CHAPTER 2

INHIBITORY POTENTIAL OF CHALCONES ON BRAIN PARTS AND SPINAL CORD ACETYLCHOLINESTERASE ACTIVITY AND MEMORY IN RATS USING Y-MAZE
Introduction

Acetylcholinesterase (AChE) is a key component of cholinergic brain synapses and neuromuscular junctions. The major biological role of the enzyme is the termination of impulse transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine (Hasan et. al., 2005). The central cholinergic pathways play a prominent role in learning and memory processes (Nabeshima, 1993). Cholinergic neurons in the central nervous system (CNS) are degenerated in patients with Alzheimer’s disease, and senile dementia severity and degree of degeneration are correlated with functional loss in this and similar disorders (Davies and Maloney, 1976; Perry et. al., 1978). Based on a cholinergic hypothesis, many attempts have been made to reverse cognitive deficits by increasing brain cholinergic activity via acetylcholinesterase inhibitors (AChEIs), acetylcholine precursors, or cholinergic agonists. It has been the prevailing view that the symptomatic efficacy of AChEIs is attained through their augmentation of acetylcholine- mediated neuron-to-neuron transmission. However, there is evidence that AChEIs may slow disease progression and
hippocampal atrophy and may have disease-modifying effects (Farlow, 2002; Giacobini, 2001). The cholinergic receptor agonists (muscarinic and nicotinic) and enhancers of the endogenous levels of acetylcholine (synthesis promoters and inhibitors of its metabolizing enzymes) have been examined as potential treatments for senile dementia of the Alzheimer’s type. Among the various approaches attempted, AChE inhibition was the most successful (Giacobini, 1996), and a selective AChE inhibitor, donepezil, has been used to treat mild Alzheimer’s disease (Doody, 1999). Thus, enzyme acetylcholinesterase (AChE) has long been an attractive target for the rational drug design and discovery of inhibitors for the treatment of Alzheimer’s disease.

Recent evidence also point to a direct role of AChEIs in the inhibition of the release of inflammatory substances from specialized cells (Giunta et. al., 2004). The role of inflammatory cytokines, including TNF α, IL-1 and IL-6, in neurodegenerative diseases has been extensively studied in the last several years (Cacabelos et. al., 1991; Fillit et. al., 1991; Grimaldi et. al., 2000; Licastro et. al., 2000; Gambi et. al., 2004; Lugaresi et. al., 2004; Reale et. al., 2004). The accumulation of the cytokines, IL-1, IL-6 and TNF in AD does not appear to be merely a consequence of the degenerative process, but may play a role in the
cascade of events including neuronal death (Paganelli et. al., 2002; Licastro et. al., 2003). Flavonoids, are known to inhibit both LPS stimulated necrosis factor alpha and interleukin-6 release which modulate pro-inflammatory molecules that have been reported in many progressive neurodegenerative disorders. Also 2'-substituted chalcones have been shown to inhibit the production of IL-1β monocytes stimulated with LPS (Batt et. al., 1993). Recently, it has been found that BChE inhibitors may also be effective for the treatment of Alzheimer and related dementias (Yu et. al., 1999). Chalcones have been shown to be the inhibitors of AChE and BChE (Ansari et. al., 2005). Thus, it prompted us to investigate the effects of variably substituted chalcones on the memory deficits induced by cholinergic disturbances using scopolamine in rats. Memory parameter was evaluated by using Y-maze and Elevated plus maze. Here, we report that out of eight tested chalcones, two have ameliorating effects on scopolamine-induced learning and memory in rats.

**Materials and Methods**

**Animals**

Adult male albino rats weighing 200-250 gram were used for the study.
The animals were procured from the central animal house facility at Jawaharlal Nehru Medical College, Aligarh. The rats were group housed in polypropylene cages (38×23×10cm) under standard laboratory conditions with natural light and dark cycle. They were allowed free excess of dry rat diet and tap water ad libitum. All procedures described were reviewed and approved by the Institutional Animal Ethics Committee.

Materials

Chalcones were prepared by the methods described in "review section". Chalcones used in the study are: 2',4',4-trihydroxychalcone (ISL, isoliquiritigenin); 2',4',3,4 tetrahydroxychalcone (BUT, butein); 2', 2'-dihydroxychalcone (DHC); 2'- hydroxy – 3, 4 -dimethoxychalcone (HDMC); 4', 4- dichlorochalcone (DCC); 4'– chloro , 4-methoxy-chalcone (CMC); 1,3-bis (4-chlorophenyl) –3 (carboxymethylthio) propan-1-one (DCCP) and 1- (4-chlorophenyl)– 3 (4-methoxyphenyl)-3- (carboxymethylthio) propan-1-one (CMCP).

(-) scopolamine hydrobromide, acetylthiocholine iodide and DTNB [5, 5'-dithiobis (2-nitrobenzoic acid)] were purchased from the
Sigma Chemical Co. All other materials were obtained from normal commercial sources and were of the highest grade available.

Methods
Rats were divided into ten groups of ten animals each. The animals in experimental (eight) groups were administered with chalcones intraperitoneally at the dose of 25mg/ kg body weight daily for 7 consecutive days. Chalcones were dissolved in DMSO-normal saline. The animals of control group received a similar volume of dimethylsulfoxide-normal saline intraperitoneally for 7 consecutive days. The final concentration of DMSO in normal saline did not exceed 0.5 %. Memory impairment was induced by the administration of scopolamine (1mg/kg, i.p). Scopolamine hydrobromide was dissolved in normal saline. Working memory performances were assessed by recording spontaneous alternation behavior in a single session in a Y- maze and transfer latency in the Elevated plus maze immediately after the administration of chalcones.

Y maze task
Spontaneous alternation behavior, which is regarded as a measure of spatial memory (Sarter et. al., 1988), was investigated using the Y- maze
test. The Y-maze is a three-arm horizontal maze (40 cm long and 3 cm wide with walls 12 cm high) in which the arms are symmetrically disposed at 120° to each other. Rats were initially placed within one arm, and the sequence (i.e., ABC CBA, etc) and number of arm entries were recorded manually for each rat over a 5 min period. An actual alternation was defined as entries into all three arms on consecutive choices (ABC, CAB, or BCA but not BAB). One hour before this test, rats were treated with the seventh and last dose of chalcones (25 mg/kg), and 30 min later memory impairment was induced by administration of scopolamine (1 mg/kg, i.p). Control group animals received vehicle only. Maze arms were thoroughly cleaned between tests to remove residual odors. Percentage alternation was determined by dividing the total number of alternations by the total number of choices minus 2 multiplied by 100 as shown in the following equation (Fraser et. al., 1997):

% Alternation = \[\frac{(\text{Number of alternations})}{(\text{Total arm entries} - 2)}\] \times 100. The number of arm entries serves as an indicator of locomotor activity. The rats were subjected to Elevated plus maze after this test.

**Elevated plus maze**

Elevated plus maze serves as the exteroceptive behavioral model to evaluate memory in mice. The procedure, technique and end point for
testing memory was followed as per the parameters described by the investigators (Itoh et al., 1990; Joshi et al., 2005). The apparatus consisted of a plus-shaped maze elevated 45 cm above the ground level with two open (10×50×10 cm) and two closed arms. There was a central square arena (10×10 cm) at the junction of open and closed arms where animal is placed at the start of the experiment. Each rat was placed at the end of an open arm, facing away from the central platform. Transfer latency (TL) was defined as the time (in seconds) taken by the animal to move from the open arm into one of the closed arms with all its four legs. The rat was allowed to explore the maze for 2 min and then returned to its home cage. Significant reduction in TL value of retention indicated improvement in its memory. One hour before this test, rats were treated with the seventh and last dose of chalcones (25 mg/kg), and 30 min later memory impairment was induced by administration of scopolamine (1 mg/kg, i.p). Control group animals received vehicle only. Maze arms were thoroughly cleaned between tests to remove residual odors.

**Estimation of acetylcholinesterase activity**

**Tissue preparation**

On the seventh day, immediately after the Y-maze and Elevated plus
maze performances rats were sacrificed by decapitation. Their brains along with spinal cords were removed quickly, and placed on the petridish, over ice. The brains and spinal cords were washed with ice-chilled normal saline repeatedly to clean. The brains were dissected out into three regions—cerebrum, cerebellum, and brain stem. The assay of AChE in brain parts and spinal cord was performed by the method of Ellman et. al. (1961).

**Principle:**

AChE estimation is based on the measurement of the rate of production of thiocholine as AChE is hydrolyzed. This is accompanied by the continuous reaction of the thiol with 5', 5-dithiobis-2-nitrobenzoate ion to produce the yellow anion of 5-thio-2- nitrobenzoic acid.

**Chemicals and reagents:**

1) Phosphate buffer (0.1 M, pH- 8.0).

2) DTNB reagent: 39.6 mg of 5', 5-dithiobis-2-nitrobenzoic acid (DTNB) was dissolved in 10 ml of 0.1 M phosphate buffer (pH- 8.0) and 15 mg of sodium bicarbonate was added to it.

3) Substrate: 0.075 M acetylcholine iodide.

4) Inhibitor: $10^{-4}$ M eserine sulphate was dissolved in 0.1 M phosphate buffer (pH- 8.0).
Procedure:

Saline cleaned and accurately weighed tissues were homogenized in 0.1 M phosphate buffer (pH- 8.0) in a concentration of 10 mg/ml and centrifuged at 1500 rpm for 5 min. 0.4 ml of the supernatant was pipetted in a cuvette containing 2.6 ml of phosphate buffer. To this 0.1 ml of DTNB reagent was added and mixed well. O.D was read at 412 nm using Beckman DU spectrophotometer and the absorbance of the suspension was set at zero. 0.02 ml of the substrate was added and the changes in the absorbance were recorded from 5th to 10th min at the interval of one minute. To determine non-specific esterase, 0.1 ml of eserine sulphate (inhibitor) was added to another cuvette containing 0.4 ml of homogenate supernatant, 2.5 ml of phosphate buffer and 0.1 ml of DTNB reagent. The changes in absorbance were recorded as described above after adding 0.02 ml of substrate. The rate of change of activity of the suspension with eserine was subtracted from that of the suspension without eserine. The enzyme activity is expressed as μmoles of substrate hydrolyzed per gm tissue per minute. Protein concentration was determined by the method of Lowry et. al. (1951).

\[ R = \frac{A}{1.36 \times 10^4} \times \frac{1}{\frac{400}{3120} C_o} = \frac{5.74 \times 10^{-4} A}{C_o} \]
where,

\[ R = \text{Rate of enzyme activity in moles of substrate hydrolyzed / gm tissue / min} \]

\[ A = \text{Change in absorbance per minute} \]

\[ C_0 = \text{Original concentration of tissue (mg/ml)} \]

**Statistical analysis**

All the data expressed in the table and figures are mean ± SEM. Data was analyzed by one way analysis of variance (ANOVA) followed by Tukey post test. Differences were considered significant at P < 0.05 or less.

**Results**

**Effects of scopolamine and chalcones on the Y- maze test**

The effects of chalcones on spontaneous alternation behavior was examined using the Y– maze test and the results showed that variably substituted chalcones exhibited different pattern of activity. Scopolamine (1 mg/ kg, i.p) injected before 30 min of the test significantly decreased [\( F_{(1,10)} = 11.231, \ P< 0.001 \] the spontaneous alternations as compared to vehicle treated control group, indicating impairment in memory.
(amnesia). Only two of the tested chalcones (ISL and BUT) increased the spontaneous alternations. The spontaneous alternations of rats treated with ISL (isoliquiritigenin) and BUT (butein) (25mg/ kg × 7 days) are significantly \( F (2, 15) = 9.975, P < 0.001 \) higher than that of scopolamine treated control group. However, rest of the tested chalcones showed no effect on the spontaneous alternation behavior. Thus chalcones exhibited no impairment on spatial working memory (Figure 2). Moreover, numbers of arm entries were similar in all experimental groups as compared to vehicle treated control group, demonstrating that general locomotor activity was not affected by chalcones (Figure 3).

**Effects of scopolamine and chalcones on the Elevated plus maze test**

Transfer latency (TL) reflected retention of learned task or memory. The rats treated with chalcones (ISL and BUT; 25mg/ kg, i.p) showed reduction of TL which is increased by scopolamine, indicating significant improvement in memory. Scopolamine (1 mg/kg, i.p) injected before 30 min of the test significantly increased \( F(1, 10) = 35.703, P < 0.001 \) the TL as compared to vehicle treated control group, indicating impairment in memory (amnesia) (Figure 4). The rats treated with ISL and BUT (25 mg/kg, i.p × 7 days) significantly reversed the memory
impairment \[ F(2, 15) = 10.779, P < 0.001 \] as compared to scopolamine treated control group.

**Effect of chalcones on acetylcholinesterase activity**

The results of variably substituted chalcones, screened for their activity towards acetylcholinesterase inhibition in the different brain parts and spinal cords are presented in Table 5. As shown in Table 5, only hydroxy substituted chalcones i.e ISL, BUT, DHC and HDMC showed inhibitory activity towards acetylcholinesterase while treatment with other chalcones such as DCC, DCCP, CMC, and CMCP caused no alteration in the acetylcholinesterase activity and were totally inactive.

ANOVA followed by Tukey post hoc test showed that intraperitoneal administration of chalcones (25mg/ kg, i.p × 7 days) significantly reduced the acetylcholinesterase activity in cerebrum \[ F(4,25) = 27.820, P < 0.001 \], cerebellum \[ F(4,25) = 21.006, P < 0.001 \], brain stem \[ F(1,10) = 27.594 , P < 0.001 \], and spinal cord \[ F(2,15) = 5.010 , P < 0.022 \] as compared to vehicle treated control group.
Structural requirements for AChE inhibition and memory improvement

From these results it is evident that the hydroxyl (OH) group present in ring A has vital role in the inhibition of acetylcholinesterase activity. Compounds having hydroxyl group at 2' position such as in ISL (isoliquiritigenin, 2',4',4- trihydroxy chalcone), BUT (2',4',3,4-tetrahydroxychalcone), DHC (2',2-dihydroxychalcone), and HDMC (2'-hydroxy-3,4,-dimethoxychalcone) showed inhibitory activity against AChE. Compounds having chloro and methoxy substituents as in DCC, DCCP, CMC, CMCP showed no inhibition and are totally inactive. Thus, it may be concluded that a hydroxyl group ortho to the side chain is very much responsible for acetylcholinesterase inhibitory activity. Substituents like chloro, methoxy, hydroxyl, carboxymethyl moiety may decrease or increase the activity of the variably substituted chalcones in the present study.

Results obtained from Y- maze and Elevated plus maze tests showed that only two of the tested chalcones i.e ISL (isoliquiritigenin, 2',4',4- trihydroxy chalcone), and BUT (2',4',3,4-tetrahydroxychalcone) having three and four hydroxy groups increased the spontaneous alternations and decreased the transfer latency respectively, indicating
improvement in memory. Thus, for the ameliorating effects of chalcones on memory deficits, presence of more than two hydroxyl groups on both the rings is necessary.
Figure 2. Effects of chalcones on Spontaneous alternation behavior of scopolamine induced amnesic rats. Data represent mean ± S.E.M of ten rats.

Cont SCP ISL BUT DHC HDMC DCC DCCP CMC CMCP SCP (1mg/kg, i.p)

abc Indicate statistical significance in comparison to vehicle treated group at P< 0.05, P < 0.01, P < 0.001, respectively with ANOVA followed by post hoc Tukey test.
Figure 3. Effects of chalcones on the number of arm entries of scopolamine induced amnesic rats. Data represent mean ± S.E.M of ten rats.
Figure 4. Effects of chalcones on Transfer latency of scopolamine induced amnesic rats. Data represent mean ± S.E.M of ten rats.

Cont SCP ISL BUT DHC HDMC DCC DCCP CMC CMCP

SCP (1mg/kg, i.p)

a-c Indicate statistical significance in comparison to vehicle treated group at P< 0.05, P < 0.01, P < 0.001, respectively with ANOVA followed by post hoc Tukey test.
Table 5. Effects of chalcones on acetylcholinesterase activity of different brain parts and spinal cord. Data are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Specific activity of AChE (μmol/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cerebrum</td>
</tr>
<tr>
<td>Control (6)</td>
<td>4.2620 ± 0.13</td>
</tr>
<tr>
<td>ISL (6)</td>
<td>2.7633 ± 0.17&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>BUT (6)</td>
<td>3.4123 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DHC (6)</td>
<td>2.5685 ± 0.13&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>HDMC (6)</td>
<td>3.3053 ± 0.11&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>DCC (6)</td>
<td>4.1655 ± 0.18</td>
</tr>
<tr>
<td>DCCP (6)</td>
<td>4.1762 ± 0.19</td>
</tr>
<tr>
<td>CMC (6)</td>
<td>4.0657 ± 0.21</td>
</tr>
<tr>
<td>CMCP (6)</td>
<td>3.9340 ± 0.21</td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate number of rats. <sup>a</sup>-<sup>c</sup> Indicate statistical significance in comparison to vehicle treated group at P< 0.05, P < 0.01, P < 0.001, respectively with ANOVA followed by post hoc Tukey test.
Discussion

In the present study, we examined the effects of variably substituted chalcones on scopolamine-induced memory impairment in the Y-maze task, and the Elevated plus maze test in rats. Scopolamine, a non-selective muscarinic antagonist blocks cholinergic signaling without changing the acetylcholine concentration, and produces memory deficits that are similar to those found in age related senile CNS dysfunction (Ebert and Kirch, 1998). Thus the scopolamine-induced amnesic murine model is useful for investigating age-related senile CNS dysfunction.

The ameliorative effects of chalcones (ISL and BUT, 25 mg/kg, i.p) on learning and memory was investigated by using the Y-maze task because spontaneous alternation behavior in the Y-maze is considered to reflect working memory. Scopolamine decreased spontaneous alternation to approx 24%. Chalcones (ISL and BUT, 25 mg/kg, i.p) increased spontaneous alternations to approx 22% by ISL (isoliquiritigenin) and 17.5% by BUT (butein) respectively of the scopolamine treated group. In the Y-maze test, no significant changes in total number of arm entries after drug treatment means that the treatment has
no effect on general locomotor activity (Sarter et. al., 1988), and the same is observed in the present study.

In order to confirm the effects of chalcones on another type of learning and memory, we used elevated plus maze. Increase in transfer latency of 64 % was observed in the scopolamine- treated group as compared to vehicle treated control group. The chalcones, ISL and BUT (25 mg/kg, i.p) treated group ameliorated memory impairment due to scopolamine by ISL to 24 % and BUT to 18.6 % respectively, i.e. decreasing the transfer latencies to vehicle-treated control group level. Collectively, these behavioral studies suggest that chalcones improve the memory in amnesic rats induced by scopolamine.

To investigate the mode of action of chalcones, their AChE inhibitory activity was assessed using rat brain parts and spinal cord homogenates. It is well known that the anti- amnesic effects of tacrine and donepezil are due to AChE inhibition in brain (Murray et. al., 1991; Dawson and Iverson, 1993). Our study showed that all the four hydroxy substituted chalcones i.e. ISL, BUT, DHC, and HDMC significantly decreased AChE activity but it is interesting to note that improvement in memory is only observed with only two chalcones i.e. ISL and BUT.
The central cholinergic system is considered to be most important neurotransmitter involved in regulation of cognitive functions (Enz et. al., 1993). Cognitive dysfunction has been shown to be associated with reduced cholinergic transmission and the facilitation of central cholinergic transmission with improved memory (Bhattacharya et. al., 1993). AChEI (acetylcholinesterase inhibitor) treatment, increasing the availability of ACh, improves cognitive function in AD (Evans et. al., 1989; Ballard, 2002; Wilkinson et. al., 2004). In the present study, chalcones when administered for 7 days showed significant reduction of brain and spinal cord acetylcholinesterase activity probably facilitating cholinergic activity by raising the level of ACh leading to improved cognitive action. The fact that in both humans and rats given anti-muscarinics apparent learning deficits are partially reversed by cholinergic agonists (e.g. AChE inhibitors; Collerton, 1986; Drachman, 1977) appears to support the notion of a specific role of ACh in learning and memory processes and the amnesia (Collerton, 1986). Our results suggest that the ameliorative activities of chalcones on memory dysfunction involve more than AChE inhibition.

Polyphenols stimulate the SIRT1 (a human deacetylase that promotes cell survival) (Tanny, 2001) protein and could be potential
regulators of aging associated processes. SIRT1 activators show promise as therapies for obesity, diabetes, age-related disease, Alzheimer’s disease and stroke (Davies and Bozzo, 2006). Also, activation of sirtuin extends lifespan and promotes longevity and healthy aging in a variety of species, potentially delaying the onset of age-related neurodegenerative disorders (Gan, 2007). According to Howitz et. al. (2003) isoliquiritigenin and butein are sirtuin activators. Storlin et. al. (1990), proposed that the activity of MAO-B (monoamine oxidase-B), appears to increase with age in various regions of the human and rat brain. This increase in MAO-B is mainly associated with the proliferation of astrocytes that accompanies the neuronal loss that occurs in aging and AD (Riederer et. al., 1986). MAO inhibitors are potential candidates as anti-Alzheimer drugs (Carreiras, 2004). Recently isoliquiritigenin was considered as MAO-B inhibitor (Kong et. al., 2000). Thus, suggesting ISL (2’, 4’, 4- trihydroxychalcone) as dual AChE/MAO inhibitors is of great interest. Angiotensin- converting enzyme inhibitors reversed the learning deficits in aged mice or those induced by scopolamine (Barnes et. al., 1989). Butein has been shown to be a potent inhibitor of angiotensin converting enzyme (Kang, 2003). However, the memory enhancing effects of chalcones has not been
described previously in animals. These data also support our present findings. The present study suggests that chalcones are responsible for the anti-amnesic activity in scopolamine-induced memory deficits in rats. These findings also suggest the possible neuroprotective role for chalcones.

Many lines of evidence suggest that uses of antioxidant or anti-inflammatory agents (Quintanilla et. al., 2005), melatonin (Jang et. al., 2005), Egb761 (Ahlemeyer and Krieglstein, 2003), blueberry (Joseph et. al., 2003), NSAIDs (Hirohata et. al., 2005) effectively alleviate the symptoms of AD. Anti-inflammatory and oxidant properties may contribute to the memory enhancement effect (Dhingra et. al., 2004; Joshi, 2007). Oxygen free-radicals are implicated in the process of age-related decline in cognitive performance may be responsible for the development of Alzheimer’s disease in elderly persons (Sinclair et. al., 1998; Berr, 2002; Butterfield and Lauderback, 2002). Moreover, chalcones have been shown to have potent antioxidative potential (Anto et. al., 1995). Recently, it has been reported that ACh in vitro, affecting the lymphocytic cholinergic system, attenuate the release of cytokines (Borovikova et. al., 2000). Overproduction of interleukin-1 within the brain is associated with Alzheimer disease and other neurological
disorders. AChE inhibitors (AChEIs) have a direct role in the inhibition of the release of inflammatory substances from specialized cells (Giunta et. al., 2004). Cyclooxygenase-2 and inducible nitric oxide synthase may be important in the prevention of memory deficits, one of the symptoms related to AD (Patil et. al., 2003). Isoliquiritigenin has been reported to suppress cyclooxygenase-2, known to play an important role in inflammation (Takahashi et. al., 2000) and also blocked pro-inflammatory cytokine-induced expression of VCAM-1, E- selectin through NF-kB signal disruption (Kwon et. al., 2007). Butein suppressed the nuclear factor-(NF)kB activation induced by various inflammatory agents and also the expression of cyclo-oxygenase (Pandey et. al., 2007). These factors may also contribute favorably to the memory enhancement effects of our chalcones.

Conclusion

The above behavioral and biochemical results suggest that chalcones (ISL and BUT) have the ability to improve or ameliorate memory dysfunction, in part, by facilitating the cholinergic transmission in brain. Moreover, our data indicate that chalcones may represent for the development of anti-amnesic.