induced by Aβ$_{25-35}$ in a dose dependent manner (Fig-5.5.G). Fluorescent microscopic analysis also substantiated the obtained quantitative data (Fig-5.5.A-F).
Fig-5.5: Confocal microscopic examination of scavenging of ROS by GT and vitexin induced by Aβ_{25-35} (A) Control (B) Aβ_{25-35} (C) GT (50 μg/ml) + Aβ_{25-35} (D) Vitexin (50 μM) + Aβ_{25-35} (E) GT alone (F) Vitexin alone. (H) Quantitative analysis of ROS scavenging by GT and vitexin (significance at p<0.05; # Control vs Aβ_{25-35}; *Aβ_{25-35} vs GT/Vitexin treated).

5.3.1.5. Effect of GT and Vitexin on Aβ_{25-35} induced RNS formation

In addition to the oxidative stress, nitrosative stress caused by NO also plays a vital role in Aβ_{25-35} induced neurotoxicity. Nitric oxide, a short-lived messenger, has been evidenced to be involved in the physiological and pathophysiological development of AD.
(Duncan and Heales, 2005). Toxicity of Aβ in neuronal cells is linked with enhancement of NO liberation, which up-regulates redox-sensitive transcription factors such as nuclear factor kappa B, an important factor responsible for oxidative and inflammatory reactions in AD progression (Padayachee et al., 2012; Pytlowany et al., 2008). In the present study, quantification of RNS level clearly showed that treatment of Neuro-2a cells with Aβ25-35 significantly ($p<0.05$) elevated the RNS production (0.29 ± 0.01 mmoles/mg of protein). However, the cells pre-treated with vitexin (50 μM) significantly ($p<0.05$) reduced the nitrite level to 0.21 ± 0.01 mmoles/mg of protein (Fig-5.6) indicating the scavenging of RNS by GT and vitexin.

![Bar graph showing inhibitory effect of GT and vitexin on Aβ25-35 induced reactive nitrogen species](image)

**Fig-5.6: Inhibitory effect of GT and vitexin on Aβ25-35 induced reactive nitrogen species (significance at $p<0.05$; # Control vs Aβ25-35; *Aβ25-35 vs GT/Vitexin treated).**

5.3.1.6. Effect of GT and Vitexin on lipid peroxidation induced by Aβ25-35

Lipid peroxidation (LPO) is one of the major outcomes of free radical-mediated injury to tissue. Several evidences have illustrated lipid peroxidation as one of the important mechanisms of neurodegeneration in AD brain. Increased levels of LPO products such as 4-hydroxynonenal (4-HNE) and 2-propenal have been observed in brain cerebrospinal fluid and plasma of individuals with AD (Skoumalova et al., 2011). The neurotoxic action of Aβ involves generation of ROS which leads to peroxidation of lipids and consequent generation of neurotoxic aldehyde 4-HNE. Toxic by-products of LPO modifies membrane transporters, alters calcium homeostasis and promotes the formation of NFT (Butterfield et al., 2010). Exposure of Neuro-2a cells to Aβ25-35 significantly elevated the level of MDA (54.37 ± 3.41 nmol/mg of protein) when compared to control.
indicating the macromolecular damages. Whereas, cells pre-treated with GT/vitexin, significantly \((p<0.05)\) reverted back the MDA level similar to that of control in a concentration dependent manner. The restoration of MDA levels might be due to the antioxidant property of GT and vitexin that effectively quenched the free radicals formed upon \(\text{A}\beta_{25-35}\) toxicity.

Fig-5.7: Inhibitory effect of GT and vitexin on \(\text{A}\beta_{25-35}\) induced lipid peroxidation (significance at \(p<0.05\); # Control vs \(\text{A}\beta_{25-35}\); *\(\text{A}\beta_{25-35}\) vs GT/Vitexin treated).

5.3.1.7. Effect of GT and Vitexin on oxidation of proteins induced by \(\text{A}\beta_{25-35}\)

Fig-5.8: Inhibitory effect of GT and vitexin on \(\text{A}\beta_{25-35}\) induced protein oxidation (significance at \(p<0.05\); # Control vs \(\text{A}\beta_{25-35}\); *\(\text{A}\beta_{25-35}\) vs GT/Vitexin treated).
Another direct consequence of free radical formation is the oxidation of proteins in the cells that lead to the formation of toxic secondary metabolites like protein carbonyls (Butterfield and Lauderback, 2002). These secondary metabolites are extremely toxic causing serious cellular damages. The effect of vitexin on Aβ25-35 induced oxidation of proteins is depicted in Fig-5.8. Exposure of Neuro-2a cells to Aβ25-35 significantly elevated the level of PCC (132.72 ± 3.28 mmoles of PCC/mg of protein) when compared to control (107.57 ± 2.29 mmoles of PCC/mg of protein) indicating the oxidation of proteins. Whereas, cells pre-treated with GT and vitexin, significantly (p<0.05) restored back the PCC level similar to that of the control in a concentration dependent manner. The restoration of PCC levels might be due to the quenching of free radicals formed upon Aβ25-35 toxicity by GT and vitexin.

5.3.1.8. Transcriptional regulation of genes involved in lipid homeostasis by GT and vitexin

Lipid peroxidation induced by Aβ has been reported to elevate cellular levels of cholesterol, disturbing normal lipid homeostasis in the lipid raft region and promoting Aβ production (Cutler et al., 2004; Pappolla et al., 2002). The regulation of candidate genes involved in lipid metabolism and lipid raft formation altered by Aβ25-35 were analyzed in this study. The expression of the liver X receptor-α (LXR-α) (0.49 fold) and its transcriptional target seladin-1 (0.79 fold) and ApoE (0.96 fold) were downregulated in Aβ25-35 treated group impairing the cholesterol efflux and homeostasis. However, another downstream target ABCA-1, which is involved in cholesterol efflux was slightly upregulated (1.08 fold) upon Aβ treatment. At the same time pre-treatment with GT (LXR-α – 0.98 fold; seladin-1 – 1.02 fold; ApoE – 1.65 fold; ABCA-1 – 2.10 fold) and vitexin (LXR-α – 2.05; seladin-1 – 3.05; ApoE – 1.35; ABCA-1 – 2.64) significantly (p<0.05) upregulated the expression of all the genes studied when compared to Aβ treated group, offering neuroprotection by maintaining lipid homeostasis (Fig-5.9). Cholesterol plays a key role in the organization of cellular membranes and studies have indicated that distorted cholesterol level in the lipid raft leads to the modification of lipid raft proteins inducing Aβ secretion (Simons et al., 1998). To maintain homoeostasis, LXR-α is activated upon changes in cholesterol level and further potentiates the expression of its target genes like ABCA-1, ApoE and seladin-1, which are involved in
cholesterol uptake, trafficking, export and regulating lipid raft formation (Fan et al., 2009). These proteins under the influence of LXR-α, are involved in reverse cholesterol transport, a mechanism by which surplus cholesterol from various tissues are send back to liver for excretion. The membrane protein ATP binding cassette transporter A-1 (ABCA-1) induces the efflux of cholesterol by loading the excess onto the acceptor apolipoprotein-E (APOE) (Hummasti et al., 2004). Also the expression of ABCA-1 has been reported to induce APOE levels and reduce the formation of amyloid plaques (Riddell et al., 2007). Earlier investigations also indicate that activation of ABCA-1 and APOE through LXR upregulation facilitates Aβ clearance (Jiang et al., 2008; Wahrle et al., 2008). Seladin-1, another direct transcriptional target of LXR-α plays a key role in cholesterologenic pathway regulating lipid raft formation and its expression has been reported to be decreased in the brain of AD patients (Crameri et al., 2006). Hence, LXR activation by synthetic compounds is emerging as one of the therapeutic strategies to combat AD. The results of our study indicate that GT and vitexin can act as LXR activators to protect neuronal cells from Aβ25-35 induced toxicity.

Fig-5.9: Transcriptional regulation of genes involved in lipid metabolism (significance at $p<0.05$; # Control vs Aβ25-35; *Aβ25-35 vs GT/Vitexin treated).
5.3.1.9. Docking analysis of vitexin with LXR-α

As gene expression analysis showed the activation of LXR-α with vitexin, molecular docking analysis was performed in order to delineate the mode of interaction of vitexin on the target site. The results showed that vitexin positively bind to the ligand binding site of LXR-α interacting with ASN371, VAL372 and GLN375 through hydrogen bonding, and PRO338, PRO370 through π-alkyl bonding with the docking score of 68.89 and binding energy of -89.38 kcal/mol.

![Molecular docking analysis](image)

Fig-5.10: Molecular docking analysis representing the mode of interaction of vitexin with LXR-α

5.3.1.10. Transcriptional regulation of genes involved in antioxidant response and mitochondrial biogenesis by GT and vitexin

Scavenging of free radicals produced in the cell is done by the cumulative effect of activation of several antioxidant defense genes and enzymes. To elucidate the antioxidant dependent protective effect of GT and vitexin against Aβ25-35 neurotoxicity, the regulation of Pgc-1α, Nrf-2 and HO-1 were analyzed by qPCR analysis (Fig-5.11). Upon Aβ25-35 induced stress, a slight increase in the expression level of Pgc-1α (1.21 fold) and a significant (p<0.05) downregulation of Nrf-2 (0.26 fold) and its downstream target HO-1 (0.24) were observed when compared to the control. Pre-treatment with 50 μg/ml
GT/50 μM vitexin effectively increased the expression of Pgc-1α, Nrf-2 and HO-1 by 1.58/1.37, 0.28/0.57 and 0.32/0.75 fold respectively compared to Aβ25-35 treated group. Peroxisome proliferator-activated receptor-γ coactivator (Pgc-1α) is an important transcription factor involved in mitochondrial biogenesis, which controls the activation of several other genes including nuclear factor erythroid 2-related factor-2 (Nrf-2), involved in the scavenging of free radicals (Sahin et al., 2011). A decline in the level of Pgc-1α has been reported in the brain of AD patients inducing mitochondrial dysfunction (Qin et al., 2009). Nrf-2 is a key regulator of oxidative stress because of its ability to modulate and co-ordinate the expression of several genes, which protect the cells from injury. Several studies have reported that Nrf-2 activation has the ability to attenuate the toxicity mediated by Aβ (Kanninen et al., 2008; Lee et al., 2013). Further, Nrf-2 mediated upregulation of HO-1 pathway and increase in level of GSH has been reported to protect neuronal cells from oxidative stress mediated toxicity (Chao et al., 2014). In the present study, the upregulation of Pgc-1α and its associated genes Nrf-2 and HO-1 is an indication that GT/vitexin protects Neuro-2a cells from Aβ25-35 toxicity through Nrf-2/HO-1 dependent antioxidant pathway. The results strongly suggest that GT/vitexin has the ability to augment mitochondrial biogenesis, induce the expression of antioxidant response genes and thereby protect the cells from Aβ25-35 induced oxidative stress.

Fig-5.11: Transcriptional regulation of genes involved in antioxidant response by GT and vitexin (significance at p<0.05; # Control vs Aβ25-35; *Aβ25-35 vs GT/Vitexin treated).
5.3.1.11. Effect of GT and vitexin on non-enzymatic antioxidant Glutathione

Glutathione is a non-enzymatic antioxidant involved in scavenging of free radicals and the synthesis of which is regulated by Nrf-2. Since Aβ25-35 induced toxicity significantly reduced the expression of Nrf-2, the level of glutathione upon Aβ25-35 toxicity was evaluated. Aβ25-35 treatment to cells significantly ($p<0.05$) reduced the level of GSH (17.12 ± 2.7 μmoles of GSH/mg protein). However, treatment with GT/vitexin augmented the level of GSH in a dose dependent manner substantiating with the results of qPCR analysis (Fig-5.12).

![Graph showing effect of GT and vitexin on non-enzymatic antioxidant glutathione upon Aβ25-35 toxicity](image)

**Fig-5.12:** Effect of GT and vitexin on non-enzymatic antioxidant glutathione upon Aβ25-35 toxicity (significance at $p<0.05$; * Control vs Aβ25-35; * Aβ25-35 vs GT/Vitexin treated).

5.3.1.12. Effect of GT and vitexin on Aβ25-35 induced mitochondrial membrane potential loss

Prolonged changes in the mitochondria cause formation of reactive oxygen species, impairment in the respiratory chain, dissipation of mitochondrial membrane potential and contributes to the neuronal cell death (Xian et al., 2012). Recent studies have implicated the involvement of mitochondrial permeability transition pore (mPTP) in both necrotic and apoptotic neuronal cell death in Aβ induced pathological changes (Du and Yan, 2010).
Fig-5.13: (A) Transcriptional regulation of Cyclophilin-D gene. Confocal microscopic examination of Rh123 fluorescence (B) Control (C) Aβ25-35 (D) GT (50 μg/ml) + Aβ25-35 (E) Vitexin (50 μM) + Aβ25-35 (F) GT alone (G) Vitexin alone. (G) Quantification of MMP loss (significance at p<0.05; # Control vs Aβ25-35; * Aβ25-35 vs GT/Vitexin treated).
Opening of the mPTP with the association of Cyclophilin D (CypD) disrupts the mitochondrial membrane potential and intensify apoptosis process by releasing apoptogenic factors like cytochrome c and apoptosis inducing factors (Du et al., 2008). Increased CypD expression upon Aβ toxicity has been positively correlated with AD pathology. As apparent to the previous studies, treatment of Aβ25-35 significantly \( (p<0.05) \) augmented the expression of Cyp-D compared to the control, whereas GT/vitexin downregulated the expression (Fig-5.13.A). The obtained results were further substantiated by analyzing the mitochondrial membrane potential. Cyp-D expression and mPTP formation causes depolarization of mitochondrial membrane potential. The loss of mitochondrial membrane potential by Aβ25-35 resulted in decrease in the intracellular fluorescence of Rh123 dye by 24.9 ± 1.3%, as shown in Fig-5.13.H. However, the cells pre-treated with GT (50 μg/ml) and vitexin (50 μM) maintained the mitochondrial integrity which could be identified from the increase in fluorescent intensity (73.28 ± 1.36% and 74.49 ± 2.21% respectively) when compared to Aβ25-35 treated group. The quantitative results were also corroborated with confocal microscopic analysis, where an increase in fluorescence was found in cells pre-treated with vitexin indicating its protective role (Fig-5.13.B-G). The reduction in expression of Cyp-D and restoration of MMP indicates that GT and vitexin can act as potent antioxidants and alleviate Aβ induced- oxidative stress related pathological changes.

5.3.1.13. Modulation of unfolded protein response genes by GT and vitexin

Endoplasmic reticulum (ER) is the principal organelle responsible for proper folding and processing of proteins. The toxic peptide Aβ has been reported to be involved in disturbing the normal homeostasis of the cell by impairing ER (Fonseca et al., 2013). Perturbation in the normal functioning of ER leads to unfolded protein response (UPR), a compensatory mechanism that tends to adapt to the changing environment. Prolonged ER stress triggers cell death with the expression of apoptotic proteins, a last measure to weed out dysfunctional cells. Cells retort to ER stress by inducing the expression of genes like Grp78 involved in protein folding, the expression of which induces cell survival. However when stress remains uncontrollable, cells undergoes apoptosis by activating apoptosis inducing gene Gadd153/CHOP (Yu et al., 1999; Takahashi et al., 2009).
$\text{A}25\text{-}35$ treatment to cells significantly ($p<0.05$) reduced the expression of $\text{Grp78}$ (0.57 fold) and upregulated the expression of $\text{Gadd153}$ (1.83 fold) when compared to the control (Fig-5.14). Pre-treatment with GT/vitexin however reversed this effect and resulted in the significant upregulation of $\text{Grp78}$ (0.69/0.84 fold) and downregulation of $\text{Gadd153}$ (1.25/0.93 fold). The results indicate that GT/vitexin prevented $\text{A}25\text{-}35$ induced ER stress mediated toxicity to cells by significantly augmenting $\text{Grp78}$ expression, and declining $\text{Gadd153}$ expression.

**Fig-5.14: Transcriptional regulation of unfolded protein response genes by GT and vitexin which were altered by $\text{A}25\text{-}35$ (significance at $p<0.05$; # Control vs $\text{A}25\text{-}35$; * $\text{A}25\text{-}35$ vs GT/Vitexin treated).**

5.3.1.14. Anti-apoptotic effect of GT and vitexin against $\text{A}25\text{-}35$ toxicity

The anti-apoptotic effect of GT and vitexin was assessed by acridine orange/ethidium bromide dual staining. Fig.5.15 illustrates the three types of nuclei belonging to live cells (green), live apoptotic cells (orange), and dead cells (red). Apoptotic and dead cells were observed in $\text{A}25\text{-}35$ treated cells which can be identified by condensed nucleus exhibiting orange-red staining. In contrast, cells pre-treated with vitexin remained alive with organized structure which can be detected from the green fluorescent staining.
Fig-5.15: Confocal microscopic examination of the cells reveal the protective effect of GT and vitexin against Aβ25-35 induced apoptosis (A) Control (B) Aβ25-35 (C) GT (50 μg/ml) + Aβ25-35 (D) Vitexin (50 μM) + Aβ25-35 (E) GT alone (F) Vitexin alone.

Fig-5.16: Inhibition of Aβ25-35 induced Caspase-3 activity by GT and vitexin (significance at p<0.05; # Control vs Aβ25-35; * Aβ25-35 vs GT/Vitexin treated).

Activation of caspase-3 plays a major role in inducing the apoptotic process upon Aβ insult (Suganthy and Devi, 2016). In accordance with the earlier reports, Aβ25-35 treated group exhibited a significant (p<0.05) increase in caspase-3 activity (1.16 ± 0.08 μmol pNA/min/mg of protein) (Fig-5.16). In contrast, the cells pre-treated with GT (50 μg/ml) and vitexin (50 μM) exhibited a significant reduction of 0.76 ± 0.02, 0.95 ± 0.1
and 0.83 ± 0.05 μmol pNA/min/mg of protein in caspase-3 activity respectively when compared to Aβ25-35 treated group.

5.3.1.15. Effect of GT and vitexin on the expression of apoptotic and anti-apoptotic proteins

The anti-apoptotic effect of GT/vitexin against Aβ25-35 was further evaluated by quantifying the expression of apoptotic (Bax) and anti-apoptotic protein (Bcl-2). The ratio of Bcl-2/Bax correlates with the extent of apoptosis taking place in cells. Aβ25-35 treatment to cells augmented the expression of Bax indicating apoptosis (Fig-5.17). On the other hand, GT and vitexin pre-treatment suppressed bax expression and induced the expression of anti-apoptotic protein Bcl-2. The Bcl-2/Bax expression ratio was found to increase in GT (0.68 and 0.69 fold for 25 and 50 μg/ml) and vitexin (0.57 and 0.74 for 25 and 50 μM) treated group in a dose dependent manner. Results prove that GT and vitexin displays potent roles in inhibiting the apoptosis induced by Aβ25-35 by downregulating Bax and upregulating Bcl-2 expression.

![Fig-5.17: Western blot analysis of anti-apoptotic protein Bcl-2 and apoptotic protein Bax in Neuro-2a cells treated with GT and vitexin](image)

5.3.1.16. STRING analysis

STRING database, which provides direct and indirect interactions between genes/proteins was used to identify the molecular mechanism behind the neuroprotective effect of vitexin. Ubiquitin-C (UBC), SIRT-1, Keap-1 and PPAR-γ are found to be some of the important interacting partners indirectly being targeted by GT and vitexin (Fig-5.18). UBC gene which codes for Polyubiquitin-C, is one of the important components in
the ubiquitin proteosome system (UPS), and is involved in removing the impaired proteins including Aβ (Hong et al., 2014). SIRT-1 has been regarded as one of the potent targets of AD. Deacetylation of histones by SIRT-1 has been showed to modulate stress response and induce longevity (Wong and Tang, 2016). Similarly, activation of KEAP-1 is involved in inducing the antioxidant response element through Nrf-2/HO-1 pathway (Yamazaki et al., 2015). PPAR-γ co-ordinates lipid metabolism, neuroinflammation and agonists of PPAR-γ were shown to reduce the Aβ burden (Jiang et al., 2008). Thus it can be extrapolated that GT and vitexin offers neuroprotection against AD by indirectly modulating the UPS for the degradation of Aβ, inducing the antioxidant system via modulating Keap-1, providing longevity through SIRT-1 and co-ordinating lipid metabolism through PPAR-γ.

Fig-5.18: Prediction of interaction network of genes/proteins that could be modulated by GT and vitexin to exert neuroprotective effect against Aβ25-35 induced toxicity
5.3.2. Neuroprotective effect of *G. tiliæfolia* and vitexin against glutamate excitotoxicity

Glutamate is a major excitotoxic neurotransmitter, the increased accumulation of which in the synaptic cleft causes neurological dysfunction including Alzheimer’s disease ([Lipton, 2007](#)). Under pathological condition, the Aβ synthesized in the brain, blocks the neuronal glutamate uptake and removes the blockade of NMDAR. These changes cause an increase in the level of glutamate (glutamate excitotoxicity) and leads to synaptic plasticity impairment ([Li et al., 2011](#)). Blockade of glutamate excitotoxicity by small molecules is emerging as one of the therapeutic strategies to combat AD. Hence in the following section, the neuroprotective effects of the Indian medicinal plant *Grewia tiliæfolia* and its active constituent vitexin were evaluated for their neuroprotective effect against glutamate induced toxicity in Neuro-2a cells.

5.3.2.1. Effect of GT and vitexin on glutamate-induced neurotoxicity in Neuro-2a cells

Initially in order to fix the toxic dose, a range of concentrations of glutamate (1-10 mM) was checked for their toxicity.

![Graph showing dose dependent cytotoxicity exerted by glutamate in Neuro-2a cells](image)

*Fig-5.19: Dose dependent cytotoxicity exerted by glutamate in Neuro-2a cells (significance at *p*<0.05; * Control vs glutamate)*
Fig-5.20: Neuroprotective effect of GT and vitexin against glutamate toxicity (significance at $p<0.05$; * Glutamate vs GT/vitexin)

Fig-5.21: Microscopic examination which reveals the protective effect of GT and vitexin against glutamate toxicity (A) Control (B) Glutamate (5 mM) (C) GT-50 $\mu$g/ml + Glutamate (D) Vitexin-50 $\mu$M + Glutamate
Glutamate showed dose dependent reduction in cell viability. Neuro2a cells exposed to 5 mM glutamate showed a significant ($p<0.05$) reduction in cell viability (47% reduction) compared to the control and hence 5 mM was fixed for further assays (Fig-5.19). However, pre-treatment with GT and vitexin for 2 h significantly restored the cell viability (91% for GT (50 µg/ml) and 93% for vitexin (50 µM)) when compared to glutamate treated group (Fig-5.20). Further cell shrinkage, membrane blebbing and death was evidenced in cells exposed to glutamate. However, GT and vitexin effectively restored the normal morphology as that of the control group (Fig-5.21.A-D). Based on the observation 25, 50 µg/ml and 25, 50 µM were used for GT and vitexin respectively, for further experimental analysis.

5.3.2.2. Effect of GT and vitexin on glutamate induced ROS and RNS formation

Formation of ROS and RNS by glutamate-induced toxicity and their scavenging effect by GT and vitexin was evaluated by using the fluorescent dye DCFH-DA. Concomitant to the previous studies glutamate treatment caused oxidative stress to neuronal cells which is evident from the ROS and RNS formation (Van Laar et al., 2015). Cells exposed to glutamate showed a significant ($p<0.05$) increase in fluorescent intensity of 1535.6 ± 35.5 AU compared to control, which can be correlated to increase in ROS formation. Neuro-2a cells pre-treated with GT (822.5 ± 26.4 AU at 50 µg/ml) and vitexin (633.4 ± 23.5 AU at 50 µM) significantly scavenged the ROS formed, which is evident from the reduction of fluorescent intensity and protected the neuronal cells from glutamate induced oxidative stress (Fig-5.22).

In the same way, glutamate treatment to cells significantly increased the nitric oxide production (0.43 ± 0.01 mmoles of nitrite/mg protein) when compared to control (0.29 ± 0.07), while GT (0.29 ± 0.08 mmoles of nitrite/mg protein at 50 µg/ml) and vitexin (0.30 ± 0.05 mmoles of nitrite/mg protein at 50 µM) treatment showed reduction in nitric oxide formation similar to that of the control further confirming their antioxidant potential (Fig-5.23). The results indicate that GT and vitexin act as antioxidants to prevent the toxicity exerted by glutamate in Neuro-2a cells.
5.3.2.3. Effect of GT and vitexin on glutamate induced macromolecular damages

As oxidative stress induced by glutamate causes oxidation of lipids and proteins, the effect of GT and vitexin on macromolecular damages were analyzed. The level of
lipid peroxidation was determined from the amount of thiobarbituric acid reactive substances (TBARS) and the level of protein oxidation was measured from the amount of...
protein carbonyl content (PCC) present in the cell lysates. Glutamate treatment increased the oxidation product of lipids in the form of MDA (83.06 ± 4.67 nmol MDA/mg protein) and proteins in the form of protein carbonyls (127.87 ± 4.58 mmoles of free carbonyls/mg protein) significantly compared to control. However, GT (50 μg/ml) and vitexin (50 μM) attenuated MDA formation to 60.65 ± 4.09, 40.16 ± 5.37 nmol MDA/mg protein and protein carbonyl content to 105.45 ± 2.41, 102.12 ± 1.39 mmoles of free carbonyls/mg protein (Fig-5.24 and 5.25). The results prove that GT and vitexin can intervene with oxidative stress process and further protect the neuronal cells from glutamate induced macromolecular damages.

5.3.2.4. Effect of GT and vitexin on glutamate induced MMP loss and the expression of Cyp-D

One of the direct implications of glutamate induced oxidative insult is the loss of membrane potential, and opening of mitochondrial permeability transition pore (Du and Yan, 2010). The alterations in the mitochondrial membrane potential (MMP) was evaluated both microscopically and fluorometrically using Rh-123 dye. Corroborating with the previous results, fluorescent microscopic observation shows a decrease in the fluorescence of Rh-123 dye in glutamate treated cells and restoration of MMP in GT/vitexin treated cells (Fig-5.26.A-F) (Ward et al., 2000). Quantification of MMP loss shows that cells treated with 5 mM glutamate showed a significant dissipation of membrane potential as evidenced from the reduction of fluorescent intensity (35.5 ± 1.8%) when compared to that of the control. Pre-treatment with GT/vitexin significantly reduced the alterations in the MMP, which is evident from the increase in fluorescence intensity (80% and 91% for GT and vitexin respectively) (Fig-5.26.G). Further gene expression analysis of cyclophilin-D, showed that GT (0.49 fold) and vitexin (0.65 fold) significantly suppressed its expression when compared to glutamate (1.78 fold) treated group (Fig-5.27). The results indicate that GT and vitexin protected the Neuro-2a cells from glutamate-induced toxicity by lowering the MMP loss, through the suppression of cyclophilin-D.
Fig-5.26: Fluorescent microscopic examination of restoration of MMP by GT and vitexin (A) Control (B) Glutamate (C) GT (50 µg/ml) + Glutamate (D) Vitexin (50 µM) + Gutamate (E) GT alone (F) Vitexin alone. (G) Quantification of MMP loss (significance at $p<0.05$; # Control vs glutamate; * glutamate vs GT/Vitexin treated).
5.3.2.5. Transcriptional regulation of stress associated genes by GT and vitexin

Antioxidant response elements (ARE’s) have been reported to play a major role in protecting the cells from stress stimuli and the suppression of which is implicated in diseased condition. Nrf-2 dependent activation of HO-1 and NQO-1 by antioxidants is regarded as one of the potential targets in disease interventions (Joshi and Johnson, 2012). The transcriptional regulation of mitochondrial stress associated genes (Nrf-2, HO-1 and NQO-1) and endoplasmic reticulum stress associated genes (Grp78 and Gadd153) were examined in the presence and absence of GT/vitexin upon glutamate induced toxicity (Fig-5.28). All the protective stress associated genes Nrf-2 (0.28 fold), HO-1 (0.3 fold), NQO-1 (0.59 fold) were significantly downregulated, whereas the apoptosis mediating gene Gadd153 was significantly upregulated (1.46 fold) when treated with glutamate. However pre-treatment with 50 μg/ml GT (0.6, 0.65, 1.3 and 0.58 folds) and 50 μM vitexin (0.73, 0.51, 1.21 and 0.57 folds) showed an upregulation in the expression of Nrf-2, HO-1, NQO-1 and Grp78 respectively. On the other hand the expression of Gadd153 is significantly downregulated upon GT (0.75 fold) and vitexin.
(0.69 fold) treatment. Endoplasmic reticulum (ER) is a principal organelle for the storage of calcium and prolonged oxidative stress leads to ER stress condition. Grp78 is an ER stress associated protein molecule, the expression of which is involved in cell survival.
Silencing of Grp78 was shown to exacerbate the neuronal cells to glutamate induced toxicity (Yu et al., 1999). To the contrary, activation of Gadd153 is related to the pathological hallmarks (Takahashi et al., 2009). The results of stress response genes show that GT/vitexin has the potential to modulate the cellular mechanisms and thereby protect the cells from glutamate neurotoxicity. The non-enzymatic antioxidant glutathione is regulated by the antioxidant response elements (Nrf-2, HO-1) and it is one of the most important endogenous antioxidant, which downregulates the associated oxidative stress. Moreover, it also minimizes the peroxidation of lipids and other oxidative stress mediated damage inside the cellular machinery (Kerksick and Willoughby, 2005). Hence the level of glutathione upon glutamate toxicity and with GT/vitexin pre-treatment was analyzed. Complementing the results of gene regulation, the level of glutathione is significantly reduced on exposure with glutamate (11.71 ± 1.56 μM of GSH/mg of protein) when compared to control (59.45 ± 2.70 μM of GSH/mg of protein), whereas pre-treatment with GT (37.84 ± 5.4 μM of GSH/mg of protein) and vitexin (44.14 ± 4.13 μM of GSH/mg of protein) restored the level (Fig-5.29).

5.3.2.6. Regulation of NMDAR, calpain and glutamate transporters by GT and vitexin

Physiologically, the excess glutamate at the synaptic cleft is taken up by the glutamate transporters like GLAST-1, GLT-1 and eliminated by the perisynaptic astrocytes (Foran and Trotti, 2009). Under disease conditions, the uptake of glutamate by the transporters is reduced due to the impairment in the expression of transporters level. It has been identified that the lipid peroxidation products could result in the loss of function of glutamate transporters (Blanc et al., 1998). The gene regulation of major glutamate transporters GLAST-1 and GLT-1, which are involved in removing the excess glutamate were analyzed by qPCR analysis (Fig-5.30). Upon glutamate exposure to Neuro-2a cells, the level of GLAST-1 (0.22 fold) and GLT-1 (0.15 fold) were significantly downregulated indicating the impairment of glutamate transporters.
Fig-5.30: Gene expression evaluation of NMDAR, Calpain and glutamate transporters on pre-treatment with GT/vitexin upon glutamate toxicity (significance at $p<0.05$; # Control vs Glutamate; * Glutamate vs GT/Vitexin treated).

On the other hand, GT and vitexin pre-treatment increased the expression of the glutamate transporters facilitating the clearance of glutamate. Similarly, the expression of NMDAR and the subsequent Calpain expression were also analyzed. Oxidative stress has been implicated in the upregulation of NMDAR and the overactivation of which is seen in excitotoxic condition resulting in the influx of calcium ions (Heurteaux et al., 1994). One of the downstream targets of NMDAR is the calcium dependent protease calpain, which is involved in several pathogenic conditions including cleavage of Bax and inducing apoptosis (Sánchez-Gómez et al., 2011). Results showed that glutamate treatment resulted in a significant 5.8 fold increase in the expression of NMDAR and 17 fold increase in Calpain indicating the occurrence of excitotoxicity mechanism. Conversely, glutamate induced increase in expression of NMDAR, calpain were reversed by GT/vitexin indicating their efficacy in interfering with modulating the excitotoxicity pathway.
5.3.2.7. Docking analysis

The ability of vitexin to bind with potential target sites of NMDAR and GSK-3β were studied by molecular docking analysis.

Fig-5.31: Molecular docking analysis showing the interaction between NMDAR and vitexin

Fig-5.32: Molecular docking analysis showing the interaction between GSK-3β and vitexin
Docking studies showed that vitexin efficiently bind to the NMDA receptor binding pocket with the dock score of 54.46 and binding energy of -87.8 kcal/mol. Vitexin formed four hydrogen bonds (with LYS318, ILE314, TYR330, SER332, MET334), pi-sigma bond (with SER332). The interaction between vitexin and NMDAR is shown in Fig-5.31. One of the downstream targets of NMDAR activation is GSK-3β, which induces the phosphorylation of tau protein resulting in the disassociation of microtubule assembly. Molecular docking of vitexin with GSK-3β showed that the compound can bind to the target site with two hydrogen bonds (GLN254, GLY253), pi-cation bond (LYS150) and pi-alkyl bond (ARG306) with the docking score of 56.36 and binding energy of -83.39 kcal/mol (Fig-5.32). In silico results confirms that vitexin can bind positively with NMDAR and GSK-3β and inhibit their overactivation.

5.3.2.8. Effect of GT and Vitexin against glutamate induced apoptosis

In order to evaluate the effect of glutamate on apoptotic and anti-apoptotic proteins, caspase-3 activity and the expressions of Bcl-2 and Bax were analyzed.

![Graph showing the effect of GT and vitexin on glutamate induced Caspase-3 activity](image)

**Fig-5.33:** Effect of GT and vitexin on glutamate induced Caspase-3 activity (significance at \(p<0.05\); \# Control vs Glutamate; * Glutamate vs GT/Vitexin treated).
Glutamate treatment showed a significant increase in the caspase-3 cleavage of the substrate AC-DEVD-pNA with the enzyme activity of $1.4 \pm 0.11$ μM of pNA/min/mg protein, while pre-treatment with GT/vitexin showed a reduction in caspase-3 level ($0.98 \pm 0.03$, $0.56 \pm 0.09$ μM of pNA/min/mg protein for GT and vitexin respectively) (Fig-5.33). Similarly the protein expression of Bax showed an increase of 1.46 fold in glutamate treated group while decreasing the Bcl-2 (0.27 fold) expression. However GT/vitexin treatment reversed the effect and increased the Bcl-2/Bax ratio indicating a reduction in the cell apoptosis due to glutamate (Fig-5.34).

5.3.2.9. STRING analysis

Identification of interacting partners with STRING database showed a wide range of genes/proteins that could be modulated by GT/vitexin. STRING analysis, which predicts the association of genes/proteins with neighbouring targets indicates that GT/vitexin could directly or indirectly regulate microtubule-associated protein tau (MAPT), β-catenin (CTNNB1), AXIN-1,2, AKT1 and JNC (Fig-5.35) and thereby inhibits the tau phosphorylation and protecs microtubule assembly. The regulating partners identified were found to be mostly associated with Wnt signaling pathway.
Fig-5.35: Prediction of interacting partners involved in glutamate excitotoxicity pathway modulated by GT and vitexin

5.4. CONCLUSION

To conclude, GT and vitexin exhibit multi-targeted action in protecting the Neuro-2a cells against Aβ25-35 and glutamate mediated toxicity. Protective effect of multifaceted GT and vitexin is mediated through

- Exhibiting dual cholinesterase inhibition
- Inhibiting oxidative stress
- Augmenting antioxidant response genes
- Stabilizing mitochondrial function
- Inhibiting macromolecular damages
- Regulating genes involved in lipid homeostasis
- Modulating glutamate transporter expression and
Inhibition of apoptosis

Our findings suggest that GT and vitexin as multiple-targets drugs holds potential for neuroprotection and therefore may be promising for the treatment of AD.

5.5. SUMMARY

Multifarious neuroprotective action of *G. tiliaefolia* and vitexin against Alzheimer’s disease