AIM AND OBJECTIVES

Chronic stress has been known to cause numerous changes in brain structure that include altering its neurochemistry, excitability, neuronal morphology and cell death (Conrad, 2006). Oxidative stress and reactive oxygen species are known to play a crucial role in the progression of stress-induced neurological diseases (Kontos and Wei, 1986). Production of free radicals after chronic unpredictable stress plays a key role in the pathogenesis of cognitive deficits (Farooq et al., 2012). Traumatic brain injury releases pro-inflammatory cytokines and results into neuroinflammation in hippocampus and other brain parts, leading to cognitive imbalance (Kontos and Wei, 1986). Oxidative damage and apoptosis are two crucial mechanisms that play a significant role in traumatic brain injury-induced neurological disorders (Bayir et al., 2003). Chronic stress is also known to increase serum corticosterone level and impairs memory retrieval process (Kurukulasuriya et al., 2004). Olfactory bulbectomy alters neurogenesis in several regions of brain, which is one of the putative pathogenic mechanisms to explain depression like state (Koo et al., 2010). The several neurodegenerative diseases associated with chronic stress mostly includes depression and memory dysfunction (Radley et al., 2004). All these forms of chronic stress are known to cause oxidative damage, mitochondrial dysfunction, neuroinflammation, and apoptosis with significant alterations in behaviour parameters (Halliwell, 2006). However, the molecular and cellular mechanism and pathways associated with these stress related problems are still not known and poorly understood.

The incidence and progression of chronic stress related complications can be halted by using targeted approach of herbal/phytochemical antioxidants and herbal derivatives. Various antioxidants have been tried for their effectiveness in reducing deleterious effects on neurons due to oxidative stress (Jara-Prado et al., 2003). Dietary and medicinal phyto-antioxidants are being used as an adjuvant therapy to limit their side effects and increase their effectiveness. Therefore, the present study has been designed to explore the
Aim and Objectives

neuroprotective mechanism of various anti-oxidants/anti-inflammatory drugs (curcumin, ginseng and quercetin) against chronic stress and related problems. Hence, the present study has been divided into following subheadings:

CHAPTER 1: NEUROPROTECTIVE EFFECTS OF CURCUMIN AND GINSENG AGAINST EXPERIMENTAL MODEL OF CHRONIC UNPREDICTABLE STRESS INDUCED COGNITIVE DEFICITS

CHAPTER 2: NEUROPROTECTIVE FUNCTIONS OF CURCUMIN AND QUERCETIN AGAINST EXPERIMENTAL MODEL OF OLFACTORY BULBECTOMY INDUCED DEPRESSION

CHAPTER 3: THERAPEUTIC ROLE OF GINSENG AND QUERCETIN AGAINST EXPERIMENTAL MODEL OF MILD TRAUMATIC BRAIN INJURY INDUCED COGNITIVE LOSS
CHAPTER 1

NEUROPROTECTIVE EFFECTS OF CURCUMIN AND GINSENG AGAINST EXPERIMENTAL MODEL OF CHRONIC UNPREDICTABLE STRESS INDUCED COGNITIVE DEFICITS

CHAPTER 1.1: NEUROPROTECTIVE ROLE OF CURCUMIN AND ITS INTERACTION WITH PIPERINE AGAINST CHRONIC UNPREDICTABLE STRESS INDUCED COGNITIVE LOSS

1.1.1 INTRODUCTION

Memory impairment is a common and usual comorbidity associated with prolonged stress (Radley et al., 2004). Chronic stress is known to influence cognitive performance in various psychiatric patients (Vanitallie, 2002). Chronic stress increases corticosterone secretion, which causes dysregulation of hypothalamic-pituitary-adrenocortical (HPA) axis and impairment of hippocampus-dependent learning and memory processes (Kurukulasuriya et al., 2004). Secretion of corticosterone also triggers an increase in oxidative stress that ultimately leads to memory deficits (Sato et al., 2010). These physiological consequences of stress depend on the intensity and duration of the stressor and on how an organism perceives and reacts to the noxious stimulus (Joels, 2006). Therefore, chronic unpredictable stress (CUS) model has been standardised to study the development and progress of stress and related problems (Willner et al., 1992).

Degeneration of cholinergic neurons is one of the major hallmarks in the brain of cognitive deficit patient (Selkoe, 1991). Along with this, study report also suggest that neuronal functions are altered by generation of reactive oxygen species which leads to oxidative stress; a prominent feature in the pathogenesis of cognitive dysfunction (Massaad and Klann, 2011). Various antioxidants have been tried for their effectiveness in reducing deleterious effects on neurons due to oxidative stress (Jara-Prado et al., 2003). Dietary
and medicinal phyto-antioxidants are being used as an adjuvant therapy to limit their side effects and increase their effectiveness. Curcumin, the yellow pigment extracted from the rhizomes of *Curcuma longa*, has been extensively studied for its antioxidant (Nafisi et al., 2009), anti-inflammatory and neuroprotective activities (Motterlini et al., 2000). Curcumin has been reported to possess free radical scavenging activity against various neurodegenerative pathologies, including Alzheimer’s disease (Mancuso et al., 2012). Earlier study from our laboratory has also suggested that curcumin restored mitochondrial enzyme complex activities and attenuated reactive oxygen species production (Kumar et al., 2011). Besides, our laboratory also demonstrated an improved learning and memory performance by curcumin treatment in experimental model (Kumar et al., 2009). Manganese complexes of curcumin exhibited a great capacity to protect brain lipids against peroxidation and enhance superoxide dismutase (SOD) activity (Vajragupta et al., 2003). Earlier, study also showed an inhibitory effect of curcuminoids on acetylcholinesterase activity against scopolamine-induced amnesia (Ahmed and Gilani, 2009). Curcumin has also been reported to reduce serum corticosterone level in restraint stress induced memory dysfunction (Xu et al., 2009). These reported pharmacological properties of curcumin clearly suggest its beneficial role against stress induced cognitive impairment.

Poor bioavailability of curcumin due to its conjugation like sulfation and glucuronidation by liver and intestinal enzymes, limits its frequent use as a therapeutic agent (Wahlstrom and Blennow, 1978). To overcome this problem, piperine, a major alkaloid obtained from black pepper (*Piper nigrum* Linn.) and long pepper (*Piper longum* Linn.) has been employed as a combination therapy in the present study since piperine is known to increase the bioavailability of many drugs (Atal et al., 1985). In light of these reports, the present study aims to investigate the protective effect of curcumin and its interaction with piperine against chronic unpredictable stress induced cognitive deficits and oxidative damage in mice.
1.1.2 MATERIALS AND METHODS

1.1.2.1 Animals

Male Laca mice (25–30 g) bred at Central Animal House (CAH) Panjab University, Chandigarh, were used. They were housed under standard laboratory conditions (25±2°C, 60–70% humidity), maintained on a 12 hour natural day–night cycle, with free access to standard food and water. Animals were acclimatized to laboratory conditions before the test. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of Panjab University (IAEC/346-356/UIPS/2) and conducted according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines of Government of India on the use and care of experimental animals.

1.1.2.2 Chronic unpredictable stress

Mice were exposed to a random pattern of mild stressors (Murua et al., 1991) daily for 28 days. The order of various stressors used in the present study is depicted below:

<table>
<thead>
<tr>
<th>Days</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<th>23</th>
<th>24</th>
<th>25</th>
<th>26</th>
<th>27</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress</td>
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<td>T</td>
<td>F</td>
<td>S</td>
<td>O</td>
<td>N</td>
<td>T1</td>
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<td>S1</td>
<td>T2</td>
<td>O</td>
<td>C2</td>
<td>N</td>
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<td>T1</td>
<td>S1</td>
<td>O</td>
<td>C1</td>
<td>N</td>
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<td>S</td>
<td>F</td>
<td>O</td>
<td>T</td>
<td>T1</td>
<td></td>
</tr>
</tbody>
</table>

C — Cold swim (8°C, 5 min); T — Tail pinch (1 min); F — Food and water deprivation (24 h); S — Swimming at room temperature (24±2°C, 20 min); O — Overnight illumination; N — No stress; T1 — Tail pinch (1.5 min); C1 — Cold swim (10°C, 5 min); S1 — Swimming at room temperature (24±2°C, 15 min); T2 — Tail pinch (2 min); C2 — Cold swim (6°C, 5 min).

1.1.2.3 Drugs and treatment schedule

Curcumin and piperine were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals used for biochemical and mitochondrial
estimations were of analytical grade. The animals were randomly divided into eight experimental groups (n=6-8). First and second group was named as naïve and control (CUS) group respectively. Curcumin (100, 200 and 400 mg/kg, p.o.) were treated as group 3–5 respectively. Piperine (20 mg/kg, p.o.) served as group 6. Co-administration of piperine (20 mg/kg) with curcumin (100 and 200 mg/kg) was categorized as group 7 and 8 respectively. Curcumin and piperine were prepared in peanut oil and administered orally on the basis of body weight (1 ml/100 g). Solutions were made fresh at the beginning of each day of the drug treatment. Drugs were administered daily 30 minutes before CUS procedure for 28 days. The entire study was conducted in multiple phases. The detailed experimental design for chronic unpredictable stress protocol has been shown in Fig. 1.1.1.
1.1.2.4 Behavioral assessments

1.1.2.4.1 Assessment of locomotor activity (Actophotometer)

The locomotor activity was assessed by using an actophotometer (IMCORP, Ambala, India). The apparatus was placed in a darkened, light-sound attenuated and ventilated testing room. Each animal was observed in a square (30 cm) closed arena equipped with infrared light sensitive photocells using digital photoactometer as shown in picture 1.1.1. Animals were placed individually in the activity chamber for a 3 min acclimatization period before performing actual activity tasks for 5 min. The motor activity was detected by infrared beams above the floor of the testing area (both x and y axis) and values expressed as total photo beam counts intersected by the animal per 5 min (Kumar and Garg, 2008).

1.1.2.4.2 Elevated plus maze paradigm

The elevated plus maze (EPM) consists of two opposite black open arms (16 × 5 cm), crossed with two closed walls of the same dimensions of 12 cm height as shown in picture 1.1.2. The arms were connected with a central square of dimensions 5 × 5 cm. The entire maze was elevated to a height of 25 cm from the floor. Acquisition and retention of memory processes were assessed as previously described (Sharma and Kulkarni, 1992). Acquisition of memory was tested on day 20 of CUS procedure. Animal was placed individually at one end of the open arm facing away from the central
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square. The time taken by the animal to move from the open arm to the closed arm was recorded as the initial transfer latency (ITL). Animal was allowed to explore the maze for 20 sec after recording the ITL and then returned to the home cage. If the animal could not enter closed arm within 90 sec, same was guided to the closed arm and ITL was given as 90 sec. Retention of memory was assessed on day 21 as first retention transfer latency (1st RTL) and on day 28 as the second retention transfer latency (2nd RTL) respectively.

1.1.2.4.3 Morris water-maze test

Morris water-maze apparatus (MWM) is most commonly used model to test spatial memory (Morris, 1984). The MWM procedure is based on the principle that animal dislikes swimming and hence when placed in a large pool of water its tendency is to escape it by searching for a platform. MWM consists of large circular pool (150 cm in diameter, 45 cm in height, filled to a depth of 30 cm with water at 28±1°C) as shown in picture 1.1.3. The tank was divided into four equal quadrants. A submerged platform (10 cm×10 cm), painted white was placed in the middle of the target quadrant of this pool, 1 cm below surface of water. The position of platform was kept unaltered throughout the training session. The tank was located in a large room where there were several brightly colored cues external to the maze; these were visible from the pool and could be used by the mice for spatial orientation. The position of the cues remained unchanged throughout the study. The water maze task was carried out for four consecutive days from day 24-27. The mice received daily four consecutive training trials, with each trial having a ceiling time of 120 sec. For each trial, individual mouse was gently put into the water at one of four starting positions, the sequence of which being selected randomly and allowed 120
sec to locate submerged platform. Then, it was allowed to stay on the platform for 20 sec. If animal failed to find the platform within 120 sec, it was guided gently onto platform and allowed to remain there for 20 sec.

**Acquisition trial** – Each mouse was subjected to four trials on each day (starting from day 24-27). A rest period of 1 hour was allowed in between each trial. Four trials per day were repeated for four consecutive days. Starting position on each day to conduct four acquisition trials was changed as described below and Q4 was maintained as target quadrant in all acquisition trials.

<table>
<thead>
<tr>
<th>Day</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day1</td>
<td>Q1</td>
<td>Q2</td>
<td>Q3</td>
<td>Q4</td>
</tr>
<tr>
<td>Day2</td>
<td>Q2</td>
<td>Q3</td>
<td>Q4</td>
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<td>Day3</td>
<td>Q3</td>
<td>Q4</td>
<td>Q1</td>
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<tr>
<td>Day4</td>
<td>Q4</td>
<td>Q1</td>
<td>Q2</td>
<td>Q3</td>
</tr>
</tbody>
</table>

Mean escape latency time (ELT) calculated for each day during acquisition trials was used as an index of acquisition.

**Retrieval trial** – On day 28, the platform was removed. Animal was placed in water maze and allowed to explore the maze for 120 sec. Mean time spent in all three quadrants, i.e. Q1, Q2 and Q3 were recorded and the time spent in the target quadrant, i.e. Q4 in search of missing platform provided an index of retrieval. Care was taken that relative location of water maze with respect to prominent visual clues was not disturbed during the total duration of study.

1.1.2.5 Serum corticosterone estimations

1.1.2.5.1 Preparation of serum

The blood was collected immediately through retro-orbital route after the last behavioral test. Blood collected in the test tubes was allowed to clot at room temperature. The tubes were then centrifuged at 2000 rpm for 10 min, the straw colored serum was separated and stored at -20°C.

1.1.2.5.2 Corticosterone assessment

The extraction of corticosterone was performed as per the modified method of Silber and its group (Silber et al., 1958). Briefly, 0.1-0.2 ml of serum
was treated with 0.2 ml of freshly prepared chloroform: methanol mixture (2:1, v/v), followed by 3 ml of chloroform instead of dichloromethane used in the procedure of Silber and its group. The step of treatment of petroleum ether was omitted. The samples were vortexed for 30 sec and centrifuged at 2000 rpm for 10 min. The chloroform layer was carefully removed with the help of syringe with a long 16 gauge needle attached to it and was transferred to a fresh tube. The chloroform extract was then treated with 0.1 N NaOH (2 ml) by vortexing rapidly and NaOH layer was rapidly removed. The sample was then treated with 3 ml of 30 N H$_2$SO$_4$ by vortexing vigorously. After phase separation, chloroform layer on top was removed using a spinal syringe as described above and discarded. The tubes containing H$_2$SO$_4$ were kept in dark for 30-60 min and thereafter fluorescence measurements carried out in fluorescence spectrophotometer (make Hitachi, model F-2500) with excitation and emission wavelength set at 472 and 523 nm respectively. The standard curve depicting the fluorescence yield versus corticosterone concentration was used for result analysis.

1.1.2.6 Biochemical assessments

Immediately after the last behavioral test, animals were randomized into two groups; one group was used for the biochemical assays and the other for assessment of mitochondrial enzyme complex activities. The animals were sacrificed by decapitation and whole brain of each animal was put on ice and weighed. A 10% (w/v) tissue homogenates were prepared in 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 × g for 15 min. Aliquots of supernatants were separated and used for biochemical estimations.

1.1.2.6.1 Assessment of oxidative stress

1.1.2.6.1.1 Estimation of lipid peroxidation

The extent of lipid peroxidation was determined quantitatively by performing the method as described by Wills and its group (Wills et al., 1966). The amount of malondialdehyde (MDA) was measured by reaction with thiobarbituric acid at 532 nm using Perkin Elmer Lambda 20
spectrophotometer (Norwalk, CT, USA). The values were calculated using molar extinction co-efficient of chromophore (1.56×10 M⁻¹ cm⁻¹).

1.1.2.6.1.2 Estimation of reduced glutathione

Reduced glutathione was estimated as per the method of Ellman (Ellman, 1959). Homogenates were precipitated with 1.0 ml of 4% sulfosalicylic acid by keeping the mixture at 4°C for 1 h and the samples were immediately centrifuged at 1200 × g for 15 min at 4°C. The assay mixture contained 0.1 ml of supernatants, 2.7 ml of phosphate buffer of pH 8 and 0.2 ml of 0.01 M dithiobisnitrobenzoic acid (DTNB). The yellow color developed was read immediately at 412 nm using Perkin Elmer lambda 20 spectrophotometer (Norwalk, CT, USA). The results were expressed as nanomoles of reduced glutathione per milligram of protein.

1.1.2.6.1.3 Estimation of superoxide dismutase

Superoxide dismutase (SOD) activity was assayed as per the method of Kono (Kono, 1978) wherein reduction of nitrazobluetetrazolium (NBT) was inhibited by superoxide dismutase. The assay system consists of EDTA 0.1 mM, sodium carbonate 50 mM and 96 mM of nitro blue tetrazolium (NBT). In the cuvette, 2 ml of the above mixture, 0.05 ml of hydroxylamine and 0.05 ml of the supernatant was added and auto-oxidation of hydroxylamine was measured for 2 min at 30 s intervals by measuring absorbance at 560 nm using Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA).

1.1.2.6.1.4 Estimation of catalase

Catalase activity was determined as per method of Luck (Luck, 1971), wherein breakdown of hydrogen peroxide (H₂O₂) is measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H₂O₂, phosphate buffer and 0.05 ml of supernatants of tissue homogenates (10%). The change in absorbance was recorded for 2 minute at 30 s interval at 240 nm using Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA). The results were expressed as micromoles of H₂O₂ decomposed per milligram of protein/min.
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1.1.2.6.1.5 Protein estimation

The protein content was estimated by biuret method (Gornall et al., 1949) using bovine serum albumin as a standard.

1.1.2.6.2 Assessment of nitrosative stress

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide was determined by a colorimetric assay with Greiss reagent (0.1% N-(1-naphthyl) ethylene diamine dihydrochloride, 1% sulphanilamide and 5% phosphoric acid) according to Green and its group (Green et al., 1982). Equal volumes of the supernatants and Greiss reagent were mixed incubated for 10 minutes at room temperature in the dark. The absorbance was measured at 540 nm using Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA). The concentration in the supernatants were determined from the standard sodium nitrite curve.

1.1.2.6.3 Estimation of acetyl cholinesterase (AChE) activity

AChE is a marker of loss of cholinergic neurons in the brain. The AChE activity was assessed as described by Ellman and its group (Ellman et al., 1961). The assay mixture contained of 0.05 ml of supernatant, 3 ml of sodium phosphate buffer (pH 8), 0.1 ml of acetylthiocholine iodide and 0.1 ml of DTNB (Ellman reagent). The change in absorbance was measured for 2 min at 30 s intervals at 412 nm using Perkin Elmer lambda 20 spectrophotometer (Norwalk, CT, USA). Results were expressed as micromoles of acetylthiocholine iodide hydrolyzed per min per mg of protein.

1.1.2.7 Mitochondrial enzyme complex activity

1.1.2.7.1 Isolation of mitochondria

Another group of animals were used for the estimation of mitochondrial enzyme complex activities. Briefly, mitochondria were isolated by differential centrifugation (Berman and Hastings, 1999). The brain samples were homogenized in 10 ml of homogenizing buffer containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1mM EGTA, 1 mg/ml BSA, pH 7.4. The homogenates were brought to 30 ml with the same buffer and centrifuged at
2000 g for 3 min at 40°C. The pellets were discarded and supernatants were divided into 2 tubes followed by centrifugation at 12000g for 10 min. The pellets containing mixture of synaptosomes and mitochondria were suspended in 10 ml of homogenization buffer containing 0.02% digitonin to lyse synaptosomes followed by centrifugation at 12000g for 10 min to settle down both extra synaptosomal and intra synaptosomal mitochondria. The mitochondrial pellets were washed twice in the same buffer without EGTA, BSA and digitonin.

1.1.2.7.2 Complex-I (NADH Dehydrogenase activity)

Complex-I was measured spectrophotometrically by the method of King and Howard (King and Howard, 1967). The method involved catalytic oxidation of NADH to NAD+ with subsequent reduction of cytochrome C. The reaction mixture contained 0.2 M glycyl glycine buffer pH 8.5, 6 mM NADH in 2 mM glycyl Glycine buffer and 10.5 mM cytochrome C. The reaction was initiated by addition of requisite amount of solubilized mitochondrial sample and followed absorbance change at 550 nm for 2 min.

1.1.2.7.3 Complex-II (Succinate Dehydrogenase (SDH) activity)

SDH was measured spectrophotometrically by the method of King (King, 1967). The method involves oxidation of succinate by an artificial electron acceptor, potassium ferricyanide. The reaction mixture contained 0.2 M phosphate buffer pH 7.8, 1% BSA, 0.6 M succinic acid, and 0.03 M potassium ferricyanide. The reaction was initiated by the addition of mitochondrial sample and absorbance change was followed at 420 nm for 2 min.

1.1.2.7.4 Complex-III (MTT ability)

The MTT assay was based on the reduction of (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-H-tetrazolium bromide (MTT) by hydrogenase activity in functionally intact mitochondria. The MTT reduction rate was used to assess the activity of the mitochondrial respiratory chain in isolated mitochondria by the method of Liu and its group (Liu et al., 1997). Briefly, 100 µl mitochondrial samples were incubated with 10 µl MTT for 3
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hour at 37°C. The blue formazan crystals were solubilized with dimethylsulfoxide and measured by an ELISA reader at 580 nm filter.

1.1.2.7.5 Complex-IV (Cytochrome oxidase assay)

Cytochrome oxidase activity was assayed in brain mitochondria according to the method of Sottocasa and its group (Sottocasa et al., 1967). The assay mixture contained 0.3 mM reduced cytochrome C in 75 mM phosphate buffer. The reaction was started by the addition of solubilized mitochondrial sample and absorbance change was recorded at 550 nm for 2 min.

1.1.2.8 Statistical analysis

All the values were expressed as Mean±SEM. All test data were analyzed using One way analysis of variance (ANOVA) followed by post hoc Tukey’s test. The criterion for statistical significance was P<0.05. All statistical procedures were carried out using sigma stat Graph Pad Prism (Graph Pad Software, San Diego, CA).

1.1.3 RESULTS

1.1.3.1 Effect of curcumin, piperine and their interaction on locomotor activity

Chronic unpredictable stress for 28 days significantly decreased locomotor activity in control group as compared to naïve group (Fig. 1.1.2). Chronic curcumin (200 and 400 mg/kg) treatment for 28 days significantly improved locomotor activity as compared to control (CUS) group. Further, curcumin (100 mg/kg) did not produce any significant effect on locomotor activity as compared to control. However, co-administration of curcumin (100 and 200 mg/kg) with piperine (20 mg/kg) for 28 days significantly potentiated their protective effects (increased locomotor activity) which was significant as compared to their effects alone in CUS treated mice (p<0.05). Further, per se treatment of curcumin (400 mg/kg) did not show any significant effect on locomotor activity as compared to naïve (data not shown) (Fig. 1.1.2).
Fig. 1.1.2 Effects of curcumin and its co-administration with piperine on locomotor activity. Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to naive group; bP <0.05 as compared to CUS; cP <0.05 as compared to C(100)+CUS; dP <0.05 as compared to C(200)+CUS; eP <0.05 as compared to P(20)+CUS (One way analysis of variance (ANOVA) followed by post hoc Tukey’s test). CUS, chronic unpredictable stress; C(100), curcumin (100 mg/kg); C(200), curcumin (200 mg/kg); C(400), curcumin (400 mg/kg); P(20), piperine (20 mg/kg)

1.1.3.2. Effect of curcumin, piperine and their interaction on latency time in elevated plus maze (EPM) test

Following training, chronic unpredictable stress (CUS) control mice performed poorly throughout the experiment and did not show any change in the retention transfer latencies (RTL) on day 21 (1st RTL) and 28 (2nd RTL) as compared to ITL on day 20, demonstrating chronic stress-induced memory impairment. Besides, curcumin (200 and 400 mg/kg) treatment for 28 days significantly decreased both 1st and 2nd RTL on day 21 and 28 respectively as compared to control (p<0.05). Further, curcumin (100 mg/kg) treatment for 28
days did not show any significant effect on retention transfer latencies (1st and 2nd RTL) as compared to control; however, co-administration of curcumin (100 and 200 mg/kg) with piperine (20 mg/kg) for 28 days significantly shortened transfer latencies as compared to their effects alone in chronic unpredictable stress (CUS) treated mice (p<0.05). Further, per se treatment of curcumin (400 mg/kg) did not show any significant effect on transfer latency time as compared to naive (data not shown) (Fig. 1.1.3).

![Fig. 1.1.3 Effects of curcumin and its co-administration with piperine on latency time in elevated plus maze test. Values are expressed as mean ± SEM. For statistical significance, \(^{*}P<0.05\) as compared to naive group; \(^{\circ}P<0.05\) as compared to CUS; \(^{\circ}P<0.05\) as compared to C(100)+CUS; \(^{\circ}P<0.05\) as compared to C(200)+CUS; \(^{\circ}P<0.05\) as compared to P(20)+CUS (One way analysis of variance (ANOVA) followed by post hoc Tukey's test).

### 1.1.3 Effects of curcumin, piperine and their interaction on escape latency time (ELT) in Morris water maze test

The change in the escape latency time to reach the hidden platform was observed in training trials. The mean latency (days 24–27) was significantly prolonged in the control group (CUS) as compared to the naive group, indicating a poorer learning performance. Curcumin (200 and 400 mg/kg) treatment for 28 days significantly shortened escape latency time as compared to control, thereby showing improvement in learning performance.
Further, curcumin (100 mg/kg) treatment for 28 days did not show any significant effect on escape latency time as compared to control. However, co-administration of curcumin (100 and 200 mg/kg) with piperine (20 mg/kg), for 28 days significantly improved learning performance (decreased escape latency time) as compared to their effect alone in chronic unpredictable stress (CUS) treated mice (p<0.05). Further, per se treatment of curcumin (400 mg/kg) did not show any significant effect on escape latency time as compared to naive (data not shown) (Fig. 1.1.4).

![Fig. 1.1.4 Effects of curcumin and its co-administration with piperine on escape latency time in Morris water maze. Values are expressed as mean ± SEM. For statistical significance, *P <0.05 as compared to naive group; **P <0.05 as compared to CUS; ***P <0.05 as compared to C(100)+CUS; ****P <0.05 as compared to C(200)+CUS; *****P <0.05 as compared to P(20)+CUS (One way analysis of variance (ANOVA) followed by post hoc Tukey’s test). CUS, chronic unpredictable stress; C(100), curcumin (100 mg/kg); C(100), curcumin (200 mg/kg); C(400), curcumin (400 mg/kg); P(20), piperine (20 mg/kg)]

1.1.3.4 Effects of curcumin, piperine and their interaction on time spent in the target quadrant (TSTQ) in Morris water maze test

Animals exposed to chronic unpredictable stress for 28 days, significantly failed to find the location of the platform, thus spending
significantly less time in the target quadrant as compared to naive group. However, curcumin (200 and 400 mg/kg) treatment for 28 days significantly increased time spent in the target quadrant as compared to chronic unpredictable stress (CUS) control (Fig. 1.1.5.). Further, curcumin (100 mg/kg) treatment for 28 days did not show any significant effect on TSTQ as compared to control. Curcumin (100 and 200 mg/kg) treatment with piperine (20 mg/kg) for 28 days significantly increased time spent in target quadrant as compared to their effects alone in chronic unpredictable stress (CUS) treated mice (p<0.01). Further, per se treatment of curcumin (400 mg/kg) did not show any significant effect on TSTQ as compared to naïve (data not shown) (Fig. 1.1.5).

**Fig. 1.1.5 Effects of curcumin and its co-administration with piperine on time spent in target quadrant in Morris water maze.** Values are expressed as mean ± SEM. For statistical significance, a P <0.05 as compared to naive group; b P <0.05 as compared to CUS; c P <0.05 as compared to C(100)+CUS; d P <0.05 as compared to C(200)+CUS; e P <0.05 as compared to P(20)+CUS (One-way ANOVA followed by Tukey’s test). CUS, chronic unpredictable stress; C(100), curcumin (100 mg/kg); C(100), curcumin (200 mg/kg); C(400), curcumin (400 mg/kg); P(20), piperine (20 mg/kg)

1.1.3.5 Effect of curcumin, piperine and their interaction on serum corticosterone (CORT) level

Chronic unpredictable stress for 28 days significantly elevated serum corticosterone (CORT) level as compared to naive group (Fig. 1.1.6.).
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Curcumin (200 and 400 mg/kg) treatment for 28 days significantly attenuated serum CORT level which was significant as compared to chronic unpredictable stress (CUS) control. However, curcumin (100 mg/kg) treatment for 28 days did not show any significant effect on serum CORT level as compared to control. Further, co-administration of curcumin (100 and 200 mg/kg) with piperine (20 mg/kg) for 28 days significantly improved serum CORT level as compared to their effects alone in CUS treated animals (p<0.01). Further, per se effect of curcumin (400 mg/kg) did not show any significant effect on serum CORT levels as compared to naive (data not shown) (Fig. 1.1.6).

Fig. 1.1.6 Effects of curcumin and its co-administration with piperine on serum corticosterone (CORT) levels. Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to naive group; bP <0.05 as compared to CUS; cP <0.05 as compared to C(100)+CUS; dP <0.05 as compared to C(200)+CUS; eP <0.05 as compared to P(20)+CUS (One-way ANOVA followed by Tukey’s test). CUS, chronic unpredictable stress; C(100), curcumin (100 mg/kg); C(100), curcumin (200 mg/kg); C(400), curcumin (400 mg/kg); P(20), piperine (20 mg/kg)
1.1.3.6 Effect of curcumin, piperine and their interaction on oxidative-nitrosative stress

Chronic unpredictable stress (CUS) for 28 days significantly increased oxidative stress markers as evidenced by increase in lipid peroxidation (LPO), and depletion of reduced glutathione (GSH), catalase and superoxide dismutase (SOD) level as compared to naive group (p<0.05). Apart from oxidative damage, CUS caused a significant increase in nitrosative stress marker as seen by increased nitrite concentration as compared to naive group (p<0.05) (Table 1.1.1.). However, curcumin (200 and 400 mg/kg) treatment for 28 days significantly attenuated oxidative-nitrosative stress markers (reduced lipid peroxidation and nitrite levels, and restored reduced glutathione, catalase and superoxide dismutase level) which was also significant as compared to CUS control. Further, curcumin (100 mg/kg) treatment for 28 days did not produce any significant effect on oxidative and nitrosative stress (lipid peroxidation, nitrite, reduced glutathione, catalase and superoxide dismutase level) as compared to control. Also, piperine (20 mg/kg) treatment for 28 days did not produce any significant effect on oxidative and nitrosative stress level as compared to control. However, co-administration of curcumin (100 and 200 mg/kg) with piperine (20 mg/kg) for 28 days significantly potentiated their protective effect on oxidative and nitrosative stress markers (reduced lipid peroxidation and nitrite levels, and restored reduced glutathione, catalase and superoxide dismutase level) which was also significant as compared to their effects alone in CUS treated animals (p<0.05). Further, per se treatment of curcumin (400 mg/kg) did not show any significant effect on oxidative and nitrosative stress markers (lipid peroxidation, nitrite, reduced glutathione, catalase and superoxide dismutase level) as compared to naïve group (data not shown) (Table 1.1.1).
Table 1.1.1 Effects of curcumin and its co-administration with piperine on lipid peroxidation (LPO), reduced glutathione (GSH), superoxide dismutase (SOD), catalase and nitrite level

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>LPO (mole of MDA/mg pr) (% of naïve)</th>
<th>GSH (µmole of GSH/mg pr) (% of naïve)</th>
<th>SOD (units/mg pr) (% of naïve)</th>
<th>Catalase (µmole of H_2O_2/min/mg pr) (% of naïve)</th>
<th>Nitrite (µg/ml) (% of naïve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>0.159±0.006 (100)</td>
<td>0.075±0.005 (100)</td>
<td>58.23±3.51 (100)</td>
<td>0.727±0.011 (100)</td>
<td>303.3±13.21 (100)</td>
</tr>
<tr>
<td>Control (CUS)</td>
<td>0.607±0.029 (381.8)</td>
<td>0.021±0.004 (28)</td>
<td>11.72±2.12 (20.1)</td>
<td>0.195±0.032 (26.8)</td>
<td>777.8±16.26 (256.4)</td>
</tr>
<tr>
<td>C(100) +CUS</td>
<td>0.546±0.027 (343.4)</td>
<td>0.028±0.003 (37.3)</td>
<td>16.43±3.43 (28.2)</td>
<td>0.243±0.022 (100)</td>
<td>716±11.23 (236.3)</td>
</tr>
<tr>
<td>C(200) +CUS</td>
<td>0.433±0.022 (273.2)</td>
<td>0.037±0.002 (49.3)</td>
<td>31.6+2.61 (54.3)</td>
<td>0.396±0.018 (33.4)</td>
<td>566.2±10.22 (186.8)</td>
</tr>
<tr>
<td>C(400) +CUS</td>
<td>0.323±0.012 (203.1)</td>
<td>0.055±0.005 (73.3)</td>
<td>46.2±3.3 (100)</td>
<td>0.528±0.026 (72.6)</td>
<td>386.6±12.16 (126.4)</td>
</tr>
<tr>
<td>P(20) +CUS</td>
<td>0.579±0.028 (203.1)</td>
<td>0.024±0.003 (32)</td>
<td>12.16±1.98 (79.4)</td>
<td>0.188±0.044 (25.8)</td>
<td>755.6±15.79 (249.2)</td>
</tr>
<tr>
<td>C(100) + P(20) +CUS</td>
<td>0.444±0.022 (279.2)</td>
<td>0.039±0.002 (52)</td>
<td>30.3±3.11 (52.1)</td>
<td>0.387±0.066 (52.6)</td>
<td>580.8±11.17 (191.4)</td>
</tr>
<tr>
<td>C(200) + P(20) +CUS</td>
<td>0.342±0.019 (215.1)</td>
<td>0.052±0.002 (69.3)</td>
<td>45.2±4.3 (77.6)</td>
<td>0.506±0.043 (69.6)</td>
<td>395.2±12.20 (130.4)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. For statistical significance, aP < 0.05 as compared to naive group; bP < 0.05 as compared to CUS; cP < 0.05 as compared to C(100)+CUS; dP < 0.05 as compared to C(200)+CUS; eP < 0.05 as compared to P(20)+CUS (One-way ANOVA followed by Tukey’s test). CUS, chronic unpredictable stress; C(100), curcumin (100 mg/kg); C(100), curcumin (200 mg/kg); C(400), curcumin (400 mg/kg); P(20), piperine (20 mg/kg).
1.1.3.7 Effect of curcumin, piperine and their interaction on brain acetylcholinesterase (AChE) activity

Chronic unpredictable stress for 28 days significantly increased acetylcholinesterase enzyme activity as compared to the naive group (Fig. 1.1.7). Curcumin (200 and 400 mg/kg) treatment for 28 days attenuated acetylcholinesterase activity which was significant as compared to control (CUS) group. Curcumin (100 mg/kg) treatment did not show significant effect on AChE activity as compared to control (CUS). However, co-administration of curcumin (100 and 200 mg/kg) with piperine (20 mg/kg) for 28 days potentiated the protective effect (decreased AChE activity) which was significant as compared to their effects alone in CUS treated animals (p<0.05). Further, per se treatment of curcumin (400 mg/kg) did not show any significant effect on AChE activity as compared to naive (data not shown) (Fig. 1.1.7).

Fig. 1.1.7 Effects of curcumin and its co-administration with piperine on brain acetylcholinesterase activity. Values are expressed as mean ± SEM. For statistical significance, *P <0.05 as compared to naive group; **P <0.05 as compared to CUS; *P <0.05 as compared to C(100)+CUS; **P <0.05 as compared to C(200)+CUS; ***P <0.05 as compared to P(20)+CUS (One-way ANOVA followed by Tukey’s test). CUS, chronic unpredictable stress; C(100), curcumin (100 mg/kg); C(100), curcumin (200 mg/kg); C(400), curcumin (400 mg/kg); P(20), piperine (20 mg/kg)
1.1.3.8 Effects of curcumin, piperine and their interaction on mitochondrial respiratory enzyme complex activity

Chronic unpredictable stress for 28 days significantly impaired mitochondrial respiratory enzyme complex activities (complex I-II) (Fig. 1.1.8.1) and (complex III-IV) (Fig. 1.1.8.2) as compared to naive group. Curcumin (200 and 400 mg/kg) treatment for 28 days significantly restored mitochondrial enzyme complex I-IV activities as compared to control (CUS). However, co-administration of curcumin (100 and 200 mg/kg) with piperine (20 mg/kg) for 28 days significantly potentiated their protective effect as compared to their effect alone in CUS treated animals (p<0.05). Further, per se treatment of curcumin (400 mg/kg) did not show any significant effect on mitochondrial respiratory enzyme complex activities (complex I-IV) as compared to naïve (data not shown).

Fig. 1.1.8.1 Effects of curcumin and its co-administration with piperine on mitochondrial respiratory enzyme complex I and II activities. Values are expressed as mean ± SEM. For statistical significance, \(^{a}P <0.05\) as compared to naive group; \(^{b}P <0.05\) as compared to CUS; \(^{c}P <0.05\) as compared to C(100)+CUS; \(^{d}P <0.05\) as compared to C(200)+CUS; \(^{e}P <0.05\) as compared to P(20)+CUS (One-way ANOVA followed by Tukey’s test). CUS, chronic unpredictable stress; C(100), curcumin (100 mg/kg); C(100), curcumin (200 mg/kg); C(400), curcumin (400 mg/kg); P(20), piperine (20 mg/kg)
Fig. 1.1.8.2 Effects of curcumin and its co-administration with piperine on mitochondrial respiratory enzyme complex III and IV activities. Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to naive group; bP <0.05 as compared to CUS; cP <0.05 as compared to C(100)+CUS; dP <0.05 as compared to C(200)+CUS; eP <0.05 as compared to P(20)+CUS (One-way ANOVA followed by Tukey's test). CUS, chronic unpredictable stress; C(100), curcumin (100 mg/kg); C(100), curcumin (200 mg/kg); C(400), curcumin (400 mg/kg); P(20), piperine (20 mg/kg)

1.1.4 DISCUSSION

There seem to be a complex relationship between stressful situations, mind and body's reaction to stress, and the onset of cognitive disturbances (Bhutani et al., 2009). Chronic administration of various uncontrollable stresses, a procedure known as chronic unpredictable stress, is generally thought to be a most reliable and valuable experimental model to study stress pathology (Willner et al., 1992). Chronic unpredictable stress (CUS) has been shown to influence brain regions which play a critical role in spatial navigation and memory (Churchwell et al., 2010). Thus in the present study, curcumin and its interaction with piperine has been tried as a drug strategy against chronic unpredictable stress induced oxidative damage and cognitive deficits in mice.
In the present study, memory performance was evaluated by Morris water maze (MWM) as well as elevated plus maze (EPM). Though elevated plus maze test is primarily used for anxiety, it can also be employed as an experimental model for evaluation of long term memory in rodents (Sharma and Kulkarni, 1992). In the present study, chronic unpredictable stress resulted in a significant impairment of cognitive performance in both Morris water maze and elevated plus maze tests as compared to naïve animals. These results are consistent with the previous finding (Hoffman et al., 2011). Curcumin treatment for 28 days significantly improved cognitive performance in both MWM and EPM indicating its therapeutic potential against chronic stress induced memory impairment. These results are in line with the previous findings from our laboratory (Kumar et al., 2009). Along with the cognitive deficits, there was a significant decrease in locomotor activity in CUS control animals as compared to naïve group. The results are in accordance with previous studies by Gronli and its group (Gronli et al., 2005) which showed a significant decrease in locomotor activity following chronic mild stress. Further, curcumin treatment significantly improved locomotor activity, suggesting its therapeutic potential against chronic stress. These results are similar to the previous report from our laboratory (Kumar and Singh, 2008).

Hippocampus has been well known to play a key role in spatial learning and memory (Bai et al., 2009). Since hippocampus has abundant inputs from the basal forebrain cholinergic system and thus acetylcholine (ACh) plays a crucial role in learning and memory (Prado et al., 2006). Acetylcholine is degraded by the enzyme acetylcholinesterase, terminating the physiological action of the neurotransmitter. Cognitive dysfunction affects cholinergic system resulting in increased activity of acetylcholinesterase (Dai et al., 2002). Stress has been well documented to induce alterations in acetylcholinesterase enzyme activity (Nijholt et al., 2004). In the present study, CUS caused a significant increase in acetylcholinesterase activity leading to memory deficits, but later was significantly attenuated by chronic curcumin treatment, implicating its role in cholinergic transmission processes. These results are in line with the earlier report from our laboratory (Kumar et al., 2009).
In addition to the behavioral abnormalities, chronic stress has also been reported to activate hypothalamic–pituitary–adrenal (HPA) axis (Landfield et al., 2007). A central feature of the HPA stress response is the synthesis and secretion of glucocorticoids (corticosterone in mice) from the adrenal cortex. Additionally, glucocorticoids secreted during stressful events are known to influence memory consolidation and retrieval (Roozendaal, 2002). In the present investigation, CUS animals showed a significant increase in serum corticosterone level as compared to naive group. However, curcumin treatment significantly attenuated serum corticosterone level, resulting in the HPA axis normalization. These results are also consistent with the previous report showing an increased corticosterone level against chronic unpredictable stress can be prevented by curcumin treatment (Li et al., 2009).

Corticosterone administration is also known to promote oxidative stress and consequently causes memory deficits (Sato et al., 2010). The role of oxygen free radicals in neurodegeneration and cognitive decline has been well described (Serrano and Klann, 2004). Earlier finding suggest that reactive oxygen species (ROS) can accumulate excessively in brain and can attenuate neuronal functions (Massaad and Klann, 2011). Oxidative stress is therefore implicated as one of the causes of cognitive dysfunction (Keller et al., 2005). Besides, chronic stress is said to promote oxidative stress and weaken antioxidant defense system in the brain (Lucca et al., 2009), responsible for impaired memory performance. In the present investigation, CUS resulted in significant oxidative damage as indicated by increase in lipid peroxidation, nitrite concentration, and depletion of reduced glutathione, catalase and superoxide dismutase levels. Curcumin being a lipophilic molecule is known to possess strong antioxidant activity (Bengmark, 2006). Curcumin is reported to inhibit iron-induced lipid peroxidation (Reddy and Lokesh, 1994), iNOS expression (Bengmark, 2006) and specifically scavenge NO-based radicals (Sreejavan and Rao, 1997). Curcumin is known to enhance the reduced glutathione levels in ethanol intoxicated animals (Rajkrishnan et al., 1999). It has been reported in literature that curcumin increases the level of SOD and catalase in irradiated mice (Koiram et al., 2007). In line with the above...
correlates, curcumin in the present study significantly attenuated these oxidative-nitrosative stress markers.

Generation of reactive oxygen species (ROS) may be associated with mitochondrial dysfunction since mitochondrial respiratory chain is the major sources of superoxide anion (O2⁻) generation (Jezek and Hlavatá, 2005). Since the energy production in mitochondria is catalysed by various membrane bound protein complexes, namely NADH-ubiquinol oxidoreductase (complex-I), succinate-ubiquinol oxidoreductase (complex-II), ubiquinol cytochrome C oxidoreductase (complex-III) and cytochrome C oxidase (complex IV) (Jezek and Hlavatá, 2005), thus imbalance in these mitochondrial enzymes may lead to severe oxidative damage. Further, mitochondria impairment may also result in Ca²⁺ dysregulation and activation of NOS. Nitric oxide (NO) and superoxide radical may react to form peroxynitrite which leads to oxidative damage in mitochondria (Clementi et al., 1998). The results of the present study indicate that CUS caused significant impairment in mitochondrial enzyme complex activities (I-IV) which is restored by curcumin treatment, suggesting a potential role for curcumin in restoring mitochondrial enzyme complex enzymes. Thus, the results of the present study support our hypothesis that memory deficits observed after CUS might have arisen as a result of mitochondria dysfunction, which is the key factor for the production of ROS generation and ultimately causing oxidative injury to neurons, which could therefore be prevented by antioxidant treatment.

Poor oral bioavailability of curcumin is known to limit its therapeutic efficacy. Earlier report has shown that curcumin gets reduced through alcohol dehydrogenase, followed by conjugations like sulfation and glucuronidation in liver and intestine (Wahlstrom and Blennow, 1978). Thus high concentrations of curcumin cannot be achieved and maintained in plasma after oral ingestion. Recent clinical reports suggest that only a small fraction of ingested curcumin reaches the plasma level in patients thereby showing its poor oral bioavailability (Baum et al., 2008; Mancuso et al., 2011). Therefore, one of strategy to overcome the poor oral bioavailability of curcumin involves use of bioavailability enhancers which could potentiate the bioavailability of curcumin. In the present study, co-administration of piperine with curcumin
enhanced its oral bioavailability. Piperine is a potent inhibitor of hepatic and intestinal glucuronidation (Atal et al., 1985), thus co-administration of piperine with curcumin prevents intestinal and hepatic metabolism of curcumin, thereby increased free form of native curcumin, responsible for its protective effect. In the present study, combination of curcumin and piperine significantly improved their locomotor activity and cognitive performance, along with attenuation of serum CORT, oxidative-nitrosative stress and mitochondrial enzyme complex alterations in CUS treated mice.

The present study clearly demonstrates the antioxidant and antiinflammatory properties of curcumin due to its multifactorial nature, which further shows elevated effects on co-administration with piperine, a known bioavailability enhancer (Fig. 1.1.9). Further these findings provide a scientific rationale for the co-administration of piperine and curcumin, which may act as a useful and potent adjuvant in the treatment of stress induced cognitive disorders.

![Possible mechanism of action of curcumin and its interaction with piperine against chronic unpredictable stress induced cognitive deficits](image-url)
1.2.1 INTRODUCTION

Stress is a major risk factor for the development of various types of neurological diseases, ranging from cardiovascular disorders to mental illness. Stress exposure induces several changes at multiple neural systems, among which activation of the hypothalamo-pituitary-adrenal (HPA) axis is considered to be critical (Shansky and Lipps, 2013). HPA axis activation provokes corticosterone secretion which further triggers the release of various oxidants involved in the impairment of cognitive performance (Sato et al., 2010). Brain neuronal functions are also altered by generation of reactive oxygen species which promotes oxidative stress (Halliwell and Gutteridge, 1985). The hippocampus region of the brain regulates cholinergic transmission responsible for learning and memory performance, and is also more prone to the oxidative damage (Epp et al., 2013). Chronic stress also activates microglia, resulting into neuroinflammation and cognitive dysfunctions (Farooq et al., 2012). Animals exposed to chronic stress shows an impairment of spatial learning and memory in different behavioural paradigms (Bian et al., 2012). Among numerous animal models of stress, chronic unpredictable stress is the most exploited and useful experimental model to study stress pathology in animals (Willner, 2007).

Nitric oxide (NO), an intracellular messenger of the brain is known to play a crucial role in various physiological and pathological processes of the body (Cahuana et al., 2004). It is a short-lived, lipophilic molecule generated from L-arginine by nitric oxide synthase (NOS) and has an important function in learning and memory processes (Bouladakis et al., 2010). NO mediated nitrergic signalling is a vital regulatory cascade of nervous system which plays an important role in brain homeostasis. Reactive nitrogen species (RNS) generated from NO leads to nitration of proteins (Guimaraes et al., 2005) and promotes carbonylation (Cahuana et al., 2004). The ability of nitric oxide to
exert cellular damage due to its reactive oxidative properties is perhaps its main neurotoxic mechanism. Oxidative stress and Nitric oxide (NO) have been proposed to interplay in neurodegenerative disorders; however, exact cellular cascades are not understood so far. This encourages researchers to further evaluate new treatment strategies against this disorder.

American ginseng (AG) (family Araliaceae), is widely consumed species of ginseng in United States for its potential effects to treat several neurodegenerative disorders. It is widely used as a nerve tonic and proved beneficial in the treatment of amnesia (Scholey et al., 2010). Ginsenosides are the saponin constituents of this herb which are responsible for enhancement of cognitive functions (Scholey et al., 2010). The antistress and neuroprotective effects ginsenosides are well documented in several studies (Lee et al., 2006; Lian et al., 2005). Ginsenosides are known to inhibit lipid peroxidation and possess antioxidant and free radical scavenging properties (Li et al., 1999). Sheikh and its group (Sheikh et al., 2007) have shown that treatment with ginsenosides restores stress induced alterations in plasma corticosterone levels. All these results explain the neuroprotective potential of ginsenosides; however, its exact cellular or molecular mechanism has not been understood so far.

With this background, the present study was designed to elucidate the possible protective effects of American ginseng against chronic unpredictable stress (CUS) induced cognitive deficits and oxidative stress and to explore the possible involvement of nitric oxide signalling pathway in the protective effects of American ginseng.

1.2.2 MATERIALS AND METHODS

1.2.2.1 Animals

Male Laca mice (25–30 g) bred at Central Animal House (CAH) Panjab University, Chandigarh, were used. They were housed under standard laboratory conditions (25±2°C, 60–70% humidity), maintained on a 12 hour natural day–night cycle, with free access to standard food and water. Animals were acclimatized to laboratory conditions before the test. The experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC)
of Panjab University (IAEC/346-356/UIPS/2) and conducted according to the CPCSEA guidelines on the use and care of experimental animals.

1.2.2.2 Chronic unpredictable stress

As per section (1.1.2.2)

1.2.2.3 Drugs and treatment schedule

American ginseng extract, L-NAME and L-Arginine were purchased from Sigma chemicals Co. (St. Louis, MO, USA). All other chemicals used for biochemical and mitochondrial estimations were of analytical grade. The animals were randomly divided into nine experimental groups (n=6-8) viz Group 1: naïve animals with no stress; Group 2: control animals received chronic unpredictable stress (CUS) along with an equivalent volume of vehicle; Group 3-5: CUS treated animals received American ginseng (AG) (50, 100 and 200 mg/kg; p.o.) respectively; Group 6-7: CUS treated animals received L-NAME (10 mg/kg; i.p.) and L-arginine (100 mg/kg; i.p.) respectively; Group 8-9: CUS treated animals received pre-treatment of L-NAME (10 mg/kg; i.p.) and L-arginine (100 mg/kg; i.p.) with AG (100 mg/kg; p.o.) respectively. American ginseng (AG) extract was prepared in peanut oil whereas L-NAME and L-arginine was dissolved in normal saline and administered orally (30 min before AG treatment) on the basis of body weight (1 ml/100 g). Solutions were made freshly at the beginning of each day of the drug treatment. Drugs were administered daily 30 minutes before CUS procedure for 28 days.

1.2.2.4 Behavioral assessments

1.2.2.4.1 Assessment of locomotor activity (actophotometer test)

As per section (1.1.2.4.1)

1.2.2.4.2 Elevated plus maze paradigm

As per section (1.1.2.4.2)

1.2.2.4.3 Morris water-maze test

As per section (1.1.2.4.3)
1.2.2.5 Serum corticosterone estimations

1.2.2.5.1 Preparation of serum
As per section (1.1.2.5.1)

1.2.2.5.2 Corticosterone assessment
As per section (1.1.2.5.2)

1.2.2.6 Biochemical assessments
As per section (1.1.2.6)

1.2.2.6.1 Assessment of oxidative stress
As per section (1.1.2.6.1)

1.2.2.6.1.1 Estimation of lipid peroxidation
As per section (1.1.2.6.1.1)

1.2.2.6.1.2 Estimation of reduced glutathione
As per section (1.1.2.6.1.2)

1.2.2.6.1.3 Estimation of superoxide dismutase
As per section (1.1.2.6.1.3)

1.2.2.6.1.4 Estimation of catalase
As per section (1.1.2.6.1.4)

1.2.2.6.1.5 Protein estimation
As per section (1.1.2.6.1.5)

1.2.2.6.2 Assessment of nitrosative stress
As per section (1.1.2.6.2)

1.2.2.6.3 Estimation of acetyl cholinesterase (AChE) activity
As per section (1.1.2.6.3)

1.2.2.7 Mitochondrial complex estimation

1.2.2.7.1 Isolation of mitochondria
As per section (1.1.2.7.1)
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1.2.2.7.2 Complex-I (NADH Dehydrogenase activity)
As per section (1.1.2.7.2)

1.2.2.7.3 Complex-II (Succinate Dehydrogenase (SDH) activity)
As per section (1.1.2.7.3)

1.2.2.7.4 Complex-III (MTT ability)
As per section (1.1.2.7.4)

1.2.2.7.5 Complex-IV (Cytochrome oxidase assay)
As per section (1.1.2.7.5)

1.2.2.8 Statistical analysis
As per section (1.1.2.8)

1.2.3 RESULTS

1.2.3.1 Effects of American ginseng (AG) and its pretreatment with nitric oxide modulators on locomotor activity

28 days chronic unpredictable stress significantly impaired locomotor activity as compared to naïve group, thereby showing the chronic stress induced impaired locomotion (Fig. 1.2.1). Treatment with AG (100 and 200 mg/kg) for 28 days significantly improved locomotor activity as compared to control (CUS) group. However, AG (50 mg/kg) treatment did not show any significant effect on locomotor activity as compared to control. However, L-NAME (NOS inhibitor) (10 mg/kg) pretreatment with American ginseng (AG) (100 mg/kg) for 28 days significantly potentiated their protective effects which was also significant as compared to their effects alone (P<0.05). Further, L-arginine (nitric oxide donor) (100 mg/kg) pretreatment with subeffective dose of American ginseng (AG) (100 mg/kg) significantly reversed the protective effect of American ginseng (AG) in CUS treated animals (P<0.05). Further, per se effect of American ginseng (AG) (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) did not show any significant effect on locomotor activity as compared to naïve (data not shown) (Fig. 1.2.1).
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Fig. 1.2.1 Effect of American ginseng (AG) and its pretreatment with nitric oxide modulators on locomotor activity. Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to naive group; bP <0.05 as compared to CUS; cP <0.05 as compared to AG(50)+CUS; dP <0.05 as compared to AG(100)+CUS; eP <0.05 as compared to L-NAME(10)+CUS (One-way ANOVA followed by Tukey’s test). CUS, chronic unpredictable stress; AG, American ginseng; L-ARG, L-arginine.

1.2.3.2 Effects of American ginseng (AG) and its pretreatment with nitric oxide modulators on transfer latency in elevated plus maze (EPM) test

Initial transfer latency (ITL) did not show any significant variations in any of the groups. Following training, transfer latency (1st RTL and 2nd RTL) of CUS group significantly (P<0.05) increased as compared to naive, demonstrating stress-induced memory impairment. Treatment with AG (100, 200 mg/kg) significantly lowered RTL as compared to CUS (P<0.05). AG (50 mg/kg) did not show any significant effect on RTL as compared to control. L-arginine (100 mg/kg) pretreatment with subeffective dose of AG (100 mg/kg) significantly (P<0.05) reversed the protective effect of AG. However, L-NAME
(10 mg/kg) pretreatment with AG (100 mg/kg) significantly (P<0.05) potentiated their protective effects which was also significant as compared to their effects alone. Further, per se effect of AG (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) for 28 days did not show any significant effect on latency time as compared to naïve (data not shown) (Fig. 1.2.2).

Fig. 1.2.2 Effect of American ginseng (AG) and its pretreatment with nitric oxide modulators on latency time in elevated plus maze test. Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to naive group; bP <0.05 as compared to CUS; cP <0.05 as compared to AG(50)+CUS; dP <0.05 as compared to AG(100)+CUS; eP <0.05 as compared to L-NAME(10) + CUS (One-way ANOVA followed by Tukey’s test). CUS, chronic unpredictable stress; AG, American ginseng; L-ARG, L-arginine.

1.2.3.3 Effects of American ginseng (AG) and its pretreatment with nitric oxide modulators on escape latency time (ELT) in Morris water maze test

The escape latency to reach the submerged platform decreased gradually in all the groups during four days of acquisition trials in Morris water maze test. However, the mean escape latency (days 24–27) of CUS treated group was significantly (P<0.05) prolonged as compared to naive group, indicating a poor cognitive performance (Fig. 1.2.3.). AG (100, 200 mg/kg) treatment for 28 days significantly (P<0.05) improved the escape latency time.
as compared to control (CUS). L-NAME (10 mg/kg) pretreatment with AG (100 mg/kg) significantly potentiated their protective effects which was also significant as compared to their effects alone (P<0.05). However, L-arginine (100 mg/kg) pretreatment with subeffective dose of AG (100 mg/kg) significantly reversed the protective effect of AG in CUS treated group (P<0.05). Further, per se effect of AG (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) did not show any significant effect on escape latency time as compared to naïve (data not shown) (Fig. 1.2.3).

Fig. 1.2.3 Effect of American ginseng (AG) and its pretreatment with nitric oxide modulators on escape latency time in Morris water maze. Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to naive group; bP <0.05 as compared to CUS; cP <0.05 as compared to AG(50)+CUS; dP <0.05 as compared to AG(100)+CUS; eP <0.05 as compared to L-NAME(10) + CUS (One-way ANOVA followed by Tukey’s test). CUS, chronic unpredictable stress; AG, American ginseng; L-ARG, L-arginine.
1.2.3.4 Effect of American ginseng (AG) and its pretreatment with nitric oxide modulators on time spent in target quadrant (TSTQ) in Morris water maze test

During retention trial (day 28), CUS treated animals spent significantly (P<0.01) less time in the target quadrant as compared to naive group. AG (100, 200 mg/kg) treatment for 28 days significantly (P<0.01) increased TSTQ as compared to CUS group (Fig. 1.2.4). Further, L-NAME (10 mg/kg) pretreatment with subeffective dose of AG (100 mg/kg) for 28 days significantly (P<0.01) increased TSTQ which was also significant as compared to their effects alone. However, L-arginine (100 mg/kg) pretreatment significantly (P<0.01) reversed the protective effects of AG (100 mg/kg) in CUS treated group. Further, per se effect of AG (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) did not show any significant effect on TSTQ as compared to naïve (data not shown) (Fig. 1.2.4).
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1.2.3.5 Effects of American ginseng (AG) and its pretreatment with nitric oxide modulators on serum corticosterone (CORT)

CUS treatment for 28 days significantly (P<0.05) increased the serum CORT level as compared to naive group. AG (100, 200 mg/kg) treatment for 28 days significantly (P<0.05) attenuated serum CORT levels as compared to CUS control (Fig. 1.2.5). Further, L-NAME (10 mg/kg) pretreatment with AG (100 mg/kg) for 28 days significantly (P<0.05) attenuated serum CORT level which was also significant as compared to their effects alone. However, L-arginine (100 mg/kg) pretreatment with subeffective dose of AG (100 mg/kg) significantly (P<0.05) reversed the protective effect of AG in CUS treated group. Further, per se effect of AG (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) did not show any significant effect on serum CORT level as compared to naive (data not shown) (Fig. 1.2.5)

Fig. 1.2.5 Effect of American ginseng (AG) and its pretreatment with nitric oxide modulators on serum corticosterone. Values are expressed as mean ± SEM. For statistical significance,  aP <0.05 as compared to naive group; bP<0.05 as compared to CUS; cP <0.05 as compared to AG(50) + CUS; dP <0.05 as compared to AG(100) + CUS; eP <0.05 as compared to L-NAME(10) + CUS (One-way ANOVA followed by Tukey's test). CUS, chronic unpredictable stress; AG, American ginseng; L-ARG, L-arginine.
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1.2.3.6. Effects of American ginseng (AG) and its pretreatment with nitric oxide modulators on oxidative-nitrosative stress markers

Chronic unpredictable stress (CUS) for 28 days significantly (P<0.05) increased oxidative stress markers as evidenced by increase in lipid peroxidation (LPO), and depletion of reduced glutathione (GSH), catalase and superoxide dismutase (SOD) level as compared to naive group (p<0.05). Apart from oxidative damage, Chronic unpredictable stress (CUS) caused a significant increase in nitrosative stress marker as seen by increased nitrite concentration as compared to naive group (p<0.05) (Table 2.2.1). Chronic treatment with American ginseng (AG) (100, 200 mg/kg) for 28 days significantly (P<0.05) attenuated oxidative-nitrosative stress markers (reduced LPO, nitrite levels, restoration of reduced GSH, SOD and catalase levels) as compared to Chronic unpredictable stress (CUS) group. American ginseng (AG) (50 mg/kg) did not show any significant effect on oxidative-nitrosative stress (Lipid peroxidation, nitrite, reduced glutathione, catalase and superoxide dismutase level) as compared to Chronic unpredictable stress (CUS) group. However, pretreatment of L-NAME (10 mg/kg) with American ginseng (AG) (100 mg/kg) for 28 days significantly potentiated their antioxidant like effect (reduced LPO, nitrite levels, and restoration of reduced GSH, SOD and catalase levels) which was also significant as compared to their effects alone. Further, L-arginine (100 mg/kg) pretreatment with subeffective dose of American ginseng (AG) (100 mg/kg) significantly (P<0.05) reversed the protective effect of American ginseng (AG) (100 mg/kg) in Chronic unpredictable stress (CUS) animals. In addition, per se treatment of American ginseng (AG) (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) did not show any significant effect on oxidative-nitrosative stress markers (Lipid peroxidation, nitrite, reduced glutathione, catalase and superoxide dismutase level) as compared to naive (data not shown) (Table 1.2.1)
Table 1.2.1 Effect of American ginseng (AG) and its pretreatment with nitric oxide modulators on lipid peroxide, reduced glutathione, superoxide dismutase (SOD), catalase and nitrite level

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>LPO (mole of MDA/mg pr) (% of naive)</th>
<th>GSH (µmole of GSH/mg pr) (% of naive)</th>
<th>SOD (units/mg pr) (% of naive)</th>
<th>Catalase (µmole of H₂O₂/min/mg pr) (% of naive)</th>
<th>Nitrite (µg/ml) (% of naive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>0.184±0.015 (100)</td>
<td>0.083±0.004 (100)</td>
<td>78.36±3.22 (100)</td>
<td>0.742±0.013 (100)</td>
<td>294.2±10.42 (100)</td>
</tr>
<tr>
<td>CUS</td>
<td>0.589±0.020 (320.1)</td>
<td>0.020±0.002 (24.1)</td>
<td>14.72±2.42 (18.8)</td>
<td>0.158±0.022 (21.3)</td>
<td>734.7±14.57 (249.7)</td>
</tr>
<tr>
<td>AG(50)+CUS</td>
<td>0.524±0.012 (284.8)</td>
<td>0.032±0.003 (38.6)</td>
<td>20.40±4.12 (25.6)</td>
<td>0.204±0.018 (27.5)</td>
<td>699.6±11.34 (237.7)</td>
</tr>
<tr>
<td>AG(100)+CUS</td>
<td>0.412±0.021 (223.9)</td>
<td>0.049±0.004 (45.3)</td>
<td>38.88±2.12 (50.1)</td>
<td>0.336±0.020 (191.2)</td>
<td>562±12.32 (191.2)</td>
</tr>
<tr>
<td>AG(200)+CUS</td>
<td>0.257±0.015 (139.7)</td>
<td>0.068±0.002 (59.1)</td>
<td>61.82±2.10 (78.9)</td>
<td>0.535±0.046 (147.9)</td>
<td>435.5±16.30 (147.9)</td>
</tr>
<tr>
<td>L-NAME(10)+CUS</td>
<td>0.561±0.012 (304.9)</td>
<td>0.024±0.005 (28.9)</td>
<td>13.87±2.98 (17.7)</td>
<td>0.168±0.034 (22.6)</td>
<td>729.6±15.46 (247.9)</td>
</tr>
<tr>
<td>L-Arg(100)+CUS</td>
<td>0.570±0.019 (309.8)</td>
<td>0.022±0.004 (26.5)</td>
<td>14.72±2.47 (18.8)</td>
<td>0.153±0.042 (20.6)</td>
<td>733.8±11.20 (249.3)</td>
</tr>
<tr>
<td>L-NAME(10)+AG(100)+CUS</td>
<td>0.282±0.022 (153.3)</td>
<td>0.064±0.003 (77.1)</td>
<td>59.87±4.12 (76.4)</td>
<td>0.518±0.024 (152.7)</td>
<td>449.6±10.32 (152.7)</td>
</tr>
<tr>
<td>L-Arg(100) AG(100)+CUS</td>
<td>0.537±0.010 (291.8)</td>
<td>0.031±0.005 (37.3)</td>
<td>21.61±2.57 (27.6)</td>
<td>0.228±0.026 (30.7)</td>
<td>680.1±14.32 (231.3)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to naive group; bP <0.05 as compared to CUS; cP <0.05 as compared to AG(50)+CUS; dP <0.05 as compared to AG(100)+CUS; eP <0.05 as compared to L-NAME(10)+CUS (One-way ANOVA followed by Tukey’s test). CUS, chronic unpredictable stress; AG, American ginseng; L-ARG, L-arginine.
1.2.3.7 Effects of American ginseng (AG) and its pretreatment with nitric oxide modulators on brain acetylcholinesterase (AChE) activity

Acetylcholinesterase (AChE) enzyme activity was significantly increased after 28 days of chronic unpredictable stress in CUS treated animals as compared to the naive group. AG (100, 200 mg/kg) treatment for 28 days significantly restored AChE activity as compared to CUS group. Further, pretreatment of L-NAME (10 mg/kg) with subeffective dose of AG (100 mg/kg) potentiated their protective effects as compared to their effects alone. However, L-arginine (100 mg/kg) pretreatment with AG (100 mg/kg) significantly (P<0.01) reversed the protective effect of AG. In addition, per se effect of AG (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) did not show any significant effect on AChE activity as compared to naive group (data not shown) (Fig. 1.2.6)
1.2.3.8 Effects of American ginseng (AG) and its pretreatment with nitric oxide modulators on mitochondrial respiratory enzyme complex activity

CUS for 28 days significantly (P<0.05) impaired mitochondrial NADH dehydrogenase (complex I), Succinate dehydrogenase (complex II) (Fig. 1.2.7.1), number of viable cells (complex III) and cytochrome C oxidase enzyme (complex IV) activity (Fig. 1.2.7.2) as compared to naive group. Treatment with AG (100, 200 mg/kg) significantly restored mitochondrial enzyme complex as compared to control. Further, pretreatment of L-NAME (10 mg/kg) with AG (100 mg/kg) significantly potentiated their protective effect; however, L-arginine (100 mg/kg) significantly (P<0.05) reversed the protective effect of AG. In addition, per se effect of AG (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) also not show any significant effect as compared to naïve (data not shown) (Fig. 1.2.7)

Fig. 1.2.7.1 Effect of American ginseng (AG) and its pretreatment with nitric oxide modulators on mitochondrial respiratory enzyme complex II and III activities. Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to naive group; bP <0.05 as compared to CUS; cP <0.05 as compared to AG(50)+CUS; dP <0.05 as compared to AG(100)+CUS; eP <0.05 as compared to L-NAME(10) + CUS (One-way ANOVA followed by Tukey’s test).
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**DISCUSSION**

The present study highlights that chronic unpredictable stress caused a significant decrease in locomotor activity along with poor performance in cognitive tasks (increased latency time in elevated plus maze and increased escape latency time and decreased time spent in target quadrant in Morris water maze). This was followed by increased oxidative-nitrosative stress markers, elevated serum corticosterone levels and alterations in mitochondrial enzyme complexes. Chronic treatment with American ginseng (AG) for 28 days significantly improved the cognitive performance in elevated plus maze and Morris water maze. In addition to maintenance of cognitive functions, a normalizing of oxidative-nitricgic stress and serum corticosterone level has also been observed in AG treated animals. On the other hand, L-NAME (non

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**Fig. 1.2.7.2 Effect of American ginseng (AG) and its pretreatment with nitric oxide modulators on mitochondrial respiratory enzyme complex III and IV activities.** Values are expressed as mean ± SEM. For statistical significance, *a* P < 0.05 as compared to naive group; *b* P < 0.05 as compared to CUS; *c* P < 0.05 as compared to AG(50)+CUS; *d* P < 0.05 as compared to AG(100)+CUS; *e* P < 0.05 as compared to L-NAME(10) + CUS (One-way ANOVA followed by Tukey’s test). CUS, chronic unpredictable stress; AG, American ginseng; L-ARG, L-arginine.
specific NOS inhibitor) pretreatment significantly potentiated the beneficial effects of AG, while L-arginine (NO donor) reversed the protective effects of AG suggesting the involvement of nitric oxide signaling pathway in protective effects of AG against chronic unpredictable stress induced behavioral, biochemical and mitochondrial alterations.

Stressful life events are common risk factors associated with cognitive disturbances. In the present study, animals were treated with a number of variable stressors, a procedure called as chronic unpredictable stress (CUS) that follows a set of repeated exposure of different types of mild stressors (e.g. forced swimming, food or water deprivation, wet bedding, over night illuminations, cage tilting etc) for several weeks along with treatment with vehicle or drugs (Willner, 2007). In the present study, cognitive functions were measured with the help of Morris water maze (MWM) and elevated plus maze (EPM) test. These tests are often used as complementary to each other in assessing memory behaviour. Though elevated plus maze test is primarily used for anxiety, it can also be employed as an experimental model for evaluation of long term memory in rodents (Sharma and Kulkarni, 1992). We found that chronic unpredictable stress paradigm resulted in significant impairment of spatial learning and memory in both Morris water maze (MWM) and elevated plus maze (EPM) task. These results are consistent with the previous finding from other laboratory (Hoffman et al., 2011). Treatment with AG significantly improved cognitive performance in both these behavioral paradigms. These findings are in line with the earlier reports from Chatterjee and its group (Chatterjee et al., 2012) who showed that American ginseng blocked ketamine induced memory impairment in passive avoidance paradigm.

Apart from the memory dysfunction, there was a significant decrease in locomotor activity in CUS treated animals as compared to naïve group. The results are in accordance with previous study by Gronli and its group (Gronli et al., 2005) which showed a significant decrease in locomotor activity following chronic mild stress. Further, in the present study ginseng treatment
for 28 days significantly improved locomotor activity in CUS treated animals suggesting its anti-stress like effects. In the present study, corticosterone levels were also measured since stress is known to activate the release of glucocorticoid at various points on the hypothalamic–pituitary–adrenal (HPA) axis, which has also been seen in stressed patients suffering from cognitive dysfunction (Landfield et al., 2007). Secretion of glucocorticoids during stressful events is known to influence memory consolidation and retrieval (Roozendaal, 2002). Similarly, in the current study, there was a significant rise in serum corticosterone levels in animals subjected to chronic unpredictable stress. Treatment with AG for 28 days significantly attenuated the increased levels of serum corticosterone and the data is supported by findings from Xu and its group (Xu et al., 2010).

Rise in the levels of corticosterone is known to participate actively in the generation of oxidative stress which further leads to memory dysfunction (Sato et al., 2010). Oxidative stress is therefore implicated as one of the causes of cognitive impairment (Keller et al., 2005). In the present study, chronic unpredictable stress caused significant elevation in oxidative stress (as indicated by rise in lipid peroxidation, nitrite concentration, and depletion of reduced glutathione levels, catalase and superoxide dismutase activity), thus strengthening the oxidative hypothesis of cognitive deficits. Generation of free radicals is also critically involved in neuroinflammatory injury (Moreira et al., 2006) which may be responsible for causing deficits in cognitive functions. Chronic treatment with AG for 28 days significantly reversed chronic unpredictable stress induced alterations in the level of antioxidants enzymes along with attenuation of enhanced nitrite level. These observations are supported by earlier study which demonstrated anti-oxidant and anti-inflammatory effects of ginsenosides (Bae et al., 2009).

Mitochondria are the major source of reactive oxygen species (ROS) production in the cells (Reddy and Beal, 2005). Mitochondria dysfunction is known to generate superoxide anion, hydrogen peroxide (H$_2$O$_2$) and hydroxyl free radical (OH) which accelerates neurodegenerative process (Zeevalk et
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Mitochondria impairment may also lead to Ca\(^{2+}\) dysregulation and activation of nitric oxide synthase (NOS) which further causes oxidative damage (Clementi et al., 1998). Several findings propose that reactive oxygen species (ROS) can accumulate excessively in the brain and attenuate neuronal functions (Halliwell and Gutteridge, 1985). The energy production in mitochondria is catalysed by membrane bound protein complexes, namely NADH-ubiquinol oxidoreductase (complex-I) and succinate-ubiquinol oxidoreductase (complex-II) (Ježek and Hlavatá, 2005), thus imbalance's both these enzymes that may leads to oxidative damage. In the present study, chronic unpredictable stress exposure caused dysfunction of mitochondrial enzyme complex activities (I-IV) leading to generation of ROS in mitochondria and causing oxidative damage. These findings are in continuation with the recent report from our laboratory demonstrating mitochondrial dysfunction induced by chronic unpredictable stress model in mice (Rinwa and Kumar, 2012). Further, in the present study, American ginseng (AG) administration significantly restored the alterations in the mitochondrial enzyme complex activities (i.e. mitochondrial NADH dehydrogenase; complex I, Succinate dehydrogenase; complex II, number of viable cells; complex III and cytochrome C oxidase enzyme; complex IV activity), thereby inhibiting generation of reactive oxygen species (ROS) and attenuating oxidative injury to the brain neurons.

Acetylcholine (ACh) is an essential neurotransmitter involved in the process of learning and memory. Acetylcholine is degraded by the enzyme acetylcholinesterase (AChE), which degrades ACh and thus terminates its physiological action in the synapse. Moreover, AChE has been well documented to induce apoptotic cell death cascade in the mature brain (Greenfield and Vaux, 2002). Stress is known to cause alterations in the AChE activity (Nijholt et al., 2004). Similarly, in the present study, chronic unpredictable stress caused a significant increase in acetylcholinesterase (AChE) activity responsible for poor cognitive performance. Further, in the present study, AG treatment for 28 days significantly attenuated AChE activity.
thereby showing improved cognitive performance in both the test paradigms. These results are in line with the earlier reports from Lee and its group (Lee et al., 2006) who proposed memory restorative effects of ginsenosides against enhanced AChE levels in scopolamine-induced memory impairment model of mice.

Nitric oxide (NO) is a known neuromodulator which play a critical role in the LTP formation. Role of LTP is well known in memory and cognitive performance (Gourgiotis et al., 2012). NO alone is not toxic but on combination with superoxide anions, it gets converted to peroxynitrite (ONOO−), a highly destructive radical moiety (Beckman and Koppenol, 1996). The resultant reactive species produces functional alterations in proteins, lipids and nucleic acids, which in turn may lead to neurodegeneration and apoptotic cell death (Beckman et al., 1994). Peroxynitrite can also target mitochondrial respiratory enzymes and causes mitochondrial dysfunction and generation of reactive oxygen species (Pall, 2000). Two isoforms of NOS, nNOS and eNOS, are expressed in different regions of brain which are responsible for the deficits in cognitive behaviour (Susswein et al., 2004). Increased expression of NOS can cause excitotoxicity in NMDA receptors, which would further initiate the process of inflammatory cascade and production of reactive oxygen species (Contestabile, 2003). Activated microglial promotes inducible nitric oxide synthase (iNOS) mediated neuronal injury through the release of NO (Graeber and Streit, 2010). All these different ways of escalating NO production would lead to increase in free radical generation, causing cell death. Study has reported nitric oxide and peroxynitrite mediated neuronal damage in several neurological diseases including cognitive disorders (Chabrier et al., 1999). Similarly, in our present study, pretreatment of L-arginine (a nitric oxide donor) with sub-effective dose of AG attenuated its protective effect. However, L-NAME (a non selective inhibitor of NOS) pretreatment potentiated its protective effect. Failure of protection by L-arginine suggests that increasing levels of nitric oxide enhanced nitrergic signaling mechanism and thus caused behavioral,
biochemical and mitochondrial deficits. However, inhibition of nitrergic signaling by L-NAME could be the possible reason for restoration of learning and memory impairment along with normalizing oxidative–nitrosative stress levels.

The findings of the current study illustrate that chronic unpredictable stress model mimics several behavioral, biochemical, mitochondrial and molecular symptoms of cognitive impairment. The findings also show the neuroprotective potentials of American ginseng against chronic unpredictable stress-induced cognitive impairment, biochemical and mitochondrial enzyme complex alterations possibly through modulating nitric oxide signaling pathway (Fig. 1.2.8).

Fig 1.2.8 Possible mechanism of action of American ginseng and its interaction with nitric oxide modulators on chronic unpredictable stress induced cognitive impairment