NOOTROPIC ACTIVITY OF L-33 – A POLYHERBAL FORMULATION

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Summary

L-33 (wilmer syrup) is a polyherbal formulation, consisting of plant ingredients of Brahmi (\textit{Bacopa monniera}), Yastimadhu (\textit{Glycyrrhiza glabra}), Tagar (\textit{Valeriana wallechii}) and Ashwagandha (\textit{Withania somnifera}). The present study was undertaken to investigate the effects of L-33 on learning and memory in experimental animals. Elevated plus-maze (EPM) and passive avoidance paradigm were employed to test learning and memory. Scopolamine (1mg/kg i.p.) and diazepam (1mg/kg i.p.) were used as interoceptive (stimulus inside the body) behaviour model. Three doses (5, 10 and 15 ml/kg p.o.) of L-33 were administered for 7-14 successive days in separate groups of animals. Elevated plus-maze (EPM) and passive avoidance paradigm model results show that dose of 15 ml/kg of L-33 significantly improved learning and memory of mice. Furthermore, this dose significantly reversed the amnesia induced by diazepam (1mg/kg i.p.) and scopolamine (1mg/kg i.p.). Since scopolamine-induced amnesia was reversed by L-33, it is possible that the beneficial effect on learning and memory was due to facilitation of cholinergic-transmission in mouse brain, also diazepam which is a GABA mimetic agent induces memory impairment and the subsequent inhibition of diazepam induced amnesia by L-33 may be due to inhibition of GABA-B receptors has been found to facilitate learning and memory.

Keywords: L-33 (Wilmer syrup), Amnesia, Learning, Memory, Nootropic.

Introduction

Memory is the ability of an individual to record sensory stimuli, events, information etc., retain them over short or long periods of time and recall the same at a later date when needed. Poor memory, lower retention and slow recall are common problems in today’s stressful and competitive world (1). Dementia is a mental disorder characterized by loss of intellectual ability sufficiently severe as to interfere with one’s occupational or social activities (2). Age has main role in the prevalence of dementia (3). Nootropics represent a new class of psychotropic agents with selective facilitatory effect on integrative functions of the central nervous system, particularly on intellectual performance, learning capacity and memory (4).
Typically these are thought to work by increasing the brain’s supply of neurochemicals (neurotransmitters, enzymes and hormones), improving brain’s oxygen supply or by stimulating nerve growth. Nootropics agents such as piracetam, aniracetam and donepezil are being used for improving memory, mood and behaviour, but the resulting side-effects associated with these agents have made their applicability limited. The central cholinergic pathways play a vital role in learning and memory processes (5). Centrally acting drugs (e.g. scopolamine, diazepam) impair learning and memory both in animals and human beings (6, 7, 8, 9). Indian ayurvedic system of medicine emphasizes use of herbs, nutraceuticals of life style changes for controlling age related neurodegenerative disorders (4).

In the Indian ayurvedic system of medicine, Brahmi (Bacopa monniera) used as a nerve tonic, antiepileptic (10,11), diuretic (12), to reduce stress induced anxiety, nootropic (13,14), sedative, anti-inflammatory (12,15), antidepressant and for adaptogenic activities (16,17), Yastimadhu (Glycyrrhiza glabra) used as a nerve tonic, peptic ulcer, rheumatoid arthritis, Tagar (Valeriana wallechii) used as a carminative, stimulant, antispasmodic and nervous disorder (18), and Ashwagandha (Withania somnifera) used as a anti-stress, sedative, hypnotic, antihelmintic and diuretic and as an immuno-modulatory agent(19).

In the present study, we have focused upon exploring the potential of an Indian ayurvedic poly-herbal formulation, L-33 (Wilmer syrup) for its efficacy in reversing the memory deficits and for its improving acquisition and memory retention in experimental animals. Brahmi (Bacopa monniera), Yastimadhu (Glycyrrhiza glabra), Tagar (Valeriana wallechii) and Ashwagandha (Withania somnifera) are ingredients of polyherbal formulation L-33.

Materials and Methods

Drugs and chemicals
L-33 (Annapurna Bio Ved Pvt. Ltd., Hyderabad, a Herbal Pharma Industry), Piracetam (‘Neurocetam syrup’, Brown and Burk. India), Scopolamine (‘Hyoscine’ German Remedies, India), Diazepam (‘Calmpose’ Ranbaxy, India), Lithium carbonate (‘Licab’ Torrent, Solan, India.) and Phenytoin (Sigma, USA), Sodium Nitrite (Sd-fine-chemicals, Mumbai) were used in the present study.

Animals
Swiss albino mice of either sex weighting 25-30 g used for the study. They had free access to food and water, and were maintained under standard laboratory conditions with alternating light and dark cycles of 12 h. The animals were fed with commercially available rat pelleted diet, and were acclimatized for at least 7 days before behavioural study. The experimental protocol was approved by the Institutional Animals Ethics Committee (IAEC No. 576/2002/bc/IAEC/CPCSEA) of N.E.T Pharmacy College, Raichur.

Determination of acute toxicity (LD50)
The acute toxicity of L-33 was determined by using albino mice (20-30g). The animals were fasted for 4 hrs prior to the experiment and up and down procedure (OECD guideline no. 425) method of CPCSEA was adopted for acute toxicity studies (20). Animals were administered with twice daily (0.1ml) of formulation and observed for its mortality during 48 hours study period (short term) toxicity. Based on the short term profile of drug, the dose for the next animals were determined. All the animals were observed for long term toxicity (0.1ml, twice daily for 25 days) and did not produce any obvious toxicity or mortality. Hence, the formulation was ensured to be devoid of any potential toxicity and obvious mortality. Further, the different doses of L-33 (5, 10,15ml/kg) were selected for study based on laboratory experience (21).
Animal models for testing learning and memory

(i) Passive avoidance paradigm (Exteroceptive Behaviour Model)
Passive avoidance behaviour based on negative reinforcement was used to examine the long-term memory. The apparatus consisted of an inverted petridish placed in the centre of the grid floor (Instruments and Chemicals Pvt. Ltd, Ambala) was used. The petridish served as the shock-free zone (SFZ). Each mouse was gently placed on the SFZ set in the center of the grid floor. When the mouse stepped down and placed all its paws on the grid floor, shocks (20V) were delivered for 15 sec and the step-down latency (SDL) was recorded. SDL was defined as the time taken by the mouse to step down from SFZ to grid floor. Animals were trained to remain on the SFZ for at least 60 sec and mice which did not meet these criteria in five trials were rejected. Observations were made for acquisition i.e. the number of trials required to reach the learning criteria and for retention of learning for 10 min at 2 h and 24 h post-training. The following retention parameters like step-down latency (SDL) in seconds, step-down error (SDE) as the number of times the animal stepped down from the SFZ and the time spent in the shock zone (TSZ) in seconds are noted (22, 23).

Group of adult Swiss male albino mice 25-30g, each consisting of six animals (n=6) were divided into following groups and animals were fasted overnight prior to the test but water was supplied ad libitum.

Group I: Normal control group: distilled water (10 ml/kg) was administered p.o. for 14 days.
Group II: Negative control group: Phenytoin alone (25mg/kg) was administered p.o. for 14 days.
Group III: Standard control group: Piracetam (standard 200 mg/kg) + Phenytoin (25 mg/kg) was administered p.o. for 14 days.
Group IV, V and VI: L-33 (5, 10 and 15 mg/kg, twice daily) + Phenytoin (25mg/kg, p.o.) was administered p.o. for 7 days i.e. 8th to 14th day.

(ii) Elevated plus-maze (Exteroceptive Behaviour Model)
Elevated plus-maze (EPM) served to evaluate learning and memory in mice. The procedure, technique and end point for testing learning and memory was followed as per the parameters described by the investigators working in the area of neuropsychopharmacology. The apparatus consisted of two open arms (16 cm × 5 cm) and two enclosed arms (16 cm×5 cm×12 cm). The arms 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 the time taken by mouse with all its four legs to move into one of the enclosed arms. TL was recorded on the first day. If the animal did not enter into one of the enclosed arms within 90 s, it was gently pushed into one of the two enclosed arms and the TL was assigned as 90 s. Retention of this learned-task was re-examined 24 h after the first day trial i.e. second day(22, 23, 24).

Group of adult Swiss male albino mice 20-25g, each consisting of six animals (n=6) were divided into following groups and animals were fasted overnight prior to the test but water was supplied ad libitum.

Group I: Control group: distilled water (10 ml/kg) was administered p.o. for 7 days. After 90 min of administration on 7th day, TL was recorded. Retention of learned task was examined after 24 h.
Group II: Standard group: Piracetam (200 mg/kg) was administered p.o. for 7 days. After 90 min of administration on 7th day, TL was recorded. Retention of learned task was examined after 24 h.
Groups III, IV and V (n = 6): L-33 (5, 10 and 15 ml/kg, twice daily respectively) was administered orally for 7 days. TL was noted after 90 min of administration on 7th day and after 24 h. significant reduction in TL value of retention indicate improvement in memory. The inflexion ratio (IR) was calculated by the formula as follows (25).

\[
\text{Inflexion ratio (IR)} = \frac{(L_0 - L_1)}{L_0}
\]

Where \(L_0\) is the initial TL (s) on first day and \(L_1\) is the TL (s) on the second day.

(iii) Diazepam-induced amnesia (Interoceptive Behaviour Model)
In the present investigation the mice were divided into different groups (control, diazepam alone, piracetam + diazepam, L-33+diazepam treated group) comprising six animals in each group. L-33 (5, 10, 15 ml/kg) was administered to mice of different groups for 7 days i.e. 8th to 14th day. These mice were exposed to the training session using elevated plus maze on 8th day after 90 min of the last dose. Retention (memory) of the learned task was recorded after 24hr i.e. on 9th day. Amnesia was induced in separate groups (interoceptive models) by diazepam (1mg/kg i.p.) on 8th day after 90 min of the last dose. Piracetam 200mg/kg i.p. was injected for 8th days to the positive control group of animals. All groups were treated respectively as mentioned above for a period of 14 days. TL and IR were calculated by using elevated plus maze model as described above.

(iv) Scopolamine-induced amnesia (Interoceptive Behaviour Model)
In the present investigation the mice were divided into different groups (control, scopolamine alone, piracetam + diazepam, L-33 + diazepam treated group) comprising six animals in each group. L-33 (5, 10, 15 ml/kg) was administered to mice of different groups for 7 days i.e. 8th to 14th day. These mice were exposed to the training session using elevated plus maze on 8th day after 90 min of the last dose. Retention (memory) of the learned task was recorded after 24hr i.e. on 9th day. Amnesia was induced in separate groups (interoceptive models) by scopolamine (1mg/kg i.p.) on 8th day after 90 min of the last dose. Piracetam 200mg/kg i.p. was injected for 8th days to the positive control group of animals. All groups were treated respectively as mentioned above for a period of 14 days. TL and IR were calculated by using EPM model as described above (26, 27).

Statistical analysis
All results were expressed as mean ± standard error of mean (S.E.M.). Data was analyzed using one-way ANOVA followed by Dunnett’s ‘t’ test.

Results

(i) Effect of L-33 on passive avoidance learning and retention in mice
Piracetam (200mg/kg) and different dose levels of L-33 (5, 10 and 15ml/kg) were tested in different groups. L-33 at a dose of (15ml/kg p.o.) showed statistically significant increased SDL and decreased TSZ and number of errors as compared to standard (Table 1, Fig 1, 2 and 3).

(ii) Effect on transfer latency (using elevated plus-maze)
TL of first day reflected learning behaviour of animals whereas, TL of second day reflected retention of information or memory. L-33 at a dose of (15ml/kg, p.o.) shown statistically significant increased in IR as compared to standard (Table 2, Fig 4).
(iii) Effect of L-33 on inflexion ratio in mice (Diazepam-induced amnesic model)
Diazepam (1 mg/kg) injected before training impaired learning significantly. Diazepam has induced dose dependent amnesia and in this amnesic model, a decrease in IR was observed when compared to normal control group. Piracetam and all doses of L-33(5, 10 and 15ml/kg, p.o.) treated groups had exhibited nootropic activity (memory enhancing) with increase in IR and reduction in TL observed with EPM and reversed the diazepam-induced amnesia. Statistically significant reduction in TL was observed at a dose of 15ml/kg, p.o. of L-33 treated groups (Table 3, Fig 5).

(iv) Effect of L-33 on inflexion ratio in mice (Scopolamine-induced amnesic model)
Scopolamine treated group of mice exhibits impairment of memory and decrease in inflexion ratio as compared to normal control group, which indicates the induction of amnesia. Piracetam and L-33 (15ml/kg, p.o.) treated groups had shown significant increased IR, reduction in TL and reversed the scopolamine-induced amnesia (Table 4, Fig 6)

Discussion
Memory enhancing drugs are thought to work by increasing the brain’s supply of neurochemicals (neurotransmitters, enzymes and hormones), improving brain’s oxygen supply or by stimulating nerve growth. Nootropics agents such as piracetam, aniracetam and choline esterase inhibitors like donepezil are being used for improving memory, mood and behaviour but not used generally because of more side effects associated with these agents have made their applicability limited (4). In the present study, we have focused upon exploring the potential of ayurvedic poly-herbal formulation, L-33 for its efficacy in reversing the memory deficits, improving acquisition and memory retention in experimental animals using passive avoidance and EPM model (28).
In the present study, L-33 administered orally improved learning and memory of mice significantly in both the exteroceptive and interoceptive behavioural models. Furthermore, pre-treatment with L-33 (5, 10, 15 ml/kg) protected the animals from learning and memory impairment produced by interoceptive stimuli (diazepam and scopolamine). These findings suggested the possible neuroprotective role for L-33.
The polyherbal formulation L-33 and piracetam when given along with phenytoin, significantly reversed phenytoin-induced amnesia, protective effect was observed with all parameters tested at a dose of 15ml/kg, p.o. of L-33. In EPM acquisition (learning) can be considered as transfer latency on first day trials and the retention/ consolidation (memory) is examined 24 h later (29). The animal shows significant decrease in TL as compared with the standard group.
Diazepam (1mg/kg) is a GABA mimetic agent prolongs TL and decreases IR. L-33 (15 ml/kg) increases IR thus confirm their nootropic activity which is statistically significant as compare to standard. The protective effect offered by L-33 and Piracetam against diazepam-induced amnesic model may be due to indirect release of Ach in the brain.
The scopolamine (1mg/kg) is a muscarinic receptor antagonist act by prolonging TL i.e. decreases IR in EPM model (30). L-33 (15ml/kg, p.o.) have reversed amnesia induced by scopolamine indicating nootropic activity which is statistically significant as compare to standard.

Conclusion
In the present investigation, the poly herbal formulation L-33 has shown a potent memory enhancing effect at a dose of 15ml/kg, p.o. in exteroceptive and interoceptive animal models.
Table 1. Effect of L-33 on passive avoidance learning and retention in mice (Mean ± Sem)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose (per Kg/p.o.)</th>
<th>No. of trials for acquisition</th>
<th>Step-Down Latency (SDL) Learning</th>
<th>Time Spent In Shock Zone (TSZ) Learning</th>
<th>Step-Down Error (SDE) Learning</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>10 ml</td>
<td>2.6</td>
<td>14.66±0.61</td>
<td>86.50±2.82</td>
<td>16.16±0.16</td>
</tr>
<tr>
<td>II</td>
<td>Phenytoin</td>
<td>25 mg</td>
<td>2.7</td>
<td>13.33±1.02**</td>
<td>85.16±2.27**</td>
<td>17.00±0.25</td>
</tr>
<tr>
<td>III</td>
<td>Piracetam</td>
<td>200 mg</td>
<td>3.1</td>
<td>92.16±1.19**</td>
<td>275.83±0.94**</td>
<td>3.16±0.16</td>
</tr>
<tr>
<td>IV</td>
<td>L-33</td>
<td>5 ml</td>
<td>2.6</td>
<td>48.33±0.42*</td>
<td>194.33±0.61*</td>
<td>6.16±0.16</td>
</tr>
<tr>
<td>V</td>
<td>L-33</td>
<td>10 ml</td>
<td>2.8</td>
<td>72.66±0.49*</td>
<td>256.16±0.30*</td>
<td>3.16±0.16</td>
</tr>
<tr>
<td></td>
<td>L-33</td>
<td>15 ml</td>
<td>3.0</td>
<td>82.66±0.66**</td>
<td>270.50±0.42**</td>
<td>3.50±0.22</td>
</tr>
</tbody>
</table>

n=6 in each group. Data is expressed as mean ±SEM. Statistical analysis by one-way ANOVA followed by Dunnett’s ‘t’ test Significance at p<0.05*, p<0.01**
Table 2. Effect of L-33 on Inflexion ratio (IR) in EPM Model, Diazepam induced amnesic model and Scopolamine induced amnesic model in mice (Mean ± Sem)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose (per Kg/p.o.)</th>
<th>IR (Mean±SEM) (EPM Model)</th>
<th>IR (Mean±SEM) Diazepam induced amnesic model</th>
<th>IR (Mean±SEM) Scopolamine induced amnesic model</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control (vehicle)</td>
<td>10 ml</td>
<td>0.2987± 0.011</td>
<td>0.3464ns± 0.05074</td>
<td>0.2681ns ± 0.02</td>
</tr>
<tr>
<td>II</td>
<td>Diazepam alone</td>
<td>1.0 mg i.p.</td>
<td>-</td>
<td>0.2821± 0.04446</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>Scopolamine</td>
<td>1.0 mg i.p.</td>
<td>-</td>
<td>-</td>
<td>0.1623 ±0.04</td>
</tr>
<tr>
<td>IV</td>
<td>Piracetam</td>
<td>200 mg</td>
<td>0.7016±0.007**</td>
<td>0.6594**± 0.02429</td>
<td>0.6570**± 0.008</td>
</tr>
<tr>
<td>V</td>
<td>L-33</td>
<td>5ml</td>
<td>0.4407±0.0156*</td>
<td>0.4218*± 0.02115</td>
<td>0.3843**± 0.03990</td>
</tr>
<tr>
<td>VI</td>
<td>L-33</td>
<td>10ml</td>
<td>0.6026±0.00902**</td>
<td>0.5113**± 0.02583</td>
<td>0.6355**± 0.04614</td>
</tr>
<tr>
<td>VII</td>
<td>L-33</td>
<td>15ml</td>
<td>0.6934±0.0299**</td>
<td>0.6395**± 0.02084</td>
<td>0.6314**± 0.03261</td>
</tr>
</tbody>
</table>

n=6 in each group. Data is expressed as mean ±SEM. Statistical analysis by one-way ANOVA followed by Dunnett’s ‘t’ test Significance at p<0.05*, p <0.01** and ns-not significant vs. control group.
Fig 1: Nootropic effect of L-33 on SDL in mice

Fig 2: Nootropic effect of L-33 on TSZ in mice

Fig 3: Nootropic effect of L-33 on SDE in mice

Fig 4: Nootropic effect of L-33 on IR in mice (EPM model)

Fig 5: Nootropic effect of L-33 on Diazepam-induced amnesia in mice (EPM Model)

Fig 6: Nootropic effect of L-33 on Scopolamine induced amnesia in mice (EPM model)
References

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