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Original Article

Original Article Nootropic Medicinal Plants; Evaluating Potent Formulation By Novelestic High throughput Pharmacological Screening (HTPS) Method

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Abstract

The principle of this method was to screen the pharmacological activity of six prepared polyphyto formulations by using high throughput screening method for their nootropic action. The study was performed in three stages using one, two and three animals, respectively in a group. Test formulations were given p.o daily at the dose of 50 and 100 mg/kg body weight. The test formulations were compared with the standard drug *Bacopa monnieri* for learning and memory enhancement. The pharmacological response shown in single experimental animal was compared with that shown in two animals by using regression analysis. R^2 value was determined by Least squares linear regression analysis to interpret as the proportion of the variance in the dependent variable that is predictable from the independent variable. Of the six formulations, three were considered for further evaluation in three groups (n=3). Finally, one formulation that showed maximum response was tested in a group of 12 animals. One way ANOVA was used for estimating the statistical significance. The best formulations FM-06 (compose of *Celastrus paniculatus, Habiscus rosasinensis, Bacopa monnieri, Convolvulus pluricaulis, Phyllanthus emblica, and Mentha piperita)* was selected by high throughput screening and results for twelve animals in a group were found to be statistically similar.

Keywords: Habiscus rosasinensis, High throughput screening, Learning and memory, *Mentha piperita*, Nootropic agent, *Phyllanthus emblica*.

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1. Introduction

Animal research has played a vital role in scientific and medical advances and continues to improve our understanding on various diseases. At the same time it causes unnecessary harm to animals. Scientist would

prefer to avoid use of animal in large number if they are provided with an alternative way for evaluation of biological activity with accuracy. Bioscience community accepts the use of animals in research only within an ethical framework. Although the use of animals in research cannot be completely replaced, but their refinement and reduction can be maximized [1]. For validating the traditional use of medicinal plants biological screening is essential which provides a scientific base [2].

A novelistic High throughput pharmacological screening is useful to reduce the number of animals. We could find only a single study report in the literature using minimal number of animals for evaluation of antihyperlipidemic activity [3]. In this method, a single animal is used to get preliminary pharmacological data to see if the test compound is therapeutically effective or not. This method can be expanded to any number of formulations or test drugs as it requires only a single animal for generating the preliminary data.

According to WHO, dementia is the highly prevalent disorder next to tropical diseases. The WHO 2012 Report "Dementia: a public health priority"estimates 35.6 million people living with dementia worldwide. The loss of memory in dementia or in Alzheimer's disease is the main challenge of treatment. Memory is the ability of the individual to record the sensory stimulant information and events, retain them over a short or a long period of time and recall the same at a later when it is needed. Poor memory, less attention, and slow learning are a quite a natural problems among students and old age people. Traditional Indian origin nootropic drugs (smart drugs), are used to improve human cognitive abilities, learning and memory function. Typically, they act by increasing the brain's supply of neurochemicals, improving perfusion of brain's oxygen or by activating nerve growth [4]. Promising effect has been found in several studies with the herbal medicine in improving and treating memory disorder.

Herbal supplements may be used as a substitute for pharmaceutical drugs or can be used in conjunction with the latter. The basic objective of preparing polyphyto formulations was to explore the utility and potentialities of natural source containing various plant parts for memory enhancing abilities. Important plants that act on the nervous system and improve memory include: ''Japapushpa'' (*Habiscus rosasinensis*), ''Tulsi'' (*Oscimum sanctum*), "Chandana" (*Santalum album*), ''Shankhapushpi'' (*Convolvulus pluricaulis*), ''Brahmi'' (*Bacopa monniera*), and "Jai" (*Avena sativa*). The

plants that act by anti-inflammatory activity is "Amla" (*Phyllanthus Emblica*), and act by reducing free radical and antioxidant property are "Bendakaya" (*Abelmoschus esculentus*), "Arjuna" (*Terminalia arjuna*) etc [5-13] . The present study provides a pharmacological evaluation test for evaluating number of polyphyto formulations containing various plant parts for its nootropic action in a single animal. The results were compared with the study performed in groups in a two different dose by novelistic approach; a high throughput screening to find out the best formulation.

2. Materials and Methods

2.1. Plant Material and Preparation of Formulation

Different parts of all plants were obtained from the authentic herbal suppliers from Dehradun, India and were identified and authenticated by department of botany, Sri Venkateswara University, Tirupati. Various Plant parts were air dried in the dark, and grounded into a fine powder and passes it through a # 100 sieves. The individual drugs were then weighed as per the quantity required and mixed geometrically using a blender. The mixed formulations were weighed and stored in airtight containers for experimental purposes. Polyphyto mixtures are prepared by mixing various plant parts possessing nootropic activity based on ideal tree/ herb/ shrub concept, the main principal involved in the above concept is mixing root, rhizome, bark, wood, fruit, flower, leaf, seed and herbs of the different plants having a notropic activity to obtain polyphyto mixture comprising of ideal tree/ herb/ shrub parts. The compositions of six polyphyto formulations are mentioned in Table 1.

2.2. Experimental Animals

Adult albino Wistar rats (120 ±20 g) of either sex were procured and were grouped randomly. The rats were acclimatized for

Table 1. Composition of 6 polyphyto formulations (FM01-06).

FM-01

BOTANICAL	SANSKRIT NAME	FAMILY	PARTS USED	100 gms
Bacopa monnieri	Brahmi	Plantaginaceae	Herbs	20
Prunus Amygdalus	Vatadha	Rosaceae	Fruit	20
Terminalia arjuna	Arjuna	Combretaceae	Bark	20
Labromia bojery	Misri	-	Misc.	20
Avena sativa	Atiyav	Poaceae	Fruit	20

FM-02

BOTANICAL NAME	SANSKRIT NAME	FAMILY	PARTS USED	100 gms
Oscimum sanctum	Tulasi	Lamiaceae	Leaf	20
Dioscorea batatas	Ratalu	Dioscoreaceae	Root	10
Glycyrrhiza glabra	Yashtimadhu	Fabaceae	Root	10
Papaver somniferum	Ahifen	Papaveraceae	Seed	10
Avena sativa	Atiyav	Poaceae	Fruit	50

FM-03

BOTANICAL NAME	SANSKRIT NAME	FAMILY	PARTS USED	100 gms
Juglans regia	Akschota	Juglandaceae	Fruit	25
Ribes nigrum	Kannada	Grossulariaceae	Fruit	25
Cinnamomum zeylanicum	Darusita	Lauraceae	Bark	25
Rosmarinus officinalis	Rusmari	Lamiaceae	Leaf	25

FM-04

BOTANICAL NAME	SANSKRIT NAME	FAMILY	PARTS USED	100 gms
Convolulus pluricaulis	Sankhapuspi	Gentianaceae	Herbs	40
Glycyrrhiza glabra	Yashtimadhu	Fabaceae	Root	20
Prunus Amygdalus	Vatadha	Rosaceae	Fruit	20
Asparagus racemosus	Shatavari	Asparagaceae	Root	10
Abelmoschus esculentus	Bendakaya	Malvaceae	Fruit	10

FM-05

BOTANICAL NAME	SANSKRIT NAME	FAMILY	PARTS USED	100 gms
Lactuca sativa	Lettuce	Asteraceae	Leaf	10
Juglans regia	Akschota	Juglandaceae	Fruit	10
Curcuma longa	Haridra	Zingiberaceae	Rhizome	20
Aloe vera	Ghrita- kumara	Xanthorrhoeaceae	Leaf	20
Cinnamomum zeylanicum	Darusita	Lauraceae	Bark	10
Piper nigrum	Maricha	Piperaceae	Fruit	10
Rosmarinus officinalis	Rusmari	Lamiaceae	Leaf	10
Asphaltum vernacular	Silajatu	-	Misc.	10

FM-06

BOTANICAL NAME	SANSKRIT NAME	FAMILY	PARTS USED	100 gms
Celastrus paniculatus	Jyotishmati	Celastraceae	Seed	10
Habiscus rosasinensis	Japapushpa	Malvaceae	Flower	20
Bacopa monnieri	Brahmi	Plantaginaceae	Herbs	28
Convolvulus pluricaulis	Sankhapuspi	Gentianaceae	Herbs	28
Phyllanthu semblica	Amla	Phyllanthaceae	Fruit	04
Mentha piperita	Paparaminta	Lamiaceae	Leaf	10

one week in the animal house facility. They were housed in polypropylene cages at ambient temperature of 25±1°C with a natural dark-light cycle. The animals had been provided standard pellet diet and water given *ad libitum.* All experiments were conducted in the daytime (9:30 AM to 5:00 PM). The study was approved by the institutional ethics committee (CPCSEA registration no. - 1156/ac/07/CPCSEA) of DIT-University, Dehradun. Handling and Maintenance of animals were performed according to the internationally accepted standard guidelines for laboratory animals.

2.3. Treatment Groups

After acclimatization, the animals were randomly divided into following groups treated with 50 and 100 mg/kg dose. All the groups received the vehicle, standard drug and the test drug one hour prior to each experiment.

a) Control group- Control animals were treated with 1 ml of the vehicle

b) Standard group- Treated with *Bacopa monnieri* (50 mg/kg body weight) PO,

c) Treatment group Ia and Ib- FM1, 50 and 100 mg/kg PO respectively.

d) Treatment group IIa and IIb- FM2, 50 and 100 mg/kg PO respectively.

e) Treatment group IIIa and IIIb- FM3, 50 and 100 mg/kg PO respectively.

f) Treatment group IVa and IVb- FM4, 50 and 100 mg/kg PO respectively.

g) Treatment group Va and Vb- FM5, 50 and 100 mg/kg PO respectively.

h) Treatment group VIa and VIb- FM6, 50 and 100 mg/kg PO respectively.

2.4. Experimental Method

The HTPS (High Throughput Pharmacological Screening) method was used for rapid screening of six formulations. At first all formulations were tested in a single animal and then all six formulations were tested again on two animals to check and confirm the results using following animal models:

- Elevated Plus Maze test (EPM)
- Morri's Water Maze Test (MWM)
- Pole Climbing Test (PCT)

Then result of different test using one animal and the two animals were compared statistically to obtain $r^2$and p value. Of these three formulations with better response were selected for further evaluation in the next stage of screening. The formulations selected were screened in three groups of animals. The result of each group was compared with the control for statistically significant. Finally, one formulation with good activity was selected for testing in a group of twelve animals to confirm the pharmacological

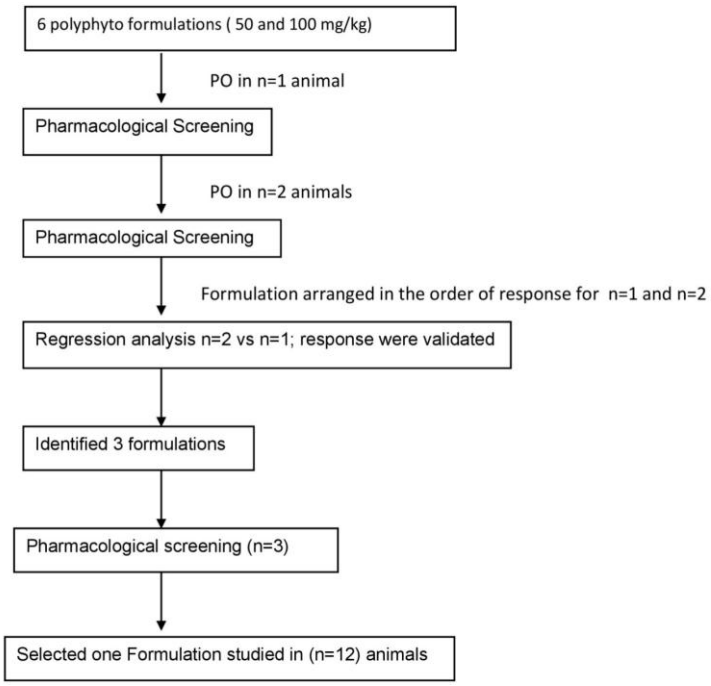

Figure 1. Highthroughput Screening of formulations based on pharmacological action.

action for enhancing learning and memory properties (Figure 1).

2.4.1. Elevated Plus Maze Test

The elevated plus maze served as the exteroceptive behavioral model (wherein the stimulus existed outside the body) to evaluate learning and memory in rats. The apparatus consisted of two open arms (50 cm × 10 cm) and two covered arms (50 cm × 40 cm ×10 cm). With the arms extended from a central platform (10 cm × 10 cm) and the maze was elevated to a height of 50 cm from the floor. On the first the day, each rat was placed at the end of open arm, facing away from a central platform [14,15]. With little modification transfer latency (TL) was taken at the time taken by the rat to move into any one of the covered arms enter with all its four legs where opposite gender of rat is placed in any one of the covered place to observe retentive memory of test rat to come faster toward that area. TL was recorded on the first day for the each animal. The rat was allowed to explore the maze for another 2 min and returned to its home cage. Retention of this learned task was examined 24 h after the first day trial [16]. The test group, control, and standard were tested for EPM test.

2.4.2. Morris Water Maze Test

The Morris water maze consisted large circular pool, 1.50 m across and 0.60 m high filled with water, which was made opaque by adding milk. Water provided a uniform intra-maze environment, thus eliminating any olfactory interference. A

28x10 cm rectangular escape platform was constructed of water resistant material and covered with material that allows the animal to remain on top when it is submerged. The platform was 28 cm in height so that it could be submerged 2 cm below the level of water surface. The water temperature was maintained at 26 ±2 °C. The animals were given a daily session of three trials per day. Latency time to reach the platform was recorded in each trial. Significant decrease in latency times from that of the first session was considered as successful learning [17]. The test group, control, and standard were tested in MWM test.

2.4.3. Pole Climbing Test

Cook's Pole Climbing Apparatus use to study cognitive function, mainly a response to conditioned stimuli during learning & its retention. The apparatus has an experimental chamber (25 × 25 × 25 cm) with the floor grid in a soundproof enclosure. Scrambled shock (6mA) is delivered to the grid floor of the chamber composed of stainless steel rods. A pole, 2.5 cm in diameter, hangs inside the chamber through a hole in the upper center of the chamber. The study rat was placed in the chamber and allowed to explore the chamber for 45 seconds. Conditioned stimulus (CS) i.e buzzer signal was turned on and unconditioned stimulus (US) i.e electric shock delivered through grid floor for 45 Sec. Animal learned to associate the buzzer with the impending foot shock and was capable of avoiding the foot shock by climbing the pole after buzzer signal. Avoidance response was defined as climbing reaction time <10 seconds only; and escape response was climbing after applying reaction time >10 seconds. Every rat was subjected to maximum 05 trials on 1st day, and 24 hrs later, rat was subjected to Relearning trials (2nd day 3 trials and on 3rd day one trial) and transfer latency was noted to check the retention of Conditioned Avoidance Response (CAR) and escape response. Animals were screened by using this model and those who demonstrated at least one escape response either on day one or two were included in the study [18]. The test group, control, and standard were tested in PC test.

2.5. Statistical Analysis

One animal and two animal response were analyzed using regression analysis to find out the linear relationship. Results were presented as the mean ± SD for best formulation. The result were compared with control and the standard using two way ANOVA, repeated measures

followed by Tukey's multiple comparisons (graphpadprism statistic software was used). P < 0.05 was set as statistically significant.

3. Results and Discussion

3.1. Effect of Transfer Latency Using Elevated Plus Maze

TL was measured for all six formulations (FM01-FM06) at 50 mg and

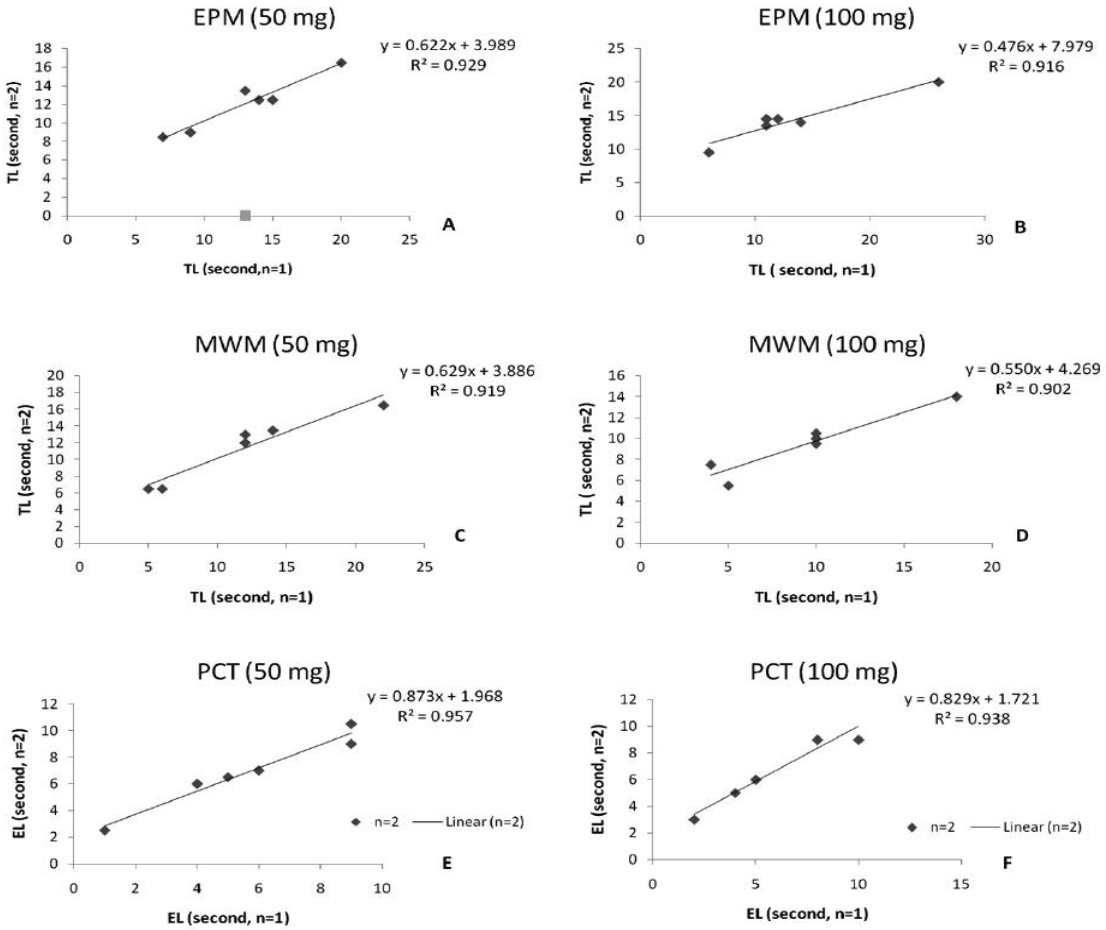

Figure 2. R2 for n=1 v/s n=2 animals (FM01 to FM06).

Table 2. Results of FM01-FM06 in animal models for learning.

FM. NO	EPM TEST (On day 2)				MWM TEST (On day 7)				PCT TEST (On day 3)			
	50 mg/kg		100 mg/kg		50 mg/kg		100 mg/kg		50 mg/kg		100 mg/kg	
	n=1	n=2	n=1	n=2	n=1	n=2	n=1	n=2	n=1	n=2	n=1	n=2
FM01	13	13.5	12	14.5	12	12	10	10	6	7	5	6
FM02	20	16.5	26	20	22	16.5	18	14	9	9	8	9
FM03	9	9	6	9.5	6	6.5	4	7.5	4	6	4	5
FM04	14	12.5	11	13.5	14	13.5	10	10.5	9	10.5	8	9
FM05	15	12.5	14	14	12	13	10	9.5	5	6.5	10	9

100 mg/kg at the time taken by the rat to move into any one of the covered arms (Table 2). The response was arranged in the decreasing order of their potency. The comparative result of an EPM test of formulations FM01 to FM06 of 50 mg/kg doses in animal (n=1 and n=2) revealed FM06>FM03>FM05>FM01>FM04>FM02. At 100 mg/kg dose the order for the n=1 and n=2 animal was FM03>FM06>FM02>FM01>FM05>FM04 and FM06>FM03>FM01>FM04>FM02>FM05, respectively. The results obtained in one animal was correlated with the responses obtained in two animals by using regression as shown in Figure 2A & 2B. The r^2 values 0.9 and above suggested a linear relationship of EPM test (n=1) with that of (n=2) result.

Formulations FM01, FM03 and FM06 were studied in n=3 animals at 50 mg/kg po and the results revealed that FM06 produced better response as compared to other two (Figure 3A).

FM06 was then compared to control groups and standard (*Bacopa monnieri*) per oral route in group of 12 animals. FM06 significantly decreased TL time in seconds during the study compared with the control and the standard group ($p<0.05$,

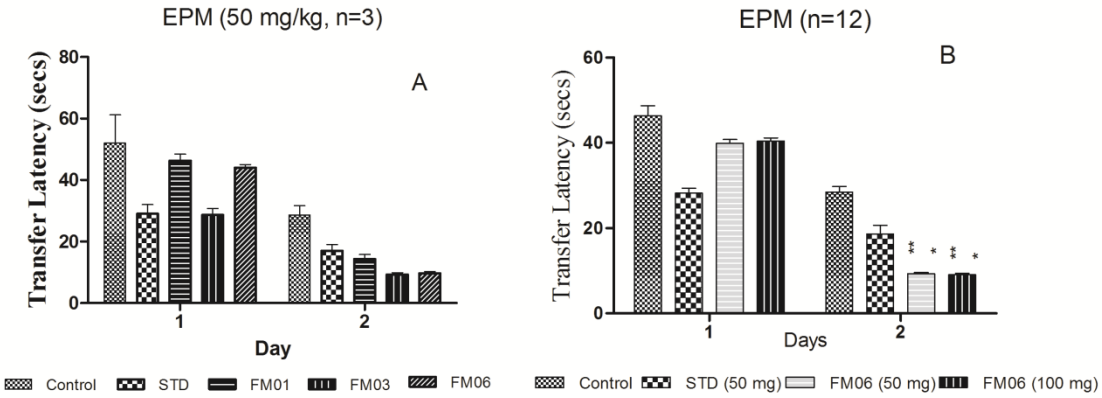

Figure 3. Mean Transfer Latency in elevated plus maze using rat***;p<0.05 vs positive and negative control.

Table 3. Effect of FM06 on EPM (n=12)

Days	Control	Std	FM06 (50 mg)	FM06 (100 mg)
Day 1	46.3± 8.23	28.25±3.82	39.91± 3.03	40.41 ± 2.46
Day 2	28.5±4.42	18.66±6.97	9.33± 0.78	9.08 ±1.17

Values are Mean ± SD

Figure 3B, Table 3).

3.2. Water Maze Test

TL was measured for all six formulations (FM01-FM06) at 50 mg and 100 mg/kg at the time taken by the rat to find an invisible platform (Table 2). The response was arranged in the decreasing order of their potency. The comparative result of the MWM test of formulations FM01 to FM06 at 50 mg/kg doses in animal (n=1 & n=2) revealed that FM06>FM03>FM01>FM05>FM04>FM02. At 100 mg/kg dose the order for n=1 and n=2 animal was FM03>FM06>FM01>FM05>FM04>FM02 and FM06>FM03>FM05>FM01>FM04>FM02, respectively. The results obtained in one animal correlated with the responses obtained in two animals by using regression as shown in Figure 2C and 2D. The r^2 values 0.9 and above suggested a linear relationship of MWM test (n=1) with that of (n=2) result.

Three formulations FM01, FM03 and FM06 were studied in n=3 animals at 50 mg/kg po and the results revealed that FM06 produced better response as compared to other two (Figure 4A).

FM06 was then compared to control groups and standard (*Bacopa monnieri*) per oral route in group of 12 animals.

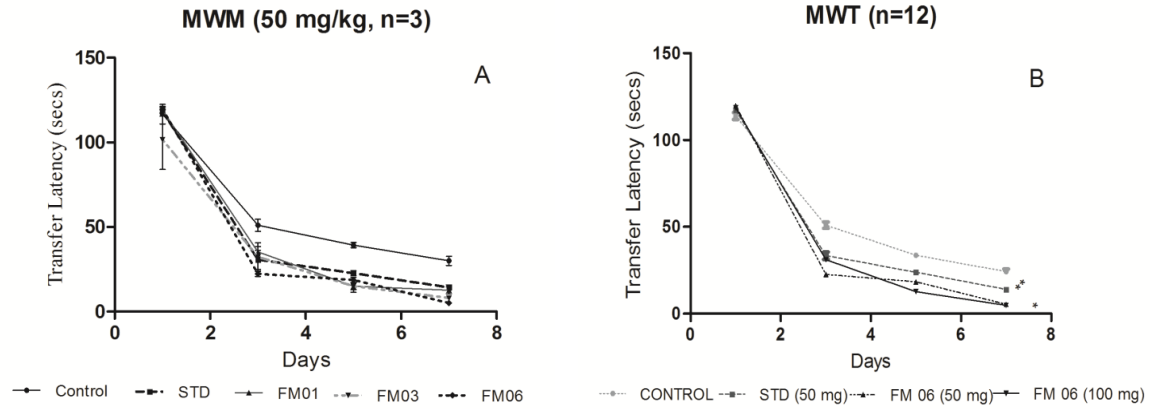

Figure 4. The transfer latency of polyphyto formulations FM06 in rat in secs using MWM;***=p<0.05vs control

Table 4. Effect of FM06 on MWM (n=12).

Days	Control	Standard	FM06 (50 mg)	FM06 (100 mg)
Day 1	113.83± 6.79	117.16±3.93	119.75±0.62	118.58±1.443
Day 3	51.00±6.37	33.42±7.34	22.58±2.46	31.08±4.03
Day 5	33.67± 4.28	23.9±3.15	18.33±1.92	12.75±1.42
Day 7	24.26±4.9	14.0±1.71	5.5±0.798	4.83±0.72

Values are Mean ± SD

FM06 significantly decrease TL time in seconds during the study compared with the control and the standard group ($p<0.05$, Figure 4B, Table 4).

3.3. Pole Clmbing Test

Escape latency (EL) was measured for all six formulations (FM01-FM06) at 50 mg and 100 mg/kg as the time taken by the rat to jump in a pole on conditioned stimuli (Table 2). The response was arranged in the decreasing order of their potency. The comparative result of a PCT test of formulations FM01 to FM06 at 50 mg/kg doses in animal (n=1 and n=2) revealed that FM06>FM003>FM01>FM04>FM05>FM02 and FM06>FM03>FM01>FM04>FM05>FM02, respectively. At 100 mg/kg dose the order for n=1 and n=2 animal was FM03>FM06>FM01>FM04>FM05>FM02. The results obtained in one animal was correlated with the responses obtained in two animals by using regression as shown in Figure 2E and 2F. The r^2 values 0.9 and above suggested a linear relationship of pole climbing test (n=1) with that of (n=2) result.

Three formulations FM01, FM03 and FM06 were studied in n=3 animals at 50 mg/kg po and the results revealed that FM06 produced better response as compared to other two (Figure 5A).

FM06 was then compared to control groups and standard (*Bacopa monnieri*) per oral route in group of 12 animals.

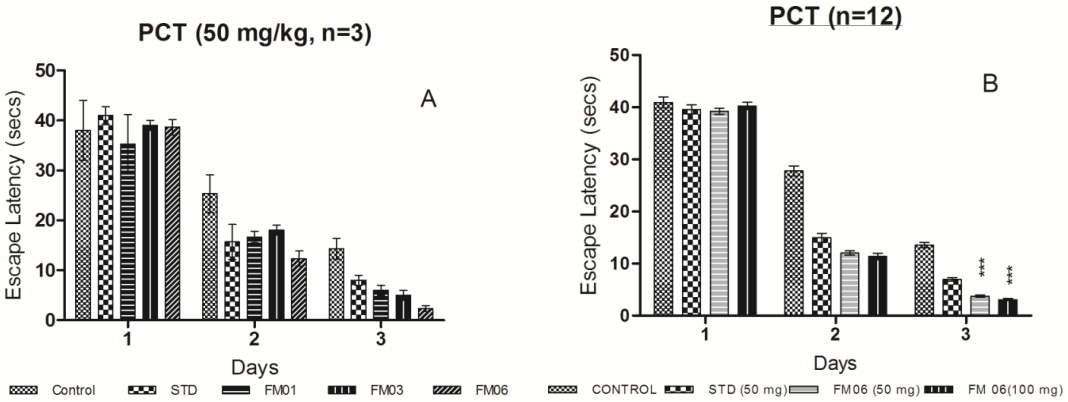

Figure 5. Bar Graph of escape latency of ratn=3 (A) and n=12 (B) insecs using Pole Climbing Apparatus; ***=p<0.05vs positive and negative control.

Table 5. Effect on PCT (n=12 animals per group)

Days	Control	Standard	FM06 (50 mg)	FM06 (100 mg)
Day 1	40.92±3.55	39.58±3.08	39.25 ±2.05	40.25±2.42
Day 2	27.75±3.38	14.92±3.08	12.00±1.41	11.3±2.18
Day 3	13.5±1.73	6.92±1.24	3.75±0.62	3.08±0.79

FM06 significantly decreased EL time in seconds during the study compared with the control and the standard group ($p<0.05$, Figure 5B, Table 5).

3.4. Discussion

In today's stressful and competitive world, poor memory, low retention, and slow recall are common problems. The allopathic treatment options are highly limited. Although nutritional and botanical therapies are available, they are under utilized. The herbal plants improve memory by preventing neuro-degeneration, reducing acetylcholine esterase activity and improving neuronal function. Thus, there is a need for further research to find drugs or formulations that could be effective for the treatment of dementia.

The use of animals in research is guided by "three Rs" principles [19]. As stated by scientists and governments, tests should be performed only when they are necessary and should cause a little suffering to them. Refinement in screening methods and reduction in the number of animals is to reduce the pain, suffering or distress caused to them due to experiments and enhance their welfare [20]. Replacement of non-animal methods over animal methods should be preferred to achieve the same scientific aim. However, this is not always possible in the case of evaluation of biological activity. High throughput screening method can be an answer to this, which allows use of a minimal number of animals for screening effective drugs. Reduction of number of animals can be achieved to obtain comparable levels of information by re-examining the findings of studies already conducted and improving animal models.

Elevated plus maze, Morris water maze (MWM) and Pole Climbing apparatus (PCA) were used to evaluate the effect of learning and memory improvement properties in rats. Morris Water Maze is a traditional tool in assessing learning and memory performance in laboratory animals. Originally designed to evaluate the antianxiety agents, elevated plus maze has also been recently extended to measure the spatial long-term memory in animals. Passive avoidance behavior is used to examine the long term memory based on negative reinforcement.

The FM06 was found to be best among six nootropic formulations and it was considered for testing on n=12 rats after a high throughput screening. In an Elevated plus maze test nootropic agent FM06 at 50 mg and 100 mg dose showed decrease in transfer latency on the second day when compared to control and standard groups indicating significant ($p<0.05$) memory improvement. In MWM test animals treated with 50 mg and 100 mg dose p.o of FM06 showed less transfer

latency time (in seconds) and was significant ($p<0.05$) when compared to control groups and *Bacopa monnieri* for its nootropic action. Similar results were obtained in Pole climbing test. The pharmacological response of a single animal study was correlated with the respons obtained in a group of 12 animals, the r^2 value was ~1. The study validate that the high throughput method can be used for screening pharmacological action of polyphyto mixture using single animal and can draw reproducible conclusion on the performance of any formulation.

The FM06 incorporated with herbal drugs like *Celastrus paniculatus, Habiscus rosasinensis, Bacopa monnieri, Convolvulus pluricaulis, Phyllanthus emblica, and Mentha piperita* has been reported good for nootropic action, example; *Celastrus paniculatus* (Jyotishmati Taila) is known for Medhya action [21] *H. rosasinnsis good for* learning and memory [22], *Bacopa monnieri* very useful in improving learning and memory [23, 24], *C. pluricaulis* is generally recommended as a brain tonic [25, 26]; *Phyllanthus emblica* is a natural remedy to improve memory function [20] and *Mentha piperita* enhance memory and alertness [27]**.**

4. Conclusion

Considering the above principles, high throughput method was designed to minimize the number of animals for screening of several polyherbal formulations for their nootropic action. The best selected formulations were studied in a group of twelve animals. The study showed that the results were reproducible when the activity was tested either in a single, two, or three animals and also statistically significant results were obtained. The statistical significance was also confirmed by regression analysis and ANOVA, which justifies the use of minimum number of animals initially for preliminary screening. The nootropic polyphyto formulations FM-06 (compose of *Celastrus paniculatus, Habiscus rosasinensis, Bacopa monnieri, Convolvulus pluricaulis, Phyllanthus emblica, and Mentha piperita)* were selected by high throughput screening and was found to have a promising effect in enhancing learning and memory. Thus, only the best formulation was tested in large number of animals. By this adoption of this method, researchers can evaluate number of bioactive components using less number of animals.

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