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Research Report

Piperine potentiates the protective effects of curcumin against chronic unpredictable stress-induced cognitive impairment and oxidative damage in mice

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ABSTRACT

Life event stressors are the major vulnerability factors for the development of cognitive disorders. A vital therapeutic for stress related disorders is curcumin, derived from curry spice turmeric. Dietary phytochemicals are currently used as an adjuvant therapy to accelerate their therapeutic efficacy. Therefore, the present study was designed to investigate the effect of curcumin and its co-administration with piperine against chronic unpredictable stress (CUS)-induced cognitive impairment and oxidative stress in mice. Male Laca mice were subjected to undergo a battery of stressors for a period of 28 days. Vehicle/drugs were administered daily 30 mins before CUS procedure. Chronic stress significantly impaired memory performance (delayed latency time to reach platform in Morris water maze as well as to reach closed arm in elevated plus maze test) and decreased locomotor activity along with sucrose consumption. Further, there was a significant impairment in oxidative parameters (elevated malondialdehyde, nitrite concentration and decreased reduced glutathione, catalase levels) and mitochondrial enzyme complex activities, along with raised acetylcholinesterase and serum corticosterone levels. Chronic treatment with curcumin (200 and 400 mg/kg, p.o.) significantly improved these behavioral and biochemical alterations, restored mitochondrial enzyme complex activities and attenuated increased acetylcholinesterase and serum corticosterone levels. In addition, co-administration of piperine (20 mg/kg; p.o.) with curcumin (100 and 200 mg/kg, p.o.) significantly elevated the protective effect as compared to their effects alone. The results clearly suggest that piperine enhanced the bioavailability of curcumin and potentiated its protective effects against CUS induced cognitive impairment and associated oxidative damage in mice.

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1. Introduction

Memory impairment is a common and usual comorbidity associated with exposure to prolonged stress (Radley et al., 2004). Chronic stress is known to influence cognitive task 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...
cortisol level in restraint stress induced memory dysfunction (Xu et al., 2003). Study also showed an inhibitory effect of curcumin, manganese complexes activities and thereby attenuated the release of reactive oxygen species (Kumar et al., 2011). These reported pharmacological properties of curcumin clearly suggest its beneficial role against stress induced cognitive impairment.

In spite of large number of reports on the beneficial effects of curcumin, there have been instances of toxicity reported with high doses of curcumin (Burgoa-Morón et al., 2010; Mancuso and Barone, 2009b). There have also been clinical reports of occurrence of side effects in patients treated with curcumin (Mancuso and Barone, 2009b). All these reports of occurrence of side effects in patients treated with curcumin clearly suggest its beneficial role against stress induced cognitive impairment.

In the given study since piperine is known to increase the bio availability of many drugs (Attal et al., 1985). In light of these reports, present study aims to investigate the protective effect of co-administration of curcumin with piperine against chronic unpredictable stress induced cognitive deficits and oxidative damage in mice.

2. Results

2.1. Effect of curcumin, piperine and their combination on locomotor activity

28 days stress paradigm significantly decreased locomotor activity in CUS control group as compared to naive group of animals (Fig. 1). Chronic curcumin (200 and 400 mg/kg) treatment dose dependently improved locomotor activity which was significant as compared to control (CUS) group. Further, curcumin (100 mg/kg) could not significantly improve locomotor activity all through the study period. However, co-administration of curcumin (100 and 200 mg/kg) with piperine (20 mg/kg) potentiated their protective effects (increased locomotor activity) which was significant as compared to their effects alone (Fig. 1) [F (9, 44) = 15.72, 39.22 (p<0.001)].

2.2. Effect of curcumin, piperine and their co-administration on sucrose preference test

As shown in Fig. 2, chronically stressed animals showed significant reduction in sucrose consumption as compared to naive group of animals. However, curcumin (200 and 400 mg/kg) treatment dose dependently and significantly increased sucrose preference of rats compared to their respective control (CUS) group. Piperine (20 mg/kg) potentiated their protective effects (increased sucrose preference) significantly as compared to control (CUS) group (Fig. 2).

![Fig. 1](image-url)  
Fig. 1 – Effects of curcumin, piperine and their combination on locomotor 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 control; ***P<0.05 as compared to C(100); ****P<0.05 as compared to C(200); *****P<0.05 as compared to P(20) (Two-way ANOVA followed by Bonferroni's post test) [9, 44] = 15.72, 39.22 for interaction of days and treatment]. 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).
improved sucrose consumption as compared to control (CUS) mice. Besides, curcumin (100 mg/kg) treatment did not show any significant effect on sucrose consumption. However, co-administration of curcumin (100 and 200 mg/kg) with piperine (20 mg/kg) potentiated their protective effect (increased sucrose consumption) and were significant as compared to their effects alone (Fig. 2). The combination effect of curcumin (200 mg/kg) and piperine (20 mg/kg) was similar to the highest dose of curcumin (400 mg/kg) \[F (9, 44)=8.67 (p<0.001)\].

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

Initial transfer latencies (ITL) on day 20 for all animals of different groups were relatively stable and showed no significant variations. Following training, CUS control mice performed poorly throughout the experiment and did not show any change in the retention transfer latencies (RTL) on days 21 and 28 as compared to pre-training latency on day 20, demonstrating chronic stress-induced memory impairment. Besides, curcumin (200 and 400 mg/kg) treated mice showed significant and dose dependent decrease in both 1st and 2nd RTL on days 21 and 28 (Table 1). Further, curcumin (100 mg/kg) treatment did not show any significant effect on retention transfer latencies; however, co-administration of curcumin (100 and 200 mg/kg) with piperine (20 mg/kg) significantly elevated their protective effects (shortened transfer latency) when compared to their effects alone (Table 1). In addition, the synergistic effect of the combination was similar to that of highest dose of curcumin (400 mg/kg). \[F (9, 44)=23.12, 72.30 (p<0.001)\].

2.4. Effects of curcumin, piperine and their combination on Morris water maze test

The change in the escape latency time to reach the hidden platform was observed in the training/acquisition trials. Although there was a downward trend in escape latency time (ELT) in water-maze training session for four days, yet the mean latency (days 24–27) was significantly prolonged in the CUS control group as compared to the naive group, indicating a poorer learning performance (Fig. 3). Curcumin (200 and 400 mg/kg) treatment for 28 days significantly shortened escape latency time in dose dependent manner as compared to control (CUS). However, curcumin (100 mg/kg) did not show any significant improvement in memory performance as compared to

Table 1 - Effect of curcumin, piperine and their interaction on latency time in elevated plus maze paradigm.

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Initial transfer latency Day 20</th>
<th>Retention transfer latency Day 21(1st RTL)</th>
<th>Day 28(2nd RTL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>64.0 ±4.57</td>
<td>15.0 ±1.71</td>
<td>11.2 ±1.68</td>
</tr>
<tr>
<td>CUS Control</td>
<td>67.0 ±3.41 b</td>
<td>73.2 ±2.66a</td>
<td>69.4 ±3.19a</td>
</tr>
<tr>
<td>C(100)</td>
<td>66.8 ±5.03 b</td>
<td>63.0 ±2.26</td>
<td>52.1 ±2.93</td>
</tr>
<tr>
<td>C(200)</td>
<td>70.4 ±5.38 b</td>
<td>45.0 ±3.44b</td>
<td>32.3 ±2.57c,d</td>
</tr>
<tr>
<td>C(400)</td>
<td>69.3 ±4.12 b</td>
<td>25.0 ±1.83c,d</td>
<td>17.2 ±1.36c,d</td>
</tr>
<tr>
<td>P(20)</td>
<td>64.2 ±3.77 b</td>
<td>70.0 ±4.77</td>
<td>66.6 ±3.44</td>
</tr>
<tr>
<td>C(100)+P(20)</td>
<td>61.6 ±3.51 b</td>
<td>47.2 ±2.77c,e</td>
<td>32.2 ±2.44c,e</td>
</tr>
<tr>
<td>C(200)+P(20)</td>
<td>63.4 ±2.22 b</td>
<td>26.2 ±1.12d,e</td>
<td>18.2 ±2.12d,e</td>
</tr>
<tr>
<td>C(100) Naive</td>
<td>65.2 ±4.12 b</td>
<td>14.2 ±1.23</td>
<td>10.4 ±1.34</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.
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).
For statistical significance,
* P<0.05 as compared to naive group.
^ P<0.05 as compared to CUS control.
" P<0.05 as compared to C(100).
" P<0.05 as compared to C(200).
" P<0.05 as compared to P(20) (Two-way ANOVA followed by Bonferroni's post test) \[F (9, 44)=23.12, 72.30 for interaction of days and treatment\].
control (CUS). Further, combination of curcumin (100 and 200 mg/kg) with piperine (20 mg/kg) showed significant improvement in the learning performance as compared to their effects alone (Fig. 3). 

Platform was removed on day 28 to estimate the retention of memory. CUS control group significantly failed to recollect 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 significantly and dose dependently increased the time spent in the target quadrant as compared to CUS control, indicating improvement in cognitive performance (Fig. 4). Curcumin (100 mg/kg) treatment did not show any significant improvement in retention of memory; however curcumin (100 and 200 mg/kg) and piperine (20 mg/kg) together significantly increased the time spent in target quadrant as compared to their effects alone (Fig. 4) [F (9, 44) = 4.42 (p<0.001)]. Further, the combination effect of curcumin (200 mg/kg) with piperine (20 mg/kg) in both acquisition and retrieval trials were comparable to the highest dose of curcumin (400 mg/kg) alone.

2.5. Effect of curcumin, piperine and their co-administration on oxidative stress parameters

Stress control animals showed significant increase in oxidative damage as evidence by increased MDA, nitrite concentration, and depleted GSH, catalase and SOD enzyme activity as compared to naive group (Table 2). However, curcumin (200 and 400 mg/kg) treatment dose dependently attenuated levels of oxidative stress which was also significant as compared to CUS control. Curcumin (100 mg/kg) treatment did not significantly improve oxidative stress levels; however co-administration of curcumin (100 and 200 mg/kg) with piperine (20 mg/kg) significantly potentiated their protective effect (decreased MDA [F (9, 71) = 52.14 (p<0.001)], nitrite concentration [F (9, 71) = 24.22 (p<0.001)], restored GSH [F (9, 71) = 62.11 (p<0.001)], SOD [F (9, 71) = 35.12 (p<0.001)] and catalase [F (9, 71) = 83.20 (p<0.001)] levels) as compared to their effects alone and was comparable to curcumin (400 mg/kg) (Table 2).

2.6. Effect of curcumin, piperine and their combination on brain acetylcholine levels

Chronic unpredictable stress for 28 days significantly increased acetylcholinesterase enzyme activity in control (CUS) animals as compared to the naive group (Fig. 5). Curcumin (200 and 400 mg/kg) treatment dose dependently attenuated acetylcholinesterase activity which was significant as compared to control (CUS) group. Curcumin (100 mg/kg) treatment did not show significant inhibition of brain acetylcholinesterase activity; however co-administration of curcumin (100 and 200 mg/kg) with piperine (20 mg/kg) potentiated the attenuation effect which was significant as compared to their effects alone (Fig. 5). The highest dose combination also proved to have similar effects with that of curcumin (400 mg/kg) [F (9, 71) = 22.75 (p<0.001)].

2.7. Effects of curcumin, piperine and their interaction on mitochondrial respiratory enzyme complex activity

Chronic stress procedure impaired mitochondrial NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II) activity which was significant as compared to naive group (Table 3). Further, it also significantly reduced the number of viable cells (complex III) and levels of cytochrome
Table 2 - Effect of curcumin, piperine and their co-administration on oxidative stress parameters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LPO (mol of MDA/mgpr)</th>
<th>GSH (pmol of GSH/mgpr)</th>
<th>Nitrite (ng/ml)</th>
<th>Catalase (nmol of H2O2/min/mgpr)</th>
<th>SOD (units/mgpr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>0.159 ± 0.006</td>
<td>0.075 ± 0.005</td>
<td>303.3 ± 13.21</td>
<td>0.727 ± 0.011</td>
<td>58.23 ± 3.51</td>
</tr>
<tr>
<td>CUS Control</td>
<td>0.607 ± 0.029a</td>
<td>0.021 ± 0.004a</td>
<td>777.8 ± 16.26a</td>
<td>0.195 ± 0.032a</td>
<td>11.72 ± 2.12a</td>
</tr>
<tr>
<td>C(100)</td>
<td>0.546 ± 0.027</td>
<td>0.028 ± 0.003</td>
<td>715 ± 11.23</td>
<td>0.243 ± 0.022</td>
<td>16.43 ± 3.43</td>
</tr>
<tr>
<td>C(200)</td>
<td>0.433 ± 0.022bc</td>
<td>0.037 ± 0.002bc</td>
<td>566.2 ± 10.22bc</td>
<td>0.396 ± 0.018bc</td>
<td>31.64 ± 2.61bc</td>
</tr>
<tr>
<td>C(400)</td>
<td>0.323 ± 0.012bcd</td>
<td>0.055 ± 0.005bcd</td>
<td>386.6 ± 12.16bcd</td>
<td>0.528 ± 0.026bcd</td>
<td>46.21 ± 3.30bcd</td>
</tr>
<tr>
<td>P(20)</td>
<td>0.579 ± 0.028</td>
<td>0.024 ± 0.003</td>
<td>755 ± 15.79</td>
<td>0.188 ± 0.044</td>
<td>12.16 ± 1.98</td>
</tr>
<tr>
<td>C(100) + P(20)</td>
<td>0.444 ± 0.022cde</td>
<td>0.052 ± 0.002cde</td>
<td>395.2 ± 12.20cde</td>
<td>0.506 ± 0.045cde</td>
<td>45.21 ± 4.30cde</td>
</tr>
<tr>
<td>C(200) + P(20)</td>
<td>0.342 ± 0.019d*e</td>
<td>0.052 ± 0.002d*e</td>
<td>395.2 ± 12.20d*e</td>
<td>0.506 ± 0.045d*e</td>
<td>45.21 ± 4.30d*e</td>
</tr>
</tbody>
</table>

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 control.
- c P < 0.05 as compared to C(100).
- d P < 0.05 as compared to C(200).
- e P < 0.05 as compared to P(20) (One-way ANOVA followed by Tukey’s test).

Fig. 5 - Effect of curcumin, piperine and their combination on brain acetylcholinesterase activity. 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 control; c P < 0.05 as compared to C(100); d P < 0.05 as compared to C(200); e P < 0.05 as compared to P(20) (One-way ANOVA followed by Tukey’s test) [F (9, 71) = 22.75]. 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).

C oxidase enzyme (complex IV) (Table 3). Curcumin (200 and 400 mg/kg) treatment significantly and dose dependently restored mitochondrial enzyme complex I, II and cytochrome C enzymes as compared to control (CUS). Further, curcumin (100 mg/kg) treatment did not show any significant effects on altered levels of mitochondrial respiratory enzymes. However, combination of curcumin (100 and 200 mg/kg) and piperine (20 mg/kg) showed a significant potentiation in their protective effect i.e. restored mitochondrial enzyme complex I [F (9, 71) = 32.11 (p < 0.001)], II [F (9, 71) = 42.30 (p < 0.001)], III [F (9, 71) = 28.21 (p < 0.001)], IV [F (9, 71) = 66.86 (p < 0.001)] activities. Further, the effect of combination of curcumin (200 mg/kg) with piperine (20 mg/kg) on mitochondrial respiratory enzyme complex activity was similar to the highest dose of curcumin (400 mg/kg).

2.8. Effect of curcumin, piperine and their combination on serum corticosterone (CORT) levels

A significant elevation in the serum CORT levels in stressed animals was noticed as compared to naive group (Fig. 6). Treatment with curcumin (200 and 400 mg/kg) dose dependently attenuated the increased levels of serum CORT which was significant as compared to CUS control. However, curcumin (100 mg/kg) treatment did not show significant inhibition of serum CORT levels. Further, co-administration of curcumin (100 and 200 mg/kg) and piperine (20 mg/kg) significantly lowered serum CORT levels as compared to their effects alone and was comparable to curcumin (400 mg/kg) [F (9, 71) = 150.20 (p < 0.001)] (Fig. 6).

3. Discussion

Stress is an unavoidable life experience that may attribute to oxidative stress leading to cognitive disturbances. 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 the most reliable and valuable experimental model to study stress pathology in animals (Willner et al., 1992). Chronic unpredictable stress (CUS) have been shown to influence different regions of brain i.e. hippocampus and prefrontal cortex (McFadden et al., 2011), which play a critical role in spatial navigation and memory (Churchwell et al.,
Table 3 - Effect of curcumin, piperine and their interaction on mitochondrial respiratory enzyme complex I, II, III and IV activities.

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Complex I (n mol of NADH oxidized/min/mg pr)</th>
<th>Complex II (n mol/mg pr)</th>
<th>Complex III (n mol/min/mg pr)</th>
<th>Complex IV (n mol/min/mg pr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(of Naive)</td>
<td>(% of Naive)</td>
<td>(of Naive)</td>
<td>(of Naive)</td>
</tr>
<tr>
<td>Naive</td>
<td>100 ± 3.2</td>
<td>100 ± 4.10</td>
<td>100 ± 5.45</td>
<td>100 ± 4.32</td>
</tr>
<tr>
<td>CUS Control</td>
<td>27.32 ± 2.1*</td>
<td>21.40 ± 1.76*</td>
<td>38.42 ± 4.45*</td>
<td>46.4 ± 3.12*</td>
</tr>
<tr>
<td>C(100)</td>
<td>34.5 ± 3.44</td>
<td>27.2 ± 5.55</td>
<td>43.5 ± 2.34</td>
<td>50.2 ± 2.45</td>
</tr>
<tr>
<td>C(200)</td>
<td>55.2 ± 1.22**</td>
<td>51.5 ± 2.63**</td>
<td>62.2 ± 4.32**</td>
<td>71.35 ± 4.26**</td>
</tr>
<tr>
<td>C(400)</td>
<td>79.2 ± 4.85**</td>
<td>70.43 ± 3.43**</td>
<td>85.2 ± 3.58**</td>
<td>89.24 ± 5.34**</td>
</tr>
<tr>
<td>P(20)</td>
<td>30.63 ± 3.48</td>
<td>24.20 ± 2.88</td>
<td>40.63 ± 2.48</td>
<td>48.20 ± 3.31</td>
</tr>
<tr>
<td>C(100)+P(20)</td>
<td>57.20 ± 2.65**</td>
<td>50.32 ± 1.95**</td>
<td>64.20 ± 4.66**</td>
<td>73.11 ± 4.23**</td>
</tr>
<tr>
<td>C(200)+P(20)</td>
<td>75.20 ± 3.15**</td>
<td>68.22 ± 2.5**</td>
<td>84.20 ± 3.45**</td>
<td>90.11 ± 4.34**</td>
</tr>
<tr>
<td>C(100) Naive</td>
<td>98.20 ± 4.8</td>
<td>100.34 ± 2.13</td>
<td>100.20 ± 3.12</td>
<td>101.44 ± 5.15</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.

Complex I [F (9, 71) = 32.11], II [F (9, 71) = 42.30], III [F (9, 71) = 28.21] and IV [F (9, 71) = 66.86]. CUS, chronic unpredictable stress; C(100), curcumin (200 mg/kg); C(100), curcumin (200 mg/kg); C(400), curcumin (400 mg/kg); P(20), piperine (20 mg/kg).

For statistical significance,
* P<0.05 as compared to naive group.
** P<0.05 as compared to CUS control.
*** P<0.05 as compared to C(100).
**** P<0.05 as compared to C(200).
***** P=0.05 as compared to C(200).
****** P<0.05 as compared to P(20) (One-way ANOVA followed by Tukey's test).

Fig. 6 - Effect of curcumin, piperine and their combination on serum corticosterone (CORT) levels. Values are expressed as mean ± SEM. For statistical significance, *P<0.05 as compared to naive group; **P<0.05 as compared to CUS control; ***P<0.05 as compared to C(100); ****P<0.05 as compared to C(200); *****P<0.05 as compared to P(20) (One-way ANOVA followed by Tukey's test) [F (8, 71) = 150.20]. 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).

2010). Thus in the present study, piperine has been tried as a drug strategy with curcumin against chronic unpredictable stress induced oxidative damage and cognitive deficits in mice.

In the present study, memory functions were evaluated by Morris water maze (MWM) as well as elevated plus maze (EPM). These two tests are often used as complementary to each other. Though elevated plus maze is primarily used for assessment of anxiety, it has also been employed as a model for evaluation of memory in rodents (Sharma and Kulkarni, 1992). In the study, chronic unpredictable stress resulted in significant impairment of cognitive tasks in both Morris water maze and elevated plus maze performance task as compared to naive animals. These results are consistent with the previous finding (Hoffman et al., 2011). Curcumin treatment significantly and dose dependently 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 also a significant decrease in locomotor activity and sucrose preference in CUS control animals as compared to naive group. The results are in accordance with previous studies by Gronli et al. (2005) which showed a significant decrease in locomotor activity and sucrose consumption following chronic mild stress. Further, curcumin in a dose dependent manner significantly restored the decrease in locomotor activity and sucrose preference. These results are similar to the reports from previous studies of our laboratory (Kumar and Singh, 2008). All these behavioral tests respond selectively to chronic curcumin treatment thus mimicking the clinical time course of memory restorative action.

Hippocampus is reported 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. Alzheimer's disease affects cholinergic system resulting in decreased activity of acetylcholinesterase (Dai et al., 2002). Stress has been well documented to induce alterations in activity of acetylcholinesterase enzyme (Nijholt et al., 2004). In the present study, CUS caused a significant decrease in the acetylcholinesterase activity leading to memory deficits, but later was significantly restored by chronic curcumin...
treatment thereby implicating retrieval and retention of memory processes. These results are in line with the earlier reports from our laboratory (Kumar et al., 2009). This could be one of the mechanistic pathways for the neuroprotective effect of curcumin in cognitive dysfunction of CUS animals. In addition to behavioral abnormalities, chronic stress is also involved in activation of hypothalamic–pituitary–adrenal (HPA) axis which has also been reported in Alzheimer's patients (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 levels as compared to the naive group. Furthermore, chronic administration of curcumin exhibited a slow but effective increase in sucrose preference, resulting in the HPA axis normalization in the CUS animals. These results are also consistent with the previous findings showing that increased corticosterone induced by chronic unpredictable stress can be prevented by chronic curcumin administration (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 reviewed (Serrano and Klann, 2004). A number of findings suggest that reactive oxygen species (ROS) can accumulate excessively in the brain and can severely attenuate the neuronal function (Massaad and Klann, 2011). Oxidative stress and increased reactive oxygen species formation summarizes the basis of cognitive impairment (Keller et al., 2005). Besides, chronic stress is said to promote oxidative stress and demolish antioxidant defense system of the brain (Lucca et al., 2009), which may form the basis for impaired memory. In the present investigation, CUS resulted in significant oxidative damage as indicated by increase lipid peroxidation, nitrite concentration, and depletion of reduced glutathione levels, catalase and superoxide dismutase activity, thus strengthening the oxidative theory of cognitive deficits and its complications. Curcumin being a lipophilic molecule is known to possess strongly 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 levels of SOD and catalase in irradiated mice (Kolram et al., 2007). In line with the above correlates, curcumin in the present study significantly and dose dependently attenuated these oxidative stress markers. Generation of reactive oxygen species (ROS) may also be associated to 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 catalyzed by various membrane bound protein complexes, namely NADH-ubiquinol oxidoreductase (complex-I), succinate-ubiquinol oxidoreductase (complex-II), ubiquinol cytochrome c oxidoreductase (complex-III) and complex IV (cytochrome C oxidase) (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. NO and superoxide radical (O₂⁻) may react to form peroxynitrite (ONOO⁻) which leads to oxidative damage in mitochondria (Clementi et al., 1998). The results of the present study indicate that CUS caused significant impairment in different mitochondrial enzyme complex activities which were later restored by curcumin treatment, suggesting a potential role for curcumin in restoring ROS generation in mitochondria. Thus the results strongly support our hypothesis that the memory deficits observed after chronic unpredictable stress 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 limits its therapeutic efficacy. Studies have reported that curcumin gets reduced through alcohol dehydrogenase, followed by conjugations like sulfation and glucuronidation in liver and intestine (Nahistrom and Blennow, 1978). Thus high concentrations of curcumin cannot be achieved and maintained in plasma and tissues after oral ingestion. This limited therapeutic potential of curcumin causes a major hindrance for its clinical development. 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 the strategies to improve the poor oral bioavailability of curcumin involves use of bioavailability enhancers which could potentiate the amount of oral curcumin reaching plasma.

In the present study, piperine was co-administered with curcumin to enhance 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 increases free form of native curcumin, responsible for its protective effect. However, there have been some reports on inhibition of drug metabolizing enzymes (CYP3A4) on combination of curcumin with piperine which could further alter the metabolism of several drugs and originate toxic effects (Mancuso and Barone, 2009a). Besides, in present study no additional drug was given with curcumin-piperine combination, thus care was taken to control reported alterations in metabolism of drugs and its associated toxic effects.

In the study we witnessed a profound increase in protective effects of curcumin when co-administered with piperine. However, in our study as well as previous literatures have suggested that piperine does not exert any antioxidant activity alone. The potentiation in the effects of combination indicates that piperine might have increased the bioavailability of curcumin possibly through inhibition of its intestinal glucuronidation resulting into increased absorption of curcumin.

The present study clearly demonstrates the memory restorative and antioxidant properties of curcumin due to
its multifactorial nature, which further shows elevated effects on combination with a bioavailability enhancer, piperine. 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 cognitive disorders.

4. Statistical analysis

All the values were expressed as mean ± SEM. The behavioral data were analyzed by Two-way analysis of variance (ANOVA) followed by Bonferroni's post test to calculate the statistical significance between various groups. All other 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 sigmasstat Graph Pad Prism (Graph Pad Software, San Diego, CA).

5. Experimental procedures

5.1. Animals

Male Laca mice (30-35 g) bred at Central Animal House (CAH) Panjab University, Chandigarh, were used. They were housed under standard (25 ± 2°C, 60-70% humidity) laboratory conditions, maintained on a 12 h 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/170-175) and conducted according to the CPCSEA guidelines on the use and care of experimental animals.

5.2. Drugs and treatment schedule

Following drugs were used in the present study. 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 nine experimental groups. First and second group was named as naive and control (CUS) group respectively. Curcumin (100, 200 and 400 mg/kg, p.o.) were treated as groups 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 groups 7 and 8 respectively. Curcumin Naive (400 mg/kg, p.o.) (without stress procedure, treatment to naive animals) served as group 9. Curcumin and piperine were prepared in peanut oil and administered orally 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 min before CUS procedure for 28 days. The doses of curcumin and piperine were selected on the basis of literature and laboratory reports (Mehla et al., 2010; Hlavackova et al., 2011). The detailed experimental design for chronic unpredictable stress protocol is shown in Fig. 7.

5.3. Chronic unpredictable stress procedures

Mice were exposed to a random pattern of mild stressors (Murua et al., 1991) daily for 28 days. The order of stressors

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**Fig. 7 – Experimental design for chronic unpredictable stress protocol.** Different sets of animals were used for estimation of locomotor activity, sucrose consumption, Morris water maze test and elevated plus maze and were studied independent of each other. At the end of the study animals were clubbed and divided into different groups for biochemical, mitochondrial and serum corticosterone estimations.
5.4. Behavioral studies

5.4.1. Locomotor activity

Animal was kept in actophotometer for the first 3 min as a habituation period before actual recording of locomotor activity for 5 min. Each animal was placed in a square (30 cm) closed arena equipped with infra-red light sensitive photocells (digital actophotometer, IMCORP, India) and values were expressed as counts per 5 min. The apparatus was placed in a darkened, light and sound attenuated and ventilated testing room (Kumar and Garg, 2008).

5.4.2. Sucrose preference test

The sucrose preference test (SPT) was conducted on last day of the study period. The mice were tested for sucrose consumption as described earlier (Bhagya et al., 2011). Animals were housed individually throughout the test duration. The mice were tested for sucrose consumption as described earlier (Bhagya et al., 2011). An animal was kept in actophotometer for the first 3 min as a habituation period before actual recording of locomotor activity for 5 min. Each animal was placed in a square (30 cm) closed arena equipped with infra-red light sensitive photocells (digital actophotometer, IMCORP, India) and values were expressed as counts per 5 min. The apparatus was placed in a darkened, light and sound attenuated and ventilated testing room (Kumar and Garg, 2008).

5.4.3. Assessment of cognitive performance

5.4.3.1. Elevated plus maze paradigm

The elevated plus maze (EPM) consisted of two opposite black open arms (16 x 5 cm), crossed with two closed walls of the same dimensions of 12 cm height. The arms were connected with a central square of dimensions 5 x 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 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 s after recording the ITL and then returned to the home cage. If the animal could not enter closed arm within 90 s, same was guided to the closed arm and ITL was given as 90 s. Retention of memory was assessed by placing the mouse again in an open arm and the retention latency was noted on day 21 and day 28 of ITL and was termed as the first retention transfer latency (1st RTL) and second retention transfer latency (2nd RTL), respectively.

5.4.3.2. Morris water-maze test

Morris water-maze apparatus (MWM) is most commonly used model to test memory (Morris, 1984). The MWM procedure is based on the principle that an 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 consisted 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). The water was made opaque with white colored dye. The tank was divided into four equal quadrants. A submerged platform (10 cm x 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 10th to day 13th. The mice received four consecutive daily training trials in the following 4 days, with each trial having a ceiling time of 120 s. 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 s to locate submerged platform. Then, it was allowed to stay on the platform for 20 s. If animal failed to find the platform within 120 s, it was guided gently onto platform and allowed to remain there for 20 s.

Acquisition trial—Each mouse was subjected to four trials on each day. A rest period of 1 h 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>
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<td>Day1</td>
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<td>Q4</td>
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<td>Day3</td>
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<td>Day4</td>
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</table>
Mean escape latency time (ELT) calculated for each day during acquisition trials was used as an index of acquisition. Retrieval trial—On fifth day (day 14th) the platform was removed. Animal was placed in water maze and allowed to explore the maze for 120 s. 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 other objects in the laboratory serving as prominent visual clues was not disturbed during the total duration of study.

5.5. Dissection and homogenization

Immediately after the last behavioral test, animals were randomized into two groups; one group was used for the biochemical assays. For biochemical analysis, animals were sacrificed by decapitation. 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 x g for 15 min and aliquots of supernatant were separated and used for biochemical estimation.

5.6. Estimations of oxidative stress parameters

5.6.1. Lipid peroxidation
The extent of lipid peroxidation was determined quantitatively by performing the method as described by Wills (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 results were expressed as nanomoles of reduced glutathione per milligram of protein.

5.6.2. Nitrite
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-(l-napththyl) ethylene diamine dihydrochloride, 1% sulfanilamide and 5% phosphoric acid) (Green et al., 1982). Equal volumes of the supernatant and the Greiss reagent were mixed and the mixture was incubated for 10 min at room temperature in the dark. The absorbance was measured at 540 nm using Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA). The values were calculated using the molar extinction coefficient (1.56 x 10 M⁻¹ cm⁻¹).

5.6.3. Reduced glutathione
Reduced glutathione in the brain was estimated according to the method of Ellman et al. (1961). The assay mixture contained 0.05 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). The results were expressed as micromoles of H₂O₂ decomposed per milligram of protein/min.

5.6.4. Superoxide dismutase activity
Superoxide dismutase (SOD) activity was assayed by the method of Kono (1978) where the reduction of nitrazobluetezolium (NBT) was inhibited by the superoxide dismutase and is measured. 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).

5.6.5. Catalase
Catalase activity was determined by Luck (1971), wherein the 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 supernatant of tissue homogenates (10%), and the change in absorbance was recorded 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.

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

5.7. Estimation of acetyl cholinesterase (AChE) activity
AChE is a marker of loss of cholinergic neurons in the brain region. The AChE activity was assayed as described by Ellman et al. (1961). The assay mixture contained 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.

5.8. Mitochondrial enzyme complex estimations
Second group of animals were used for mitochondrial enzyme complex isolation as described in the method of Berman and Hastings (1999). The whole brain was homogenized in isolated buffer. Homogenates were centrifuged at 13,000 g for 5 min at 4 °C. Pellets were re-suspended in isolation buffer with ethylene glycol tetracetic acid (EGTA) and spun again at 13,000 g at 4 °C for 5 min. The resulting supernatants were transferred to new tubes and topped off with isolation buffer with EGTA and again spun at 13,000 g at 4 °C for 10 min. Pellets containing pure mitochondria were re-suspended in isolation buffer without EGTA.

5.8.1. Complex-I (NADH dehydrogenase activity)
Complex-I was measured spectrophotometrically by the method of King and Howard (1967). The method involves catalytic oxidation of NADH to NAD⁺ with subsequent reduction in 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 solubilised mitochondrial sample and followed absorbance change at 550 nm for 2 min.

5.8.2. Complex-II (succinate dehydrogenase activity)
Complex-II was measured spectrophotometrically according to 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.

5.8.3. Complex-III (MTT activity)
The MTT assay is 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 et al. (1997). Briefly, 100 µl mitochondrial samples were incubated with 10 µl MTT for 3 h at 37 °C. The blue formazan crystals were solubilised with dimethylsulfoxide and measured by an ELISA reader at 580 nm filter.

5.8.4. Complex IV (cytochrome c oxidase)
Cytochrome oxidase activity was assayed in brain mitochondria according to the method of Sottocasa (Sottocasa et al., 1967). The assay mixture contained 0.3 mM reduced cytochrome C in 70 mM phosphate buffer. The reaction was started by the addition of solubilized mitochondrial sample and the changes in absorbance were recorded at 550 nm for 2 min.

5.9. Serum corticosterone estimations

5.9.1. Preparation of serum
Animals were sacrificed and blood was collected immediately thereafter between 8:00-9:00 AM. Blood collected in the test tubes was allowed to clot at room temperature. The tubes were then centrifuged at 2000 rpm for 10 min and the straw colored serum was separated and stored frozen at −20 °C.

5.9.2. Corticosterone assessment
For extraction of corticosterone the method of Silber et al. (1958) was modified as described. 0.1-0.2 ml of serum were 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 et al. The step of treatment of petroleum ether was omitted. The samples were vortexed for 30 s and centrifuged at 2000 rpm for 10 min. The chloroform layer was carefully removed with the help of 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 by vortexing rapidly and NaOH layer was rapidly removed. The sample was then treated with 3 ml of 30 N H2SO4 by vortexing vigorously. After phase separation, chloroform layer on top was removed using a syringe as described above and discarded. The tubes containing H2SO4 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 523.2 nm respectively. The standard curve depicting the fluorescence yield versus corticosterone concentration was used for result analysis.

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