Neuroprotective, anti-amnestic and anti-anxiety potentials of MEMJCM-2 in mice

Hanumantachar Joshi1, Charan C S2, Maged Abdo Alkanad3

1Principal, 2,3Assistant Professor, 1,2Dept. of Pharmacognosy, 2Dept. of Pharmacology, Sarada Vilas College of Pharmacy, Mysore, Karnataka, India

*Corresponding Author:
Email: amanjoshi17@yahoo.com

Abstract
Introduction: Memory impairments are the most disabling features of many disorders, impairing the normal daily activities of the patients and profoundly affecting their families. The current study was undertaken to ascertain the effects of MEMJCM-2 as a memory enhancing agent.

Materials and Methods: Elevated plus maze and Morris water maze were employed to evaluate acquisition and retention parameters. MEMJCM-2 (2, 5 and 10 mg/kg, p.o.) was administered to both young and aged mice. Scopolamine, diazepam and normal aging induced amnesia were the interceptive behavioral models.

Result: MEMJCM-2 (2, 5 and 10 mg/kg, p.o.) significantly improved acquisition and retention in young mice and also reversed the amnesia induced by diazepam (1 mg/kg, i.p.), and scopolamine (0.4 mg/kg, i.p.). It also significantly reversed aging induced amnesia due to natural aging of mice. MEMJCM-2 (10 mg/kg, p.o.) was found to possess potential anti-anxiety effects. MEMJCM-2 (10 mg/kg, p.o.) profoundly increased whole brain acetyl cholinesterase inhibition activity.

Conclusion: MEMJCM-2 can be a useful potential memory restorative formulation in the treatment of dementia seen in the elderly.

Keywords: MEMJCM-2, Amnesia, Acquisition, Retention, Dementia, Anxiety.

Introduction
Alzheimer’s disease (AD) is a multifactorial neurodegenerative disorder and there is no effective cure for this devastating disease till date. AD is characterized by progressive loss of neurons mainly in hippocampus and cortex, which results in dysfunctions of cognition and emotion.1 AD affects people aged 65 and older most commonly.2,3 Developed regions have become “aged society,” and the number of adults with AD is increasing.4 The main pathological features of AD are extracellular deposits of amyloid β-proteins, neurofibrillary tangles, neuronal injury and synapse loss.3,5 However, the exact etiology of AD is still controversial. The cholinergic hypothesis of AD is well established,6 which implied that the cholinergic system is important for learning and memory processes.7 In clinical, donepezil (DON) and galantamine, which could elevate the level of acetylcholine (ACh), are now used for AD therapy.8,9 However, there are still some limitations, such as low efficacy, adverse effects for the long-term use10. Since AD is a multi-factorial disease of the central nervous system,11 multi-component and multi-target drugs, such as traditional Chinese medicine, might be useful for AD.12,13

MEMJCM-2 is a phytochemical based formulation comprising of Gangetin and pharmaceutical adjuvants. This suspension was prepared in our research laboratory using Gangetin. Other ingredients of the preparations were ascorbic acid, cardamom oil, methyl paraben, propyl paraben, propylene glycol, sodium carboxy methyl cellulose and purified water.

Materials and Methods
Preparation of MEMJCM-2: MEMJCM-2 suspension was prepared using ethyl acetate fractions of methanolic extract of dried roots of Asparagus adscendens and ethyl acetate fraction of dried leaves of Minususops elengi, methyl paraben, propyl paraben, propylene glycol, sodiumcarboxy methyl cellulose and purified water.

Drugs and Reagents: Scopolamine hydrobromide (Sigma Aldrich, USA), diazepam (Calmpose®, Ranbaxy, India) and piracetam (Nootropil®, UCB India Pvt. Ltd., India) were diluted in normal saline and administered intra peritoneally. Volume of administration was 1 ml/100 g. All the drugs were administered in the morning session i.e. 8 AM-9 AM on each day. 5, 5′-dithiobis nitrobenzoic acid (DTNB, Ellman’s reagent, Sigma, USA) and acetyl thiocholine (Sigma, USA) were used.

Acute Toxicity Studies: Acute toxicity studies were performed according to OECD/OCDE 421 guidelines,5 Male Swiss mice selected by random sampling technique were employed in this study. The animals were fasted for 4 h with free access to water only. The experimental protocol was approved by the IAEC, Sarada Vilas College of Pharmacy, Mysuru.

Laboratory Models for Testing Memory
Elevated Plus-maze: Elevated plus-maze served as the exteroceptive behavioral model to evaluate learning and memory in mice. The procedure, technique and end point for testing learning and memory was followed as reported earlier.14,15 The elevated plus maze for mice
consisted of two open arms [16 cm × 5 cm] and two covered arms [16 cm × 5 cm × 12 cm] extended from a central platform [5 cm × 5 cm], and the maze was elevated to a height of 25 cm from the floor. On the first day, each mouse was placed at the end of an open arm, facing away from the central platform. Transfer latency [TL] was defined as the time taken by the animal to move from the open arm into one of the covered arms with all its four legs. TL was recorded on the first day for each animal. The mouse was allowed to explore the maze for another 2 minutes and then returned to its home cage. Retention of this learned-task was examined 24 h after the first day trial.

**Morris Water Maze:** The MWM test was employed to assess learning and memory of the animals. MWM is a swimming model where the animals learn to escape on to a hidden platform. It consisted of a circular water tank (150 cm diameter, 45 cm height), filled with water (30 cm depth) maintained at 25°C. Water was made opaque with a white-coloured non-toxic dye. The tank was divided into four quadrants with the help of two threads, fixed at right angle to each other on the rim of the pool. A platform (10 cm²) of 29 cm height was located in the centre of one of these four quadrants which one referred as the target quadrant. The position of the platform was kept unaltered throughout the training sessions. In the present study the target quadrant was Q4. All the trials were completed between 09:00 and 17:00 hours.16-19

**Interoceptive Behavioral Models**

**Scopolamine induced amnesia:** Amnesia was induced by administration of scopolamine hydrobromide (0.4 mg/kg, ip) on 8th day and the TL recorded. Retention was recorded after 24 hr. MEMJCM-2 and piracetam (200 mg/kg) were administered for 8 days successively. On 8th day, after 45 min of administration of doses, scopolamine was administered and TL was noted after 45 min.

**Diazepam Induced Amnesia:** Diazepam, 1 mg/kg, ip was administered to young mice and TL was noted after 45 min of injection on 8th day and after 24 hr. MEMJCM-2 and piracetam (200 mg/kg, i.p.) were administered for 8 successive days. After 60 min of administration of the last dose on 8th day, diazepam (1 mg/kg, ip) was administered. TL was noted after 45 min of administration of diazepam and after 24 hr.21

**Estimation of Brain Acetyl Cholinesterase (AChE) Activity:** The animals were sacrificed by cervical decapitation under light anesthesia on the 8th day, 90 mins after administration of the last dose of. Immediately after decapitation whole brain was carefully removed from the skull. For preparation of brain homogenate, the fresh whole brain was weighed and transferred to a glass homogenizer and homogenized in an ice bath after adding 10 volumes of 0.9% w/v sodium chloride solution. The homogenate was centrifuged at 3000 rpm for 10 min and the resultant cloudy supernatant liquid was used for estimation of brain acetyl cholinesterase activity spectroscopically using the Ellman method.22

**Laboratory models for testing anxiolytic effects**

**Elevated plus Maze Model:** The plus-maze apparatus, consisting of two open arms (16 x 5 cm) and two closed arms (16 x 5 x 12 cm) having an open roof. The MEMJCM-2 (2, 5 and 10 mg/kg, p.o.) and vehicle were administered for 8 days once daily p.o. and the last dose was given on the 9th day, 60 min prior to experiment. The standard drug Diazepam was given at a dose of 2 mg/kg i.p. 60 min before starting the experiment. After proper treatment each mouse was placed at the center of the maze with its head facing the open arm. During the 5 min experiment, the behavior of the mouse was recorded as: the number of entries into the open or closed arms and time spent by the mouse in each of the arms. An arm entry was defined as the entry of all four paws into the arm. After each trial, the elevated plus-maze apparatus was wiped clean with ethanol (10%) solution.19,23

**Light and Dark Box Test:** Light-Dark Box Test Crawley and Goodwin procedure (1980) was done to assess the anxiolytic activity of the compounds (light-dark box test).21 The apparatus consisted of a light compartment and a dark compartment. Light dark box is a rectangular box of 46 x 27 x 30 cm (l x b x h), which is divided into 2 compartments. A central opening (7 x 7 cm) on the floor level is placed for the joining of the two compartment. For this experiment, albino mice were divided into four groups, each group comprising of four animals. Vehicle (distilled water 10 ml/kg), standard (diazepam 1 mg/kg), and MEMJCM-2 (2, 5 and 10 mg/kg, p.o.) were administered p.o. one hour after administration, each mouse was placed individually in the illuminated part of the light/dark box. During the test session of 5 min., latency (the time it takes for the animal to move into the dark compartment for the first time), number of entries into the light and dark compartments, total time spent in the light compartment were recorded.

**Open Field Test:** The mouse were treated with MEMJCM-2 (2, 5 and 10 mg/kg, p.o) or diazepam 1 mg/kg, ip). After the treatment-Sixty minutes for aqueous extract and 30 min for Diazepam- the animals were placed individually in the center of the arena and were subjected to a 5-min period to the open field test. The open field apparatus was an opaque plexiglass cage (72 x 72-cm) with walls 35-cm in height where the floor was divided with white lines by 16 squares (18 x 18-cm) of identical dimension. A digital video camera was installed above the cage to record the activity of the mice. The entire room, except the OF was kept dark during the experiment. Total number of crossings and central area rearing were measured.24

**Statistical Analysis:** All the results were expressed as mean ± standard error (SEM). The data was analyzed
using one-way ANOVA followed by Tukey Kramer’s test. P values <0.05 were considered as statistically significant.

Results

Acute Toxicity Studies: All the doses (2, 5, 50, 100 and 1000 mg/kg, p.o.) of MEMJCM-2 did not produce any mortality even with the highest dose (1000 mg/kg, p.o.) employed. Three submaximal doses (2 and 5 mg/kg, p.o.) were selected for further psychopharmacological and biochemical studies.

Effect on locomotor activity: In the present study, MEMJCM-2 (2, 5 and 10 mg/kg, p.o.) did not show any significant change in the locomotor function of animals (score 220±0.9, 217±1.3 and 210±10) as compared to control group (score 215±4±11) when tested using a photoactometer.

Effect on transfer latency (TL) using elevated plus maze: MEMJCM-2 (2, 5 and 10 mg/kg, p.o.) showed dose-dependent reduction in TL of 8th day and 9th day, indicating remarkable improvement in learning ability and memory of the young and aged mice as compared to respective control groups (Graph 1). Diazepam (1 mg/kg, i.p.) and scopolamine (0.4 mg/kg, i.p.) significantly increased (P < 0.01) the TL of 9th day indicating impairment in memory (amnesia). MEMJCM-2 (2, 5 and 10 mg/kg, p.o.) successfully (P<0.001) reversed the amnesia induced by both diazepam and scopolamine (Graph 2).

Effect on Escape latency and TSTQ: In MWM extreroceptive model, there was a significant fall in ELT of standard drug and MEMJCM-2 treated groups as compared to control group which infers the improvement in learning ability (acquisition) of mice. Further there was a significant rise in TSTQ of standard drug and MEMJCM-2 (10 mg/kg) on 25th day compare D to TSTQ of normal control group. Even though there was a profound decrease in ELT and increase in TSTQ of MEMJCM-2 (2 mg/kg and 5 mg/kg, p.o) was not significant as compared to normal control group (Table 1).

Effect on Brain Cholinesterase Activity: MEMJCM-2 (2, 5 and 10 mg/kg, p.o.) showed a remarkable reduction in the brain acetyl cholinesterase activity in young and aged mice, as compared to respective control groups. Whereas, phenytoin (12 mg/kg, p.o.) significantly (P<0.01) increased the acetyl cholinesterase activity (Graph 3).

Light and Dark Box Test: Diazepam (0.5 mg/kg) significantly increased the time spent in light compartment (P<0.001) compared to normal group (Table 2). Significant increase in the time spent in the light compartment P<0.05 was seen with administration of MEMJCM-2 (2 and 5 mg/kg, p.o.) as compared to normal.

Open Field Test: MEMJCM-2 (5 mg/kg, p.o.) showed good anxiolytic activity as compared with normal mice. There was marked decrease in locomotion activity in animals treated with MEMJCM-2 (2 and 5 mg/kg, p.o.) as the number of squares crossed in the perimeter was decreased between the MEMJCM-2 treated groups and differed significantly from the control groups. The frequency of rearing also decreased significantly (Table 3). Treated groups and differed significantly from the control groups. The frequency of rearing also decreased significantly (Table 3).

Table 1: Effect of MEMJCM-2 on Escape Latency time (ELT) & Time Spent in Target Quadrant (TSTQ) in young mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose</th>
<th>ELT  (21 day)</th>
<th>ELT  (24 day)</th>
<th>TSTQ  (25 day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal Control</td>
<td>10 ml/kg p.o</td>
<td>91.06±0.57</td>
<td>49.13±0.91</td>
<td>59.05±0.4</td>
</tr>
<tr>
<td>II</td>
<td>Piracetam (Std)</td>
<td>400 mg/kg i.p.</td>
<td>73.10±1.9*</td>
<td>34.05±1.8*</td>
<td>70.69±1.1*</td>
</tr>
<tr>
<td>III</td>
<td>MEMJCM</td>
<td>2 mg/kg p.o</td>
<td>80.79±0.1</td>
<td>47.15±0.4 a</td>
<td>53.36±0.5a</td>
</tr>
<tr>
<td>IV</td>
<td>MEMJCM</td>
<td>5 mg/kg p.o</td>
<td>76.65±0.6a</td>
<td>42.45±0.2a</td>
<td>49.52±0.1a</td>
</tr>
<tr>
<td>V</td>
<td>MEMJCM</td>
<td>10 mg/kg, p.o</td>
<td>70.11±1.6*</td>
<td>32.94±150</td>
<td>59.13±1.2b</td>
</tr>
</tbody>
</table>

Each values represents mean±S.E.M. **P<0.001 compared to normal control. One-way ANOVA followed by Tukey’s post test

Table 2: Effect of MEMJCM-2 on number of squares crossed in open field apparatus

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (p.o.) mg/kg</th>
<th>Number of squares crossed</th>
<th>Rearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1 ml/kg</td>
<td>139±1.57</td>
<td>22.5±0.87</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.5</td>
<td>73.5±1.08a</td>
<td>8.12±0.56a</td>
</tr>
<tr>
<td>MEMJCM-2</td>
<td>2</td>
<td>112.11±5.8</td>
<td>17.45±0.8</td>
</tr>
<tr>
<td>MEMJCM-2</td>
<td>5</td>
<td>56.40±12.80</td>
<td>9.75±0.12a</td>
</tr>
<tr>
<td>MEMJCM-2</td>
<td>10</td>
<td>22.15±1.7</td>
<td>7.19±0.23a</td>
</tr>
</tbody>
</table>

p values a<0.001, b<0.01 as compared to normal treated group. Statistical test employed was ANOVA followed by Tukey-Kramer multiple comparison tests.
Table 3: Effect of on MEMJCM-2 time spent by mice behavior in social interaction test

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (p.o.) mg/kg</th>
<th>Time spent (sec) in social interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1 ml/kg</td>
<td>36.14 ±2.7</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.5 mg/kg</td>
<td>71.45± 2.14a</td>
</tr>
<tr>
<td>MEMJCM-2</td>
<td>2</td>
<td>47.16 ±5.2a</td>
</tr>
<tr>
<td>MEMJCM-2</td>
<td>5</td>
<td>56.13±1.78b</td>
</tr>
<tr>
<td>MEMJCM-2</td>
<td>10</td>
<td>50.57±0.16 b</td>
</tr>
</tbody>
</table>

p values a <0.001, b<0.01 as compared to normal treated group. Statistical test employed was ANOVA followed by Tukey-Kramer multiple comparison tests.

Graph 1: Effect of on MEMJCM-2 on transfer latency of young and aged mice in elevated plus maze

Values are mean ±S.E.M. (n=6). * indicates P< 0.01 as compared to control group of young mice. a indicates P< 0.001 as compared to control group of young mice. b indicates P< 0.01 as compared to control group of aged mice. c indicates P< 0.001 as compared to control group of aged mice. (One way ANOVA followed by Tukey-kramer multiple comparison tests)

Graph 2: Effect of on MEMJCM-2 on transfer latency of diazepam and scopolamine induced amnesic mice

Values are mean ±S.E.M. (n=6); * indicates P< 0.01 compared to control (young); a indicates P< 0.01 compared to diazepam group alone; b indicates P< 0.001 compared to diazepam (group alone. c indicates P< 0.01 compared to scopolamine group alone; d indicates P< 0.001 compared to scopolamine group alone;
Graph 3: Effect of on MEMJCM-2 on acetyl cholinesterase activity in mice

Values are mean ±S.E.M. (n=6); * indicates P< 0.01 compared to control (young); a indicates P< 0.001 compared to control (young); b indicates P< 0.01 as compared to control (aged); c indicates P< 0.001 compared to control (aged mice);

Discussion

Cognitive disorders are often the most disabling feature of many disorders, impairing the normal daily activities of the patients and profoundly affecting their families. The ancient Ayurvedic physicians had understood the delicate cellular mechanisms of the body and the deterioration of the functional efficiency of the body tissues. This revitalization and rejuvenation is known as the ‘rasayanyakshita’ (rejuvenation therapy). Rasayana drugs act inside the human body by modulating the neuro-endocrino-immune systems and have been found to be a rich source of antioxidants. Brahmirasayana, Trikutuchurna were reported to exhibit significant decrease in AChE activity in whole brain homogenates of mice, indicating their anti-cholinesterase potential. They had also reversed diazepam, scopolamine and ageing-induced impairment in learning and memory in mice. Glycyrrhiza glabra and ascorbic acid were proved to be memory enhancers in earlier studies, from our laboratory. MEMJCM-2 successfully reversed scopolamine, diazepam or ageing-induced amnesia, when administered for successive 8 days. Piracetam, the established nootropic agent was used in the present study for comparison because, it improves memory as a net result of several protective actions such as increased resistance to adverse conditions, brain protection against physical and chemical injuries and enhancement of reserve energy stores. Piracetam also increased choline uptake in cholinergic nerve endings, thereby facilitating cholinergic transmission. Plant extracts of Zingiber officinale, Nardostachys jatamansi, Foeniculum vulgare, Hibiscus sabdariffa, Desmodium gangeticum and Piper nigrum have been found to possess nootropic effects and they had significantly lowered the whole brain AChE activity. MEMJCM-2 exhibited highly significant anticholinesterase activity in both young and aged mice. Thus, it is possible that enhanced cholinergic transmission resulting from increased acetylcholine synthesis in brain due to abundant availability of choline and reduction of brain cholinesterase activity in young and aged mice may explain the memory improving effect exhibited by MEMJCM-2.

Conclusion

MEMJCM-2 can be of enormous use in the preliminary management of early symptoms of cognitive dysfunctions such as Alzheimer’s disease and dementia. Further investigations using human volunteers are warranted for further confirmation of nootropic potential. The possible involvement of other neurotransmitters like glutamate, GABA, catecholamines, serotonin etc. in the pathogenesis of cognitive disorders.

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