Influence of Unani polyherbal formulation on learning and memory retention in mice

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1. INTRODUCTION

The nootropic drugs belong to the class of psychotropic agents with selective facilitatory effect on intellectual performance, learning and memory. Dementia is a mental disorder characterized by loss of intellectual ability sufficiently severe as to interfere with one’s occupational or social activities. Dementia is of several types and it invariably involves impairment of memory. The most common cause of dementia is Alzheimer’s disease, which is a progressive neurodegenerative disorder associated with loss of neurons in distinct brain areas. The central cholinergic pathways play a prominent role in learning and memory processes. Centrally acting antimuscarinic drugs

ABSTRACT

The drug discovery should not be always limited to discovery of a single molecule and current belief one disease one drug approach may be untenable in the future and that rationally designed polyherbal formulations could also be investigated as an alternative in multi-target therapies and prophylaxis. Considering this the influence of Unani Polyherbal Formulation (UPF) was investigated for its nootropic activity. To investigate nootropic activity of the UPF various experimental paradigms of learning and memory were used including transfer latency (TL) on elevated plus-maze, spatial memory evaluation using radial arm maze, passive avoidance response (PAS) and object recognition test. Mice were divided into four groups viz vehicle treated, UPF 200 mg/kg, UPF 400 mg/kg and piracetam 150 mg/kg as standard. The investigation reported that UPF 200 and 400 mg/kg significantly reduced the TL on 2nd and 9th day and significantly increased the step down latency in the PAS at acquisition and retention test. In radial arm maze task UPF 200 and 400 mg/kg showed significant decrease in the days to make the mice learned and latency to find food in reference as well as working memory. UPF also attenuated scopolamine induced memory deficit. Furthermore the UPF 200 and 400 mg/kg increased discrimination index in the object recognition test indicating nootropic activity. To conclude UPF showed significant facilitatory effect on aversively motivated learning and memory in mice, spatial learning and memory and improvement of memory in absence of cognitive deficit.

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(e.g. scopolamine) impair learning and memory both in animals\(^6\) and human beings\(^4\). A number of drugs including piracetam have introduced in therapy to ameliorate cognitive deficits\(^5\).

Since allopathic system of medicine is yet to provide a radical cure, it is worthwhile to look for new directions, which would minimize the memory loss seen in elderly patients\(^6\). Recently it has been suggested that the drug discovery should not be always limited to discovery of a single molecule and current belief one disease one drug approach may be untenable in the future and that rationally designed polyherbal formulations could also be investigated as an alternative in multi-target therapies and prophylaxis\(^7\). Development of standardized, safe and effective herbal formulations with proven scientific evidence can also provide an economical alternative in several disease areas\(^8\).

Plant drugs and herbal formulation are frequently considered to be less toxic and free from side effects than synthetic one\(^9\). Unani Medicine is a form of traditional medicine practiced in middle-east & south-asian countries. Many formulations have been used in the Unani system of medicine since hundreds of years for treatment of central nervous system disorders\(^10\). We used one such Unani polyherbal formulation composed of Emblica officinalis, Delphinium denudatum Wall, Phoenix dactylifera, Prunus amygdalus Batsch, Benincasa hispida, Trapa bispinosa, Centella asiatica, Paeonia officinalis, Evolvulus alsinoides, Pistacia lentiscus, Sphaeranthus indicus and rose water. All of the above mentioned herbs are reported for neuropharmacological activity\(^11\)-\(^22\). Even though this formulation has been used in the Unani system of medicine, there is need to validate its efficacy in using modern scientific parameters. Therefore, the present study was carried out to evaluate the influence of Unani polyherbal formulation (UPF) on learning and memory using experimental models of learning and memory.

2. MATERIALS AND METHODS

2.1. Unani polyherbal formulation (UPF)

A Unani polyherbal formulation composed exclusively of herbal medicines which are also quoted in all classical Ayurvedic literature and references was prepared. Its main ingredients include the following:

- Emblica officinalis 100 g,
- Delphinium denudatum Wall 10 g,
- Phoenix dactylifera 10 g,
- Prunus amygdalus Batsch 10 g,
- Benincasa hispida 10 g,
- Trapa bispinosa 10 g,
- Centella asiatica 5 g,
- Paeonia officinalis 2 g,
- Evolvulus alsinoides 2 g,
- Pistacia lentiscus 2 g,
- Sphaeranthus indicus 2 g

and rose water. All of the above mentioned herbs were received as gift sample from Registered Medical (Unani) Practitioner from Pune, India.

2.2. Chemicals and drugs

Piracetam syrup (Nootropil\(^®\)) from UCB, India and scopolamine injection (Buscopan\(^®\)) from Cadila Healthcare, India purchased from a local market.

2.3. Animals

Swiss male albino mice (18-22g) obtained from our animal house was used. They were maintained at 25 ± 2°C and relative humidity of 45 to 55% and under standard environmental conditions (12 h light 12 h dark cycle). The animals had free access to food and water. Institutional Animal Ethical Committee approved the protocol. All experiments were carried out between 12:00-16:00 h. Institutional Animal Ethics Committee (IAEC) approved the protocol (CPCSEA/IAEC/PC-10/12) and entire study has carried out as per standard guideline of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India and IAEC.

2.4. Acute toxicity test

Healthy adult male albino mice (18-22g) were subjected to acute toxicity studies as per guidelines (OECD 423) suggested by the organization for economic co-operation and development (OECD)\(^23\). The mice were observed continuously for 2 h for behavioral and autonomic profiles and for any sign of toxicity or mortality up to a period of seven days\(^11\).
2.5. **Object recognition test**

The apparatus fabricated locally consisted of white colored plywood (70 × 60 × 30 cm) with a grid floor. It was illuminated by a 40 W lamp suspended 50 cm above the apparatus. The object to be discriminated was also made of plywood in two different shapes of 10 cm height and colored black. One day before the test, mice were allowed to explore the box without any object for 2 min. Mice were divided into four groups each group consisting six animals. First group served as negative control and received vehicle (10 mL/kg). Second and third group served as treatment group and received UPF 200 and 400 mg/kg. Fourth group served as positive control and received piracetam 150 mg/kg. On the day of test, in the first trial (T1) conducted 60 min after administration of vehicle (10 mL/kg) or UPF (200 and 400 mg/kg) or piracetam (150 mg/kg) two identical objects were presented in opposite corners of the box and the time taken by each mouse to complete 20 s of object exploration was recorded (Exploration was considered as directing the nose at a distance less than 2 cm to the object and/or touching with nose). Second trial (T2) was performed 90 min after first (T1) and a new object replaced one of the objects presented in T1 and mice were left in the box for next 5 min. The time spent for exploring the familiar (F) and the new object (N) was recorded separately and discrimination index (D) was calculated as (N-F)/(N+F). The object was changed randomly and apparatus was cleaned with hydrogen peroxide after each trial to avoid place preference and the influence of olfactory stimuli respectively.

2.6. **Radial arm maze**

Locally fabricated wooden radial arm maze elevated 50 cm above the floor consisted of an octagonal central hub 36 cm in diameter with eight radial arms. Each arm 43 cm long, 15 cm wide with 12 cm sides, had small black plastic cups mounted at 30 cm from the central hub.

Each mouse maintained at 85% of its total diet had one acquisition trial per day followed by drug treatment. Animals were classified in four groups each consisting of six mice viz vehicle (10 mL/kg), UPF (200 and 400 mg/kg) and piracetam (150 mg/kg) used as reference standard.

The evaluation consisted of two different trials with different animals, where in a food pellet was placed in a fixed arm for spatial reference memory evaluation and in the variable arm for evaluation of spatial working memory. Each mouse placed on the central hub was allowed to choose any of the arms freely to get the food. The session was considered complete when the mouse had entered all arms and had looked into all cups or spent 5 min in the maze, the mouse was taken off the maze and brought back to its home cage.

During the trial, entry into an arm which mouse had not entered yet, and looked into the cup, was recorded as a correct entry; the reentry which is defined as entry where in it looked into the cup without turning back was counted as an error. The acquisition training was completed and mouse was labeled as learned mice when mouse found the food with only one reentry for three consecutive days.

The number of days required making for the mice learned, latency to find the food and number of initial correct entries (i.e. before first reentry) of learned mouse were considered to evaluate radial maze task performance. One-h interval was kept between the spatial reference and spatial working memory evaluation. The apparatus was cleaned with either hydrogen peroxide or damp cloth after each trial to avoid place preference and the influence of olfactory stimuli.

2.7. **Transfer latency in elevated plus-maze (EPM)**

This test was used to assess the retention of learning and memory. Animals were classified in four groups each consisting six mice viz vehicle (10 mL/kg), UPF (200 and 400 mg/kg) and piracetam (150 mg/kg) used as reference standard. Locally fabricated elevated plus maze consisting of two opposite open arms 35 × 6 cm, crossed with two enclosed arms, of the same dimensions with 15 cm high walls. The maze was kept in a dimly lit room elevated 40 cm above floor level.
On day 1, mice were individually placed on the end of one of the open arms, facing away from the centre, and the time taken by the animal to enter one of the closed arms (transfer latency \((TL)\) day 1) was recorded with the help of a stop watch. The mouse was left in the enclosed arm for 10 - 15 s and returned to its home cage. On day 2, the procedure was repeated and the day 2 TL was recorded. Similarly after an interval of 1 week, on day 9, the TL was again recorded.

2.8.1. Scopolamine induced amnesia:
This test was used to assess the memory functions. Scopolamine hydrobromide (1 mg/kg, s.c.) was administered immediately after the learning trial on day 1 and retention of the previously learned task was scored on day 2 and 9.

2.8. Step down type of passive avoidance response

2.8.1. Preselection test: In the preselection trial, each mouse was gently placed on the shock free zone (SFZ), when the mouse stepped down from the platform and placed all its paws on the grid floor. An electric shock (5mA, 20 V, AC) was delivered maximum for 15 s and step down latency (SDL) was noted down. Mice showing SDL between 02 - 25 s were selected for the study.

2.8.2. Step down type of passive avoidance response: The above selected mice were allowed to habituate in a shock chamber 10 min daily for next 3 days without the application of shock. From the 4th day, mice were treated with the UPF for a period of 10 days. The step down type of passive avoidance response was studied on 10th day. Mice were divided into four groups each group consisting six animals. First group served as negative control and received vehicle (10 mL/kg). Second and third group served as treatment group and received UPF 200 and 400 mg/kg. Fourth group served as positive control and received piracetam 150 mg/kg.

On the 10th day, 30 min after the last dose, training session consisting of two trials with 60 s inter-trial interval was conducted. Acquisition test was conducted 60 min later with the upper cut off time of 60 s. The step down latency was noted when the mouse stepped down before 60 s and electric shock was delivered for maximum 15 s and retention test was carried out after 24 h in a similar manner with upper cut off of 300 s without electric shock. Evaluation was carried out by comparing step down latency to that of vehicle treated control mice.

2.8.3. Scopolamine induced memory disruption: On the 10th day, 60 min after the last dose, acquisition trial was conducted for step down passive avoidance response as per the procedure. Thirty min thereafter, all mice were subjected to the subcutaneous injection of scopolamine (1 mg/kg) to induce memory disruption. These mice were tested 24 h later for step down passive avoidance response. The effect of the UPF was evaluated by comparing the step down latency of treatment group against respective control.

2.9. Statistical Analysis
The results are expressed as mean ± sem. Comparison between the groups was made by one way analysis of variance (ANOVA) followed by Dunnett’s test by using GraphPad InStat version 3.01, GraphPad software, San Diego California, USA. The p-value is compared with the desired significance level of our test and, if it is smaller (p < 0.05), the result is significant.

3. RESULTS

3.1. Acute oral toxicity test
All mice were free of any toxicity up to the dose of 2 g/kg. However sedation was noted above the dose of 1 g/kg. From this data, two different doses 200 and 400 mg/kg were selected for further study.

3.2. Object recognition test
UPF in a dose of 200 mg/kg did not produce any significant change in discrimination index. UPF 400 mg/kg treated mice showed significant increase in discrimination index (p<0.01) when compared against vehicle treated mice. Piracetam (150 mg/kg) was also significant (p<0.01) in both the tests.
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3.3. Radial arm maze

3.3.1. Latency to find food (spatial reference): The latency of vehicle treated control mice to find food was 47.55 ± 1.33 s. Pretreatment with UPF 200 and 400 mg/kg significantly reduced the latency to find food to 41.11 ± 1.05, and 37.21 ± 1.38 s, respectively. UPF 400 mg/kg were more effective (p<0.01) than UPF 200 mg/kg (p<0.05) in this regard. Mice pretreated with piracetam 150 mg/kg required 36.53 ± 1.45 s to find the food.

3.3.2. Latency to find food (spatial working): The latency of vehicle treated control mice to find food was 49.83 ± 1.75 s. UPF 200 and 400 mg were equally effective (p<0.01) than UPF 200 mg/kg (p<0.05) in reducing the latency to find food to 42.09 ± 1.65 and 42.23 ± 1.69 s, respectively. Piracetam 150 mg/kg treated mice required 36.86 ± 1.45 s to find the food.

3.3.3. Days required to make the mice learned (Spatial reference): The number of days required to make the mice learned for vehicle treated control mice were 11.56 ± 0.89. The pretreatment with UPF 200 and 400 mg/kg resulted in a dose dependent decrease (09.86 ± 0.52 and 08.79 ± 0.85) the number of days to make the mice learned (p<0.05). The piracetam 150 mg/kg treatment required 09.74 ± 0.39 days to make the mice learned. The results suggested the facilitation of spatial learning.

3.3.4. Days required to make the mice learned (Spatial working): Number of days required to make the mice learned for vehicle treated control mice was 12.13 ± 0.48. The pretreatment with UPF 200 and 400 mg/kg significantly (p<0.05) reduced the number of days to make the mice learned to 09.80 ± 0.63 and 09.43 ± 0.56. The mice pretreated with piracetam 150 mg/kg required 09.65 ± 0.58 days to make the mice learned. This reduction compared to control group was significant (p<0.05). The result suggested the facilitation of spatial learning by the pretreatment with UPF 200, 400 mg/kg and piracetam 150 mg/kg.

Figure 1. Effect of UPF on object recognition test. Values are expressed as mean ± standard error of mean. Data were analyzed by one way analysis of variance (ANOVA) followed by Dunnett test. P values: * < 0.05; ** < 0.01.
3.4. Transfer latency using EPM

On 2nd day the TL was significantly (p<0.01) reduced in mice pretreated with piracetam 150 mg/kg when compared to those of control mice. The TL in mice pretreated with UPF 200 and 400 mg/kg was insignificant. On the 9th day, in mice pretreated with UPF 200, 400 mg/kg and piracetam 150 mg/kg TL was significantly (p<0.01) reduced. Moreover UPF 200, 400 and piracetam 150 mg/kg significantly (p<0.01) attenuated scopolamine induced memory impairment on 9th day.

3.5. Step down type of passive avoidance response

The step down latency of vehicle treated control group was 120.1 ± 3.53 s. The step down latency of mice pretreated with UPF 200 and 400 mg/kg and piracetam 150 mg/kg was 138.6 ± 3.56, 169.34 ± 4.61 and 185.34 ± 4.76 s, respectively. In the above results, UPF 200 and 400 mg/kg exhibited significant increase in the step down latency when compared to those of control mice at acquisition as well as retention test indicating facilitation of learning followed by retention of learned task.

The step down latency in vehicle treated control mice after scopolamine injection was 42.95 ± 1.62 s while the pretreatment with UPF 200 and 400 mg/kg and piracetam 150 mg/kg observed step down latency as 97.29 ± 3.63, 105.71 ± 3.82 and 157.63 ± 4.22 s, respectively.

**Table 1** Effect of UPF on radial arm maze and step down type of passive avoidance response

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle 1 mL/kg</th>
<th>UPF 200 mg/kg</th>
<th>UPF 400 mg/kg</th>
<th>Piracetam 150 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency to find food- spatial reference (Second)</td>
<td>47.55 ± 1.33</td>
<td>41.11 ± 1.05*</td>
<td>37.21 ± 1.38**</td>
<td>36.53 ± 1.45**</td>
</tr>
<tr>
<td>Latency to find food- spatial working (Second)</td>
<td>49.83 ± 1.75</td>
<td>42.09 ± 1.65*</td>
<td>42.23 ± 1.69*</td>
<td>36.86 ± 1.45**</td>
</tr>
<tr>
<td>Days required to make the mice learned- spatial reference</td>
<td>11.56 ± 0.89</td>
<td>09.86 ± 0.52*</td>
<td>08.79 ± 0.85**</td>
<td>09.74 ± 0.39*</td>
</tr>
<tr>
<td>Days required to make the mice learned- spatial working</td>
<td>12.13 ± 0.48</td>
<td>09.80 ± 0.63*</td>
<td>09.43 ± 0.56*</td>
<td>09.65 ± 0.58*</td>
</tr>
<tr>
<td>Step down latency (Second)</td>
<td>120.1 ± 3.53</td>
<td>138.6 ± 3.56</td>
<td>169.34 ± 4.61**</td>
<td>185.34 ± 4.76**</td>
</tr>
<tr>
<td>Step down latency after scopolamine induced memory impairment (Second)</td>
<td>42.95 ± 1.62</td>
<td>97.29 ± 3.63**</td>
<td>105.71 ± 3.82**</td>
<td>157.63 ± 4.22**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard error of mean. Data were analyzed by one way analysis of variance (ANOVA) followed by dunnett test. P-values: * < 0.05; ** < 0.01
4. DISCUSSION

Various mazes are used conventionally to assess the learning and memory paradigms in laboratory animals. Radial arm maze task performance is an appetitively motivated task and is also useful to assess the preferential as well as working memory performance and agents that affect these processes. The difference in the significance of individual parameters of sessions do not necessarily relate particular aspect of preferential or working memory, however significant improvement in all parameters is an indication of more cognition enhancer effect of the drug. The dose showing significant improvement in all parameters could be considered as the most effective dose.

Thus the present study documented cognition enhancer activity of UPF and thereby indicated its possible use as a supportive adjuvant therapy to treat poor learners and patients with impaired memory functions. Moreover, it may be employed as a buffer against rapid age related decline in mental functions. The reported difference of the effectiveness of the extract towards preferential and working memory in these paradigms may be due to factors like experimental conditions, experimental protocol employed and modulation of specific neurotransmitters, neurochemicals involved.

In EPM, on the ninth day mice pretreated with UPF 200, 400 mg/kg and was significantly (p<0.01) reduced the TL. UPF 400 mg/kg significantly attenuate scopolamine induced memory impairment on second day while on ninth day UPF 200 and 400 mg/kg was significant in this regard. UPF significantly (p<0.01) cause improvement in discrimination index by 400 mg/kg proved that UPF met major criteria for nootropic activity, improvement of memory in absence of cognitive deficit. This observation has been strengthened by the finding that UPF has shortened the transfer latency in the elevated plus maze model indicating improvement in memory, which is in accordance with the hypothesis of Itoh J, et al.

Testing of laboratory animals for avoidance behavior is a classic method for the assessment of learning and memory processes. The UPF 400 mg/kg significantly increased step down latency in acquisition as well as retention test. Step down type of passive avoidance response involved training rodents to avoid punishment (electric shock) by curbing normal exploratory behavior. UPF also significantly (p<0.01) attenuate scopolamine induced memory impairments.

The above results suggested the significant facilitation of short term learning and consolidation of learned task by the UPF. The facilitation of learning and retention of learned task is useful in poor learners, while antiamnesic action is required once. The neuroprotective agent would be an ideal which partly or completely reverse such disruption.

In this study scopolamine, a muscarinic antagonist which induces transient impairment in an avoidance test was used. This is a widely cited model to evaluate antiamnesic potential of test drug. It has been reported that most of the drugs that prevent this impairment of memory mostly act via cholinergic modulation. Moreover, recent behavioral and neurobiological studies have provided evidences for cholinergic involvement in learning and memory. Cholinergic system involving acetylcholine and acetylcholinesterase activity is believed to affect the learning abilities. Earlier reports have also found that, drug affecting learning and memory function have modulated Ach content and or AchE activity. It is further found to play an important role in various diseases of central nervous system (CNS) like Alzheimer, Parkinson and dementia wherein impaired learning abilities and loss of memory are commonly observed manifestations. In the aforementioned results, effectiveness of UPF towards the facilitation of learning and retention of learned task and prevention of scopolamine induced memory disruption suggested the cholinergic modulation by the UPF.
5. CONCLUSION

UPF showed significant effect on spatial learning and memory behavior as well as aversion induced learning and memory behavior. The attenuation of scopolamine induced memory disruption may suggest possible use of drug against neurodegenerative disorder like Alzheimer disease. The effect may be due to various phytochemical present in the formulation. Moreover further study is necessary for detail understanding of mechanism and role of phyto constituent responsible for action.

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