Chapter - V

Assessment of neuroprotective potential of Gelidiella acerosa benzene extract on Aβ 25-35 treated Swiss Albino mice
CONTENTS

7.1. Abstract

7.2. Introduction

7.3. Materials and Methods

7.3.1. Animals
7.3.2. Preparation of *G. acerosa* benzene extract
7.3.3. Intracerebroventricular (ICV) injection of Aβ 25-35 peptide in brain
7.3.4. Experimental groups
7.3.5. Behavioral studies
  7.3.5.1. *Y-maze test*
  7.3.5.2. *Water maze test*
  7.3.5.3. *Step-down inhibitory avoidance test*

7.3.6. Preparation of tissue homogenate

7.3.7. Evaluation of antioxidant potentials of *G. acerosa* benzene extract
  7.3.7.1. Catalase assay
  7.3.7.2. SOD assay
  7.3.7.3. GSH level measurement
  7.3.7.4. Measurement of GSH reductase
  7.3.7.5. Measurement of Glutathione –S- Transferases
  7.3.7.6. Measurement of Glutathione peroxidase

7.3.8. Macromolecular damage assessment in mice (treated with Aβ 25-35 and/or extract) brain tissue homogenate
  7.3.8.1. Lipid peroxidation assay
  7.3.8.2. Measurement of protein carbonyl content

7.3.9. Cholinesterase inhibitory assay
7.3.10. Measurement of caspase-3 activity
7.3.11. β-secretase assay
7.3.12. Determination of monoamine oxidase B (MAO B) activity
7.3.13. Western Blot analysis
7.3.14. Statistical analysis

7.4. Results and Discussion

7.4.1. Evaluation of protective effect of *G. acerosa* benzene extract on Aβ 25-35 induced memory impairment in mice by behavioral tests
  7.4.1.1. Assessment of protective effect of *G. acerosa* on Aβ 25-35 induced memory impairment by *Y-maze test*
  7.4.1.2. Assessment of protective effect of *G. acerosa* on Aβ 25-35 induced memory impairment by *Water-maze test*. 
7.4.1.3. Assessment of protective effect of *G. acerosa* on Aβ 25-35 induced memory impairment by Step-down inhibitory avoidance test.

7.4.2. Assessment of effect of *G. acerosa* benzene extract on Aβ 25-35 induced alteration in the levels of antioxidant enzymes in mice

7.4.3. Evaluation of protective effect of *G. acerosa* benzene extract on Aβ 25-35 induced macromolecular damage in mice

7.4.4. Effect of *G. acerosa* benzene extract on Aβ 25-35 induced increase in cholinesterase activity

7.4.5. Effect of *G. acerosa* benzene extract on β-secretase activity

7.4.6. Protective effect of *G. acerosa* benzene extract on Monoamine oxidase activity

7.4.7. Evaluation of anti-apoptotic potential of *G. acerosa* benzene extract on Aβ 25-35 injected mice

7.5. Conclusion

7.6. Summary of the results
7.1. ABSTRACT

Alzheimer’s Disease (AD) is a multifaceted and progressive neurodegenerative disorder characterized by loss of memory and cognition. Currently, efforts are being made to develop potent drug that targets multiple pathological mechanisms involved in AD. In the present study, the protective effect of G. acerosa benzene extract was evaluated in Aβ 25-35 peptide treated albino mice. The effect of benzene extract on peptide induced cognitive decline was assessed by behavioral studies. The antioxidant activity of G. acerosa benzene extract was evaluated by measuring the level of antioxidant enzymes. Protection against macromolecular damage was assessed by measuring the amount of thiobarbituric acid reacting substances (TBARS) and protein carbonyl content (PCC). Inhibitory effect of benzene extract was evaluated by AChE and BuChE assays. Assessment of inhibitory effect of the extract on β-secretase and MAO-B was performed by in vitro assays. Anti-apoptotic potential of extract was evaluated by measuring the level of caspase-3 activity and the expression of the proteins involved in apoptotic cascade such as Bax and BCl-2. The results demonstrated that the G. acerosa benzene extract restores the level of antioxidant enzymes significantly (P<0.05). The reduction in TBARS and PCC level in the extract treated group suggests that the extract prevents lipids and proteins from getting oxidized due to oxidative stress. In addition, the extract exhibited significant (P<0.05) AChE and BuChE inhibitory activities in peptide treated mice thereby protecting the mice from cholinergic deficit. Presence of inhibitory effect of G. acerosa benzene extract on β-secretase and MAO-B suggests that the extract protects the cells from Aβ peptide mediated toxicity and oxidative stress mediated cell damage. Reduction (P<0.05) in the level of caspase-3 activity and Bax expression suggests that the extract protects the cells from apoptosis mediated cell death. Hence the results suggest that, G. acerosa benzene extract possess excellent neuroprotective potential against Aβ 25-35 mediated toxicity under in vivo conditions.
7.2. INTRODUCTION

Alzheimer’s disease is a neurodegenerative disorder characterized by cognitive impairment (clinically) and by the appearance of senile plaques and neurofibrillary tangles (pathologically) (Cho et al., 2009). The Aβ peptides are the major components of senile plaques, which are derived from the altered proteolytic processing of APP. BACE-1 (β-secretase) is the enzyme, which is responsible for the generation of toxic Aβ fragments from APP, thereby it promotes the toxicity in brains of AD (Zhao et al., 2013). In addition to the presence of these neuropathological hallmarks, several neurotoxic mechanisms are involved in the disease occurrence and progression. Recently, the role of oxidative stress has been greatly implicated in Aβ-induced neurotoxicity and AD pathogenesis. Moreover, Aβ has been reported to produce hydrogen peroxide and peroxides of lipids in neurons (Cho et al., 2009). In addition to Aβ, monoamine oxidase (a flavin containing enzyme) is responsible for oxidative deamination of neurotransmitters and exogenous amines and produces large amount of hydrogen peroxides, which in turn creates a severe oxidative imbalance in AD brain. Moreover, for unknown reasons, the level of MAO was elevated in AD affected brains (Riederer et al., 2004). Apart from oxidative stress, cholinergic deficit has been found to be a consistent pathological factor, which is associated with memory loss and other neuronal dysfunctions (Kim et al., 2006). The peptide also induces neuronal degeneration through the process of programmed cell death or apoptosis. Presence of high levels of caspase-3 activity and other enzymes belonging to caspase family in AD brain suggests that apoptosis plays a central role in neuronal death in AD (Luo et al., 2002). Since AD involves several pathological factors (which can be better studied under in vivo conditions), the present study aims at the evaluation of neuroprotective potential of G. acerosa in Swiss albino mice.
7.3. MATERIALS AND METHODS

7.3.1. Animals

Healthy male Swiss albino mice (5-6 weeks of age) weighing 22-25 g were employed for the present study. The animals were housed in plastic cage in a ventilated room with 12 h cycle of day and night and temperature was maintained around 25°C. They were fed with standard pellet diet (Hindustan Lever Ltd, Mumbai). All the procedures followed were approved by the Institutional Animal Ethics Committee of C.L. Baid Metha College of Pharmacy, Chennai, India (Protocol No: IAEC/321/06/CLBMCP/2011; dated on 22/06/2011).

7.3.2. Preparation of G. acerosa benzene extract

The benzene extract of G. acerosa was prepared by the method as mentioned in the section 3.3.2.

7.3.3. Intracerebroventricular (ICV) injection of Aβ 25-35 peptide in brain

Aβ (25-35) (1 mM) was dissolved in sterile saline and allowed to aggregate by incubating at 37°C for 4 days. ICV injection of Aβ 25-35 was performed in mice according to the procedure established by Laursen and Belknap (1986) (by identifying the bregma point) using stereotaxic apparatus (Fig. 7.1). A 26-gauge and 3 mm (length) stainless steel needle was used for microinjection. The needle was inserted unilaterally 1 mm to the right of the middle point equidistant from each eye, at an equal distance between ears and perpendicular to the plane of the skull.

Figure 7.1: Stereotaxic apparatus
7.3.4. Experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Vehicle control</td>
<td>1% Carboxymethyl cellulose (CMC) + PBS</td>
</tr>
<tr>
<td>II</td>
<td>Aβ 25-35 treatment</td>
<td>10 µM Aβ 25-35 in PBS</td>
</tr>
<tr>
<td>III</td>
<td>Aβ 25-35 + Extract treatment (Low dose)</td>
<td>10 µM Aβ 25-35 in PBS + 200 mg/kg BW of G. acerosa benzene extract in CMC</td>
</tr>
<tr>
<td>IV</td>
<td>Aβ 25-35 + Extract treatment (High dose)</td>
<td>10 µM Aβ 25-35 in PBS + 400 mg/kg BW of G. acerosa benzene extract in CMC</td>
</tr>
<tr>
<td>V</td>
<td>Extract alone</td>
<td>400 mg/kg BW of G. acerosa benzene extract in CMC</td>
</tr>
<tr>
<td>VI</td>
<td>Aβ 25-35 + Donepezil</td>
<td>10 µM Aβ 25-35 in PBS + 1 mg/kg BW of donepezil in CMC</td>
</tr>
<tr>
<td>VII</td>
<td>Donepezil alone</td>
<td>1 mg/kg BW of donepezil in CMC</td>
</tr>
</tbody>
</table>

The animals were subjected to mild anesthesia and Aβ 25-35 (10 µg/10 µL/ICV site) (dissolved in saline) was administered through ICV injection. The peptide was administered to II, III, IV and VI groups on 21st day of the pre-treatment of G. acerosa benzene extract. After the peptide administration, the extract treatment was continued for 8 consecutive days. Neither the insertion of the needle nor injection of saline induced any significant changes on survival, behavioral response or cognitive functions. After the peptide injection, the animals were subjected to behavioral tests for eight days. On day 30, all the animals were sacrificed, brain were removed and used for further experimental studies.
7.3.5. Behavioral studies

7.3.5.1. Y-maze test

Y-maze test was performed to assess the short-term memory in mice (Maurice et al., 1996). The maze was constructed in such a way that each arm was 40 cm long, 12 cm high and 3 cm wide at the bottom and 10 cm wide at the top. The maze was wood painted in black and the arms were converged at an equilateral triangular central area, which was 4 cm at its longest axis. The entire apparatus was placed on the floor of the experimental room and was illuminated with 100 W bulb from 200 cm above (Fig. 7.2). Each mouse was placed at the end of one arm and allowed to move freely in a standard dimension through the maze for about 8 minutes and the series of arm entries including the possible returns into the same arm was recorded visually. The ability of the mouse to remember the arm, which it had already visited, is its ability to alternate. Alternation can be defined as the number of successive entries into all the three arms on consecutive occasions. Percentage of alternation was calculated using the following formula:

\[
\text{\% of Alternation} = \left[ \frac{\text{Number of alternations}}{\text{Total arm entries} - 2} \right] \times 100
\]

7.3.5.2. Water maze test

Water maze test was performed (to assess the spatial learning and memory) according to Morris (1984) with modifications. The apparatus was made of circular water tank (100 cm diameter and 35 cm height) containing water at 28°C to a depth of 15 cm and the water was made opaque by adding titanium oxide. The water pool in the tank was divided into four equally spaced quadrants and the platform (4.5 cm diameter
Chapter V

163

and 14.5 cm height) was placed in such a way that its top was submerged 2 cm below the water surface in one particular quadrant of the maze (Fig. 7.3). The mice were not allowed to swim in the pool before training. During the training trials, the time required to escape into the hidden platform in one of the quadrant was recorded. The escape latency to reach the platform was measured in four training sessions for 4 days, corresponding to third, fourth, fifth and sixth day after ICV injections. The latencies were calculated as mean of total time spent in four trials of each day. The number of times the platform was not found was also recorded.

![Figure 7.3: Water maze apparatus](image)

7.3.5.3. Step-down inhibitory avoidance test

The step-down inhibitory avoidance test was performed to evaluate the non-spatial long-term memory. The study was performed according to the method of Sakaguchi et al. (2006) with certain modifications. The study apparatus consisted of 50 cm × 25 cm × 25 cm acrylic box whose floor was 1 cm apart. A 7 cm wide, 2.5 cm high and 25 cm long platform occupied the centre floor. During the training sessions (on the seventh day after ICV injection), the animals after stepping down, they were allowed to place their paws on the grid, which received 0.4 mA scrambled foot shock for 2 seconds (Fig. 7.4). In the test session (on the eighth day after ICV injection), no foot shock was given and step-down latency was used as a measure of retention of memory. One-trial step-down inhibitory avoidance involves the activation of two separate memory types, a short-term memory (STM) and long-term memory (LTM) system. Hence the retention tests were carried out for 90 minutes (STM) and 7 days (LTM). The same mice were
used for both the tests, as testing for STM did not affect LTM scores. Each mouse was placed again on the platform and the step-down transfer latency time was recorded.

![Figure 7.4: Apparatus for step-down inhibitory avoidance](image)

### 7.3.6. Preparation of tissue homogenate

After completing the behavioral tests, all the animals were sacrificed and the whole brains were collected and subjected to homogenization. The homogenate was prepared using 100 mM Phosphate buffer (pH 7.4) containing 1% Triton X-100. Centrifugation was performed at 5000 rpm for 20 min at 4°C. The supernatant was collected separately and stored at -20°C and used for all the biochemical assays. Protein estimation was done for all the samples using the method followed by Lowry et al. (1951).

### 7.3.7. Evaluation of antioxidant potentials of *G. acerosa* benzene extract

The antioxidant potential of *G. acerosa* benzene extract was assessed by measuring the activity of antioxidant enzymes in mice brain homogenate. The activity of endogenous antioxidants such as catalase, SOD, glutathione (reduced), glutathione reductase, glutathione-S-transferase, glutathione peroxidase were monitored by the methods (6.3.5.1, 6.3.5.2, 6.3.5.3, 6.5.3.4, 6.5.3.5, 6.5.3.6 respectively) given in chapter-4.
7.3.8. Macromolecular damage assessment in mice (treated with Aβ 25-35 and/or extract) brain tissue homogenate

7.3.8.1. Lipid peroxidation assay (Yagi and Rastogi, 1979)

The lipid peroxidation assay was performed according to the method mentioned in the section 6.3.6.1. The same protocol was followed except the use of tissue homogenate instead of cell homogenate. The results were expressed as μM of TBARS/mg of protein.

7.3.8.2. Measurement of protein carbonyl content (Levine et al., 1990)

The amount of protein carbonyl content present in the tissue homogenate was determined by the method given in the section 6.3.6.2. The results were expressed as mM of free carbonyl content/mg of protein.

7.3.9. Cholinesterase inhibitory assay (Ellman et al., 1961)

AChE and BuChE inhibitory effect of G. acerosa benzene extract on Aβ 25-35 treated mice was evaluated according to the method given in the section 6.3.8. The results were expressed as U/mg of protein.

7.3.10. Measurement of caspase-3 activity (Ochu et al., 1998)

The level of caspase-3 activity was measured in tissue homogenate of all the experimental animals according to the protocol given in section 6.3.9.1. In order to determine the caspase-3 activity, tissue homogenate was prepared using a specific homogenizing buffer, which consisted of 50 mM Tris-Cl (pH 7.4), 1 mM EDTA, 10 mM EGTA, 1 mM DTT and 0.1 M phenazine methosulfate. The tissue sample in buffer solution was centrifuged at 15000 rpm at 4°C for 20 min. Protein estimation was done for the homogenate by the method of Lowry et al. (1951). The homogenate was used as the enzyme source and the enzyme activity was calculated from the standard curve plotted using p-nitroanilide (25-200 μM). The results are expressed as Units/mg of protein.

7.3.11. β-secretase assay

Principle

β-secretase (BACE-1) activity was assessed by BACE-1 activity assay kit (Biovision, USA). The assay involves the use of BACE-1 specific peptide conjugated to
two reporter molecules EDANS and DABCYL. In the uncleaved form, the fluorescent emission from EDANS was quenched by the physical proximity of the DABCYL moiety. β-secretase cleaves the conjugated peptide, which results in the separation of EDANS and DABCYL that allows the release of fluorescent signal. The level of fluorescent intensity is proportional to the level of β-secretase activity.

**Assay kit components**

- β-secretase extraction buffer - 25 mL
- β-secretase reaction buffer (2X) - 10 mL
- β-secretase substrate (in DMSO) - 200 µL
- Active β-secretase (Lyophilized) - Reconstituted in 10 µL of H2O
- β-secretase inhibitor (in DMSO) - 10 µL

**Procedure**

β-secretase activity was measured using 96-well (Nunc F16 Black MaxiSorp TM) black polystyrene microplate. The brain tissues obtained from the experimental animals were used for the preparation of homogenate. Ice-cold homogenizing buffer was added to the tissue samples and incubated in ice for 10 min. The mixture was centrifuged at 10,000 g for 5 min and the supernatant was collected separately and stored. 50 µL of brain tissue homogenate from different experimental groups were added separately to each well of 96-well plate. 2 µL of active β-secretase was used as positive control and was added to 50 µL of extraction buffer. 2 µL of β-secretase inhibitor was added to the sample and was considered as negative control. 50 µL of reaction buffer was added to all the wells and incubated for 5-10 min at 37°C for 1 h. After incubation, the fluorescent intensity was measured using a multilabel reader (Molecular Device Spectramax M3, equipped with Softmax Pro V5 5.4.1 software) with the excitation and emission wavelength of 335 and 495 nm respectively.

**Calculation**

The β-secretase activity was expressed as relative fluorescence units (RFU)/µg of protein.
7.3.12. Determination of monoamine oxidase B (MAO B) activity

**Principle**

The method involves the use of kynuramine, which is a common substrate for MAO B. The enzyme catalyzes the spontaneous cyclization of the intermediate aldehyde formed by the oxidative deamination of kynuramine to form 4-hydroxyquinoline as the end product. The formation of 4-hydroxyquinoline can be measured fluorimetrically (Krajl, 1965).

![Chemical diagram](image)

**Reagents**

- 0.2 M Phosphate buffer (pH 7.4)
  - Solution A – K₂HPO₄ - 3.48 g in 100 mL of d.H₂O
  - Solution B – KH₂PO₄ - 2.72 g in 100 mL of d.H₂O
  - 81 mL of solution A was mixed with 19 mL of solution B and the pH was adjusted to 7.4.

- 500 µM Kynuramine - 0.8 mg in 5 mL of d.H₂O

- 1 mM Clorgyline
  - Sub-stock – 10 µM
  - Working concentration - 1 µM

- 10 mM Kynurenic acid - 1.89 mg in 1 mL of d.H₂O
  - Working concentration – 25 µM to 100 µM

- 10% Zinc sulphate - 10 g in 100 mL of d.H₂O

- 1 N sodium hydroxide

**Procedure**

Brain tissue homogenate (200 µg) was incubated with 1 µM of clorgyline (MAO -A inhibitor) for 15 min. After incubation, 750 µL of phosphate buffer (pH 7.4)
and 200 µL of kynuramine (500 µM) was added and incubated at 37°C for 30 min. The reaction was terminated by the addition of 10% ZnSO₄ (250 µL), 1 N NaOH (50 µL) and centrifuged at 3000 rpm for 5 min and the supernatant was collected separately. 0.7 mL of supernatant was mixed with 1.4 mL of 1 N NaOH and the fluorescent intensity was measured at the excitation wavelength of 315 nm and emission wavelength of 380 nm using fluorescence spectrometer. The amount of 4-hydroxyquinoline formed was determined from the standard curve plotted using kynurenic acid (25-100 µM).

**Calculation**

The results were expressed as U/mg of protein (1 Unit = nmol of 4-nitroquinoline formed /min).

**7.3.13. Western Blot analysis**

Brain tissue homogenate was used for western blot analysis in which the protein concentration was determined according to the method of Lowry et al. (1951). The proteins were separated in sodium dodecyl sulfate – polyacrylamide gel and transferred to PVDF membrane. The membrane after transfer was subjected to blocking with 5% non-fat dry milk and was incubated overnight at 4°C with primary antibodies (Bax and BCl-2). Then the membrane was incubated with respective secondary antibodies. The proteins were detected using chromogenic substrate.

**7.3.14. Statistical analysis**

Statistical analysis was performed using SPSS 17.0 software package. The results of all the experiments were represented as Mean ± S.D of triplicates employed. The statistical difference between the groups was analyzed by one-way ANOVA. Comparisons were made between Aβ 25-35 treated group Vs control group (II Vs I) and also between extract/ donepezil treated group Vs Aβ 25-35 treated group (III, IV, V, VI, VII Vs II). P <0.05 were regarded as significant.
7.4. RESULTS AND DISCUSSION

7.4.1. Evaluation of protective effect of G. acerosa benzene extract on Aβ 25-35 induced memory impairment in mice by behavioral tests

7.4.1.1. Assessment of protective effect of G. acerosa on Aβ 25-35 induced memory impairment by Y-maze test.

Experimental mice were examined three days after ICV administration of Aβ 25-35, for spontaneous alternation behavior in Y-maze, which is an index of spatial working memory. Administration of Aβ 25-35 resulted in a significant (P<0.05) decrease in percentage of alternation behavior (54.33 ± 1.50) when compared to the control group (69.83 ± 8.08). The results were in agreement with the previous findings of Lu et al. (2009), where the peptide treated mice reduced the alternation behavior when compared to control. Treatment with G. acerosa benzene extract resulted in an increase in alternation behavior percentage (56.33 ± 1.21) at the concentration of 400 mg/mL (Group IV) (Fig. 7.5). In a similar study, Bagheri et al. (2011) demonstrated that the alternation score was higher for genistein (a soy isoflavone and a phytoestrogen) treated Aβ-injected rats. Donepezil, a standard anti-Alzheimer drug did not induce any significant increase in the percentage of alternation behavior in peptide treated mice (Group VI).

![Figure 7.5: Effect of G. acerosa benzene extract on Aβ 25-35 induced short term memory by Y-maze test. The results are expressed as Mean ± SD. *P<0.05 (Comparisons were made between groups II Vs I & III, IV, V, VI, VII Vs II).](image-url)
7.4.1.2. Assessment of protective effect of G. acerosa on Aβ 25-35 induced memory impairment by Water-maze test.

The effect of G. acerosa benzene extract on memory improvement was analyzed through water-maze test. All the animals were treated with G. acerosa benzene extract for 21 days followed by ICV administration. The animals were trained (to locate the platform) in three trials per day for 2 days. The experiment was performed three days after ICV administration of Aβ 25-35. Their spatial learning scores were recorded as escape latency in seconds. The results of the experiment suggests that, a significant ($P<0.05$) increase (71.33 ± 3.14) in escape latency was observed in Aβ 25-35 peptide treated group, when compared to control (39.66 ± 4.03). Treatment with 200 mg/kg and 400 mg/kg of extract exhibited a significant decrease in the escape latency ($P<0.05$), when compared to peptide treated group (Fig. 7.6). Similar results were obtained in mice treated with Gomisin A, where the compound effectively shortened the escape latencies, which was prolonged by scopolamine treatment (Kim et al., 2006). Treatment with the standard drug, Donepezil also caused a significant decrease ($P<0.05$) in escape latency, when compared to the peptide treated group.

![Figure 7.6: Protective effect of G. acerosa benzene extract on Aβ 25-35 induced memory impairment by water-maze test. $^*P<0.05$ (Comparisons were made between groups II Vs I & III, IV, V, VI, VII Vs II).](image-url)
7.4.1.3. Assessment of protective effect of G. acerosa on Aβ 25-35 induced memory impairment by Step-down inhibitory avoidance test.

The experiments were performed in mice seven days after administration of Aβ 25-35. Treatment with Aβ 25-35 resulted in significant ($P<0.05$) decrease in STM (85.33 ± 3.55) and LTM (109.5 ± 3.61), when compared to the control animals (150.33 ± 11.70 and 152.5 ± 8.96 for STM and LTM respectively) (Fig. 7.7). The data was in agreement with the previous finding, where a decrease in the step down latency was observed in Aβ 25-35 peptide treated animals (Maurice et al., 1996). Interestingly, treatment with G. acerosa benzene extract results in a significant ($P<0.05$) increase in both STM and LTM, which was verified by the increase in step-down latency. The standard drug Donepezil also increases the step-down latency significantly ($P<0.05$) in the peptide treated animals. Hence the results of the behavioral assays suggest that G. acerosa benzene extract significantly improves both STM and LTM, which was otherwise impaired due to the administration of Aβ 25-35.

![Figure 7.7: Effect of G. acerosa benzene extract on Aβ 25-35 induced memory impairment in mice by step-down inhibitory avoidance test. *$P<0.05$ (Comparisons were made between groups II Vs I & III, IV, V, VI, VII Vs II).](image)

7.4.2. Effect of G. acerosa benzene extract on Aβ 25-35 induced alteration in the levels of antioxidant enzymes in mice

Oxidative stress has been regarded as the key component in AD pathological cascade. Several modifications such as peroxidation of lipids, protein oxidation and
DNA damage has been found to occur as a result of increased accumulation of ROS in the patients of AD brain. Moreover, Aβ peptide induced oxidative stress has been greatly implicated in AD. The peptide induced toxicity is mediated by free radicals, which causes severe macromolecular damage through the inhibition of antioxidants. Hence for reducing the ROS mediated damage in AD, antioxidant therapies particularly, antioxidants from natural source have been proposed for AD treatment (Kim et al., 2011). Therefore, in the present study the effect of G. acerosa benzene extract, which has the antioxidant activity, was evaluated on Aβ 25-35 induced alteration in the levels of various endogenous antioxidant defense systems in mice.

Treatment with Aβ 25-35 significantly \((P<0.05)\) reduced the catalase activity \((0.0033 \pm 0.00037 \text{ U/mg of protein})\) in peptide treated group (Group II), when compared to the control group \((0.00477 \pm 0.0004 \text{ U/mg of protein})\) (Group I) [Fig. 7.8 – (A)]. This decrease in catalase levels might be due to the increase in the production of peroxides in the brain of peptide treated animals. Interestingly, the level of catalase was increased \((0.0040 \pm 0.00038 \text{ U/mg of protein})\) in the extract treated group (200 mg/mL) as that of control group. Animals (treated with Aβ 25-35) treated with Donepezil did not show any effect on catalase activity. Similarly, the effect of G. acerosa benzene extract on the levels of SOD was evaluated. The results suggests that Aβ 25-35 induces the levels of SOD significantly \((P<0.05)\) in peptide treated group (Group II). This increase in SOD activity might be due to the compensatory response, in order to scavenge Aβ 25-35 induced production of superoxides. Interestingly, the level of SOD was restored to normal levels as that of control, in the extract treated group (Group III and IV) [Fig. 7.8 –(B)], which might be due to the radical scavenging potential of G. acerosa benzene extract. Similar results were obtained in the study led by Jhoo et al. (2004), where the increase in SOD level was restored towards normal levels by α-tocopherol in peptide treated mice.
Figure 7.8: *G. acerosa* benzene extract restores the alteration in the level of catalase (A) and SOD (B) activity in mice treated with Aβ 25-35. *P<0.05 (Comparisons were made between groups II Vs I & III, IV, V, VI, VII Vs II).

In addition to catalase and SOD levels, the alteration in the levels of other important antioxidant enzymes such as glutathione-S-transferase, glutathione peroxidase and glutathione was also analyzed. The results show that, a significant (P<0.05) increase in glutathione-S-transferase, glutathione peroxidase and glutathione activity was observed in Aβ 25-35 peptide treated group (1.39 ± 0.054 U/mg of protein; 0.000012 ± 1.1237E-06 U/mg of protein and 0.176 ± 0.030 µM/mg of protein respectively), when compared to control group (0.56 ± 0.038 U/mg of protein; 2.459E-06 ± 4.154E-07 U/mg of protein and 0.06 ± 0.009 µM/mg of protein respectively) [Fig. 7.9- (A&B)]. The result was in agreement with the previous study of Jhoo et al. (2004), where a remarkable increase in the level of glutathione peroxidase was observed, upon treatment with Aβ1-42 in cortex and hippocampus region of mice. Treatment with *G. acerosa* benzene extract restores the level of these antioxidants towards the normal levels, which suggests that benzene extract, through its antioxidant potential reduces the Aβ peptide induced alteration in the level of antioxidant enzymes. The level of glutathione reductase was decreased significantly (P<0.05) upon treatment with Aβ 25-35 (0.052 ± 0.00055 U/mg of protein), when compared to the control group (0.1353 ± 0.0179 U/mg of protein). Treatment with benzene extract restores the levels towards normal in Aβ treated mice [Fig 7.9 – (B)]. Hence the above results suggest that the
extract possess excellent antioxidant potential, as it restores the alteration (induced by Aβ 25-35) in the level of endogenous antioxidant enzymes in mice.

Figure 7.9: Effect of *G. acerosa* benzene extract on Aβ 25-35 induced alteration in the level of glutathione-S-transferase and glutathione peroxidase (A), glutathione reductase and glutathione (B). *P<0.05* (Comparisons were made between groups II Vs I & III, IV, V, VI, VII Vs II).
7.4.3. Evaluation of protective effect of G. acerosa benzene extract on Aβ 25-35 induced macromolecular damage in mice

The effect of *G. acerosa* benzene extract on the levels of MDA content was evaluated in Aβ 25-35 treated mice brain tissue homogenate. A significant increase (*P<0.05*) in the level of TBARS (2.27 ± 0.27 µM TBARS/mg of protein) was observed in brain of Aβ 25-35 injected mice, when compared to control group (0.323 ± 0.049 µM TBARS/mg of protein) (Fig 7.10). The results were in accordance to the previous report, where a significant increase in the peroxidation products was observed in mice brain treated with Aβ 25-35 (*Hiramatsu et al., 2010*). Treatment with 400 mg of *G. acerosa* benzene extract (Group IV) resulted in a significant decrease (*P<0.05*) in the level of TBARS (0.58 ± 0.036 µM), which suggests that *G. acerosa* prevents Aβ 25-35 mediated peroxidation of lipids. Similar studies, were carried out in mice infused with Aβ 25-35, where oligonol (a proanthocyanidin) obtained from lychee fruits were found to reduce lipid peroxidation levels (*Choi et al., 2014*).

Fig 7.10: Effect of *G. acerosa* benzene extract on Aβ 25-35 induced lipid peroxidation in brain. The values are expressed as Mean±SD. *P<0.05* (Comparisons were made between groups II Vs I & III, IV, V, VI, VII Vs II).

In addition to lipid peroxidation, oxidation of proteins has been considered as an important factor in aging and age-related neurodegenerative disorders. These oxidative modifications causes altered and non-specific protein functioning. The protein oxidation can be indexed by measuring the amount of protein carbonyls present in the system.
The protein carbonyls are formed from the direct free radical attack on vulnerable amino acid side chains or from the products of glycation, glycoxidation and lipid peroxidation reactions with that of protein. Moreover, the protein carbonyls have also been found to impart neurodegeneration in AD brain (Jhoo et al., 2004). Therefore, in the present study, the amount of protein carbonyls formed due to Aβ induced toxicity was evaluated. A significant increase ($P<0.05$) in the level of protein carbonyls (67.48 ± 1.11 mM/mg of protein) was observed in the brains of Aβ 25-35 treated mice, when compared to control group (22.53 ± 2.71 mM/mg of protein) (Fig. 7.11). Jhoo et al. (2004) also demonstrated a similar result, where the peptide treatment increases the amount of protein carbonyl production in mice administered with Aβ 1-42. Treatment with G. acerosa benzene extract prevented Aβ mediated protein carbonyl content production, which was verified by the amount of protein carbonyls formed in the extract treated group. Hence the results suggest that G. acerosa benzene extract protects the macromolecules such as lipids and proteins from Aβ 25-35 mediated oxidation.

Figure 7.11: Effect of G. acerosa benzene extract on Aβ 25-35 induced protein carbonyl content production in mice brain. The values are expressed as Mean±SD. ‘*’ $P<0.05$ (Comparisons were made between groups II Vs I & III, IV, V, VI, VII Vs II).

7.4.4. Effect of G. acerosa benzene extract on Aβ 25-35 induced increase in cholinesterase activity

Since the cholinergic deficit has been greatly implicated in the pathogenesis of AD, the present study involves the evaluation of inhibitory effect of G. acerosa benzene
extract on Aβ 25-35 induced increase in cholinesterase activities (both AChE and BuChE). The results of the study suggest that Aβ 25-35 induces the level of AChE and BuChE in mice brain (0.155 ± 0.007 U/mg of protein and 0.015 ± 0.0012 U/mg of protein respectively), significantly \(P<0.05\) when compared to the control group (0.072 ± 0.0013 U/mg of protein and 0.008±0.0009 U/mg of protein for AChE and BuChE respectively). *G. acerosa* benzene extract (at 400 mg concentration) treatment (Group IV) resulted in a significant reduction in the level of these cholinesterases (in peptide treated mice), where the enzyme activity was 0.116 ± 0.0088 U/mg of protein and 0.011 ± 0.0014 U/mg of protein for AChE and BuChE respectively (Fig. 7.12). Hence the results suggest that the benzene extract of *G. acerosa* protects the mice brain from cholinergic deficit through the inhibition of ChEs. Similar study was carried out in Aβ 25-35 administered rat brain, where Ginkgolides (major component of Ginkgo extract) effectively enhances the release of the neurotransmitter ACh from the hippocampus (*Lee et al., 2004*). Huperzine A (a lycopodium alkaloid) was also reported to exhibit excellent ChE inhibition in rat brain administered with the neurotoxin AF64A (*Cheng and Tang, 1998*).

![Figure 7.12: Inhibitory effect of *G. acerosa* benzene extract on Aβ 25-35 induced increase in AChE and BuChE activities in mice brain tissue homogenate. The values are expressed as Mean±SD. *P<0.05* (Comparisons were made between groups II Vs I & III, IV, V, VI, VII Vs II).]
7.4.5. Effect of *G. acerosa* benzene extract on β-secretase activity

Since Aβ peptide plays a crucial role in the onset and progression of AD, the current therapeutic target is to reduce the production of these toxic peptides in CNS of the affected individuals. The suppression of these toxic peptides can be achieved only by inhibiting the activity of the enzyme β-secretase, which is responsible for amyloidogenic processing of APP in AD brain (*Lee et al.*, 2009). Therefore, in the present study, the inhibitory effect of *G. acerosa* benzene extract on β-secretase was evaluated in Aβ 25-35 administered mice. The results of the assay demonstrated that Aβ 25-35 injection causes significant ($P<0.05$) increase in the activity of β-secretase (1.817 ± 0.028 RFU/µg of protein), when compared to the control group (0.586 ± 0.035 RFU/µg of protein) (Fig. 7.13). The results were in agreement with the previous study, where Aβ induced the expression of β-secretase activity in cortex and hippocampus of the peptide treated mice brain (*Lee et al.*, 2009). Treatment with *G. acerosa* benzene extract (400 mg) reduced the peptide induced β-secretase activity significantly ($P<0.05$) with the RFU values of 0.9233 ± 0.029 RFU/µg of protein. In a similar study, the β-secretase inhibitory effect of icariin (a major component of the traditional Chinese herb Epimedium) was evaluated and the results suggest that the compound effectively inhibits the β-secretase activity in the hippocampus of AD transgenic mice model (*Zhang et al.*, 2014).

![Figure 7.13: Inhibitory effect of *G. acerosa* benzene extract on Aβ 25-35 induced increase in β-secretase activity in mice brain tissue homogenate. The values are expressed as Mean ± SD. *P<0.05* (Comparisons were made between groups II Vs I & III, IV, V, VI, VII Vs II).]
7.4.6. Protective effect of *G. acerosa* benzene extract on Monoamine oxidase activity

Monoamine oxidase in the brain catalyzes the oxidative deamination of biogenic amines and other neurotoxins. Two isozymes are present such as MAO-A and MAO-B, which is distinguished by their differences in substrate and inhibitor selectivity. MAO-B has been found to play a vital role in CNS and peripheral organs. Altered activities of MAO-B is implicated in neurological disorders such as AD, as the oxidation mechanism of MAO-B leads to accumulation of hydrogen peroxide, which in turn causes oxidative stress mediated cytotoxicity. Hence the development and identification of MAO-B inhibitors are of great interest in the field of drug discovery against AD (Viña et al., 2012). In line with that, the present study involves the evaluation of MAO-B inhibitory activity of *G. acerosa* benzene extract in Aβ 25-35 injected mice. The results of the assay show that, a significant increase ($P<0.05$) in the levels of MAO-B was observed in peptide treated mice (3572.47 ± 183.47 nM of 4-hydroxy quinoline/min/mg) when compared to the control group (724.28 ± 46.59 nM of 4-hydroxy quinoline/min/mg) (Fig. 7.14). Interestingly, treatment with *G. acerosa* benzene extract (400 mg) reduced the activity of MAO-B (1346.75 ± 239.00 nM of 4-hydroxy quinoline/min/mg) in peptide injected mice significantly ($P<0.05$). Lee et al. (2001) demonstrated that the compound Sanguinarine, (a major component of the herb of *Chelidonium major*) possess excellent MAO inhibitory activity in mice. In addition to this study, from the past decades several medicinal plants have been reported to possess potential MAO inhibitory activities (Herraiz and Chaparro, 2006; Kong et al., 2004; Lin et al., 2003).
7.4.7. Evaluation of anti-apoptotic potential of *G. acerosa* benzene extract on Aβ 25-35 injected mice

The process of programmed cell death or apoptosis plays an important role in the degeneration of neuronal cells, which has been routinely observed in AD brain (Blasko et al., 2000). The AD brain tissues were found to possess deposits of activated caspase-3, which promotes the apoptotic cascade mechanism in neuronal cells (Luo et al., 2002). Hence, the development of compounds with anti-apoptotic potential might be an effective therapeutic strategy in minimizing the apoptosis mediated cell death. In line with that, in the present study, the protective effective of *G. acerosa* benzene extract was evaluated against Aβ 25-35 mediated apoptosis in peptide treated mice. The results show that mice treated with Aβ 25-35 causes a significant (*P*<0.05) increase in caspase-3 activity (34.52 ± 3.62 mM of PNA/min/mg of protein), when compared to untreated control group (0.86 ± 0.14 mM of PNA/min/mg of protein). This increase in the level of caspase-3 activity was reverted to normal levels upon treatment with *G. acerosa* benzene extract (0.81 ± 0.12 mM of PNA/min/mg of protein) (Fig. 7.15).
Figure 7.15: Inhibitory effect of *G. acerosa* benzene extract on Aβ 25-35 induced increase in caspase-3 activity in mice brain tissue homogenate. The values are expressed as Mean±SD. *P<0.05 (Comparisons were made between groups II Vs I & III, IV, V, VI, VII Vs II).

In addition to caspase-3 activity, the anti-apoptotic effect of *G. acerosa* was also assessed by measuring the expression of pro-apoptotic (Bax) and anti-apoptotic proteins (BCl-2) by western blot analysis. The results show that Bax protein expression was increased in Aβ 25-35 peptide group, which suggests that the peptide promotes or induces neuronal death through the process of apoptosis. The results were in agreement with the previous finding, where treatment with Aβ 25-35 up-regulated the expression of Bax in rat hippocampus tissues *(Wang et al., 2013)*. *G. acerosa* benzene extract at the concentration of 200 mg and 400 mg reduced the expression level of Bax protein (Fig. 7.16). In addition to that, the expression pattern of BCl-2 was also assessed and the results suggest that treatment with Aβ 25-35 induces the expression of BCl-2, which could be a compensatory response of the cellular machinery in order to prevent the apoptosis mediated cell death. However, *G. acerosa* benzene extract did not affect the expression pattern of BCl-2, which suggests that the extract exerts its anti-apoptotic activity only by down-regulating the expression level of pro-apoptotic protein (Bax).
Figure 7.16: Representative western blot images showing the effect of *G. acerosa* benzene extract on the expression levels of Bax and BCl-2 in mice brain tissue homogenates (A). Quantified results of the expression pattern of Bax (B) and BCl-2 (C).
7.5. CONCLUSION

In the present study, the neuroprotective effect of *G. acerosa* benzene extract was evaluated on Aβ 25-35 treated Albino mice. The results of the experiments suggest that *G. acerosa* benzene extract protects the mice brain from Aβ 25-35 mediated cognitive decline and it also restores the peptide induced alteration in the level of activities of antioxidant enzymes. Moreover, the benzene extract exhibit its protective effect by preventing the cells from macromolecular damage. Reduction in the activities of AChE and BuChE suggests that the extract protects the mice from cholinergic deficit. The extract also possesses inhibitory effect on β-secretase and hence it could be employed as a potential anti-amyloidogenic compound. The reduction in the level of caspase-3 activity and expression pattern of Bax protein suggests that the extract has excellent anti-apoptotic activity. Overall, the outcome of the study suggests that *G. acerosa* benzene extract holds multi-faceted neuroprotective potential against Aβ 25-35 mediated toxicities.
7.6. SUMMARY OF THE RESULTS

- APP processing
- Non-Amyloidogenic pathway
- Amyloidogenic pathway
- Oxidative stress
- Endogenous antioxidants imbalance
- AChE/BuChE
- Neurodegeneration
- Cognitive deficit

Inhibition by *G. acerosa*

Restoration by *G. acerosa*

Prevention by *G. acerosa*