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Cell line toxicity study and pharmacological screening of effective nootropic herbal formulation in rat

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The polyphyto drugs FM27; compose of *Mentha piperita* (10%), *Ribes nigrum* (20%), *Cinnamomum zeylanicum* (20%), *Aloe vera* (10%), *Zingiber officinalis* (5%), *Glycine max* (5%) and *Avena sativa* (30%) on memory acquisition and retention was studied using EPM, MWM and PCA test in rat using two different routes, one is per oral ($FM27^{O}$) and another one is transcranial ($FM27^{T}$). The test drug studied for *in vitro* safety profile screening by MTT assay in a different cell line. As per the result, cell line toxicity confirmed that formulation FM27 was non toxic in SH-SY-5Y human neuroblastoma cell line, Vero monkey normal kidney epithelial cells, and Chang liver cell line. Test drug $FM27^{O}$ studied at a dose of (PO 50 and 100 mg/kg), showed graded dose response and it was observed significantly ($p < 0.01$) effect of memory improvement comparing standard group containing *Bacopa monnieri* (PO 50 and 100 mg/kg), whereas transcranially applied $FM27^{T}$ (10 mg/kg) showed a significant ($p < 0.001$) effect of memory improvement comparing control group and observed similar effect like standard drug. Herbal formulations FM27 was found to be potent for treating dementia to improve memory. The cell line toxicity study report confirmed it was safe in higher dose also.

Keywords: *Aloe vera*, *Cinnamomum zeylanicum*, Dementia, Memory, *Mentha piperita*, *Ribes nigrum*, *Zingiber officinalis*.
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Introduction

Memory is the ability of an individual to record sensory stimuli, events, and information; retain them over short or long periods of time and recall the same at a later date when needed. The drug use for the treatment of Alzheimer's disease is effective for short term and symptomatic only. There are several studies and alternative therapies that offer ways to slow the onset and progression of Alzheimer's disease in some patients. In Ayurveda; herbs that promote the intelligence are called 'medhya' drugs, use to improving memory from a long time in India as traditional therapy and there is a possibility to modify formulation or drug based on new research to support memory improvement by preventing the neurodegeneration due to different etiology.

Bacopa monnieri[1], *Convolulus pluricaulis*[2], *Withania somnifera*[3], *Curcuma longa*[4], *Areca catechu*[5], *Oscimum tenuiflorum*[6], *Terminalia arjuna*[7], *Zingiber Officinale*[8] etc are reported for nootropic agents and frequently used for memory support traditionally. Preclinical and clinical studies have shown that *Bacopa monniera* improves memory and mental function[9]. Herbal drug act same as like allopathic medicine, a recent study reveals B. monniera extract is able to reduce amyloid levels in PSAPP mice which is a transgenic mice expressing the "Swedish" amyloid precursor protein and M146L presenilin-1 mutations[10].

Soybean *(Glycine soja)* is a species of legume is a rich nutrient source of protein and containing Phospholipids (45-60%) in particular phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol; approved for raised levels of cholesterol, also used for poor concentration, cerebral and nerve conditions, and general debility[11]. The different scientific research revealed the medicinal value of the soy components against metabolic disorders (cardiovascular diseases, diabetes and obesity, etc.) also it helpful for other chronic diseases (menopausal syndrome, osteoporosis, anaemia and cancer, etc.) and reported for improving memory [12].

Zingiber officinalis was demonstrated that B-Amyloid (βA) induced oxidative stress in AD can be prevented significantly, increased whole

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brain acetyl cholinesterase inhibition activity using *Z. officinale*[13]. *Z. officinale* has the ability to increase neuronal density in the hippocampus, enhance cerebral blood flow and showed the protective effect against focal cerebral ischemia induced by the occlusion of right middle cerebral artery[14-15].

Cinnamomum zeylanicum is used as a good brain tonic, it boosts the activity of the brain; it helps in reducing nervous tension and improving memory. *Cinnamon zeylanicum* (aqueous extract) inhibit tau aggregation and filament formation, one of the important pathophysiological causes of Alzheimer s disease (AD)[16].

Ribes nigrum contains a high percentage of phenolic compounds. Black currant seed oil was found to contain linoleic acid, alpha-linolenic acid, gamma-linolenic acid, and stearidonic acid. Black currant fruit and juice contain vitamin C 2000 mg/kg, as well as rutin and other flavonoids[17]. It possesses antioxidant, anti-inflammatory[18], antithrombotic actions, and cardioprotective effect**.**

Mentha piperita- Dried peppermint approximately presented with 0.3-0.4% of a volatile oil whereas menthol content is 7-48%. Peppermint oil also contains small amounts of various additional compounds including 1,8-cineol, limonene, pulegone, caryophyllene and pinene. The aroma of peppermint (*Mentha piperita*) has been found to enhance memory and alertness[19]**.**

Avena sativa contains natural antioxidants such as tocopherols, alk (en) ylresorcinols, and phenolic acids and their derivatives, and a unique source of avenanthramides (Ncinnamoylanthranilate alkaloids) and avenalumic acids (ethylenic homologues of cinnamic acids)[20]. Cinnamic acid found to improve spatial memory and condition avoidance memory[21]. They stimulate the immune system, modulating humoral and cellular immunity, and thereby have a beneficial effect in fighting infections (bacterial, viral, fungal and parasitic) in an "Alive publishing group" states that Oats (*Avena sativa*) are useful for rebuilding nervous tissue and brain tissue[22]**.** In a clinical study, the *wild green oat extract* observed that it can increase attention or alertness, speedy thinking ability, executive function, and working memory, other way enhanced dopaminergic transmission in brain[23,24].

Aloe vera is used as an antifungal, antidiabetic, anti-inflammatory, analgesic, anticancer, antimicrobial, antiproliferative, gastric mucosal protection, hepatoprotective, neuroprotective, hypolipidaemic, immunomodulatory, antimutagenic, antileishmanial, radioprotective, wound healing and for antioxidant properties[25]. In a study activation of sodium nitrite induced memory impairment and cholinergic muscarinic receptors sensitization appraise the role of Aloe vera gel for inflammatory memory disorders like Alzheimer's[26].

The basic objective of preparing polyphyto formulations is to explore the utility and potentialities of natural source containing various plant parts have promising memory enhancing abilities. Herbal drugs or as a supplement may be used as a substitute for pharmaceutical drugs or can be used in conjunction with the latter. The formulation was prepared by using the herbs can improve memory either by action on the hippocampus and other parts of the brain; eg. *Cinnamomum zeylanicum*, *Avena sativa, Mentha piperita, Glycine max,* or by scavenging free radical or antioxidant & antiinflammatory activity; eg. *Glycine max, Zingiber officinalis*, *Ribes nigrum, Aloe vera.*etc. The preparation of herbal oil containing nootropic substances can be used easily as hair oil. The oil therapies of *Ayurveda* using the head include shirodara, shiroabyanga, shiropitchu, shirovasthi and shiropralepa in which drugs are delivered by the transcranial route[27-28] proven effective for a neurological disorder. Dementia is a global epidemic; the aim of the current study was to examine the safety and efficacy of a new combinational polyphyto drug; having nutrient and home remedies substances for its learning and memory enhancing properties by oral and transcranial route of drug delivery.

Materials and methods

Plant material and preparation of the formulation

All herbs and plant parts were collected and authenticated by the Department of Botany, Sri Venkateswara University, Tirupati. For preparing $FM27^{O}$ all herbs and plant parts (Table 1) were air dried under shade and powder then passed it through a # 100 sieve, mix all the components. For topical application, an oil $FM27^{T}$ was prepared by heating with sesame oil for 10 minutes, then ultrasonicated for 3 mins repeated 3 times, finally it centrifuged and clear supernatant oil formulation was separated.

Experimental animals

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

Table 1 — Medhya formulation FM27

Plant (scientific name)	Sanskrit name	Family	Plant parts	100 g
Mentha piperita	Pudina	Lamiaceae	Leaf	10
Ribes nigrum	Kannada	Grossulariaceae	Fruit	20
Cinnamomum zeylanicum	Darusita	Lauraceae	Bark	20
Aloe vera	Ghrita- kumara	Xanthorrhoeaceae	Leaf	10
Zingiber officinalis	Sunthi	Zingiberaceae	Rhizome	5
Glycine max	Soyabean	Fabaceae	Seed	5
Avena sativa	Atiyav	Poaceae	Fruit	30

rats were acclimatized for one week in the animal house facility. They were housed in polypropylene cages in an 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).

Treatment groups

All the groups received the vehicle, standard drug and the test drug one hour prior to each experiment. Animals were selected and divided into groups (n=6). It was studied for elevated plus maze test (EPM), Morris water maze test (MWM), pole climbing test (PCT). Group classifications are as follows:

Group 1: Control (po vehicle/ or tc oil)

Group 2: Standard; *Bacopa monniera* (po 50 mg/kg)

Group 3: Standard; *Bacopa monniera* polyphyto oil (tc 10 mg/kg)

Group 4: Test $FM27^{O}$; two dose (po 50 and 100 mg/kg)

Group 5: Test $FM27^{T}$ polyphyto oil (tc 10 mg/kg)

Method of transcranial drug administration

The hair of the scalp of the rat was trimmed without injuring the skin for the transcranial application. The test group was treated with $FM27^{T}$ polyphyto oil by applying on to the hair trimmed bald area of the scalp followed by 'rubbing in' for 1 minute with gentle massage by which it was to facilitate the oil solution come into contact with the skin and its appendages of the scalp properly. Similarly, group2 consider as standard oil containing *Bacopa monnieri* applied transcranially.

Acute toxicity study:

To evaluate the acute toxicity of drugs after a single oral dose, Swiss albino rats were fasted for 6 hours with only water provided ad libitum. Rats were divided into experimental groups (n=6) and were treated orally at doses of 300, 1000 and 2000 mg/kg. The animals were then allowed free access to food and water. The animals were observed for any abnormal behaviour, changes of body weight and mortality was noted for 14 days after the oral administration of formulation for the acute toxicity[29]. The control group was treated with normal saline (1mL/kg, i.p.). Both $FM27^{O}$ and $FM27^{T}$ were found to be safe.

Cell line toxicity study-MTT assay principle

This is a colourimetric assay that detects the reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT absorbed into the cells and enter into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then treated with an organic solvent (eg. isopropanol) and the released, solubilized formazan reagent is determined spectrophotometrically. Since the reduction of MTT can only be possible in metabolically active cells the level of activity is a measure of the viability of the cells [30].

Twenty-four hours after cell seeding, cells were incubated with varying concentrations of water extracts of the FM27 formulation at the different concentration for 48 hours at 37 °C. Following removal of the plant extracts from each well, cells were washed in phosphate-buffered saline. The cells were then incubated in serum-free DMEM to which MTT (0.5 mg/mL) was added to each well (100 µL), and incubated for a further 4 hours. Then the medium was removed and the cells were incubated for 15 minutes with 100 µL of acidic isopropanol (0.08 N HCl) to dissolve the formazan crystals. Cell line toxicity was performed to observe toxicity in cellular level; SH-SY-5Y human neuroblastoma cell line, Vero monkey normal kidney epithelial cell line and Chang liver cell line[31].

Experimental methods

Elevated plus maze test

The elevated plus maze served as the exteroceptive behavioural 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). 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. With little modification transfer latency (TL) was noted as 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 kept in the covered place to observe retention memory of test rat to come faster toward that area. On the first day, each rat was placed at the end of the open arm, facing away from a central platform[32-33]. TL was recorded on the first day for each animal for both the formulations $FM27^{O}$ and $FM27^{T}$ including control and standard. The rat was allowed to explore the maze for another 2 minutes and returned to its home cage. Retention of this learned task was examined 24 hours after the first day trial[34].

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 intramaze environment, thus eliminating any olfactory interference. A 28×10 cm rectangular escape platform was constructed of water resistant material and covered with a 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[35]. After treating with $FM27^{O}$, and $FM27^{T}$ of the respective group all the animals were given a daily session of three trials per day and tested for seven days. 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.

Pole climbing test

Cook's Pole Climbing Apparatus use to study cognitive function, mainly a response to conditioned stimuli during learning and its retention. The apparatus has an experimental chamber (25 × 25 × 25 cm) with the floor grid in a soundproof enclosure. Scrambled shock (6 mA) 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 the buzzer signal. Avoidance response was defined as climbing reaction time <10 sec only; and escape response was climbing after applying reaction time >10 sec. Every rat was subjected to maximum 05 trials on the 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[36-37].

Statistical analysis

All results were expressed as mean±standard error of mean (SD). Data were analyzed using one way ANOVA and two way repeated measures followed by Tukey's multiple comparisons and student's unpaired t-test using the GraphPad prism statistic software. $p < 0.05$ was set as statistically significant.

Results

Cell line toxicity study

The colourimetric assay was performed to determine the reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase during test of *in vitro* cell line toxicity study of nootropic herbal formulation FM27 in a different cell lines at four different concentration (25, 50, 100 and 200 µM/µg). The absorbance of the MTT formazan was determined at 540 nm in an enzyme-linked immunosorbent assay (ELISA) reader. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells. The result for cell line toxicity confirmed that formulation FM27 was non toxic in SH-SY-5Y human neuroblastoma (HN) cell line, Vero- monkey normal kidney epithelial (MNKE) cells, and Chang liver cell line (Table 2).

Table 2 — Cell line toxicity study of FM27 herbal formulation

S.No	Conc (µM/µg)	SH-SY-5Y (HN) cell line	Vero MNKE cell line	Chang Liver cell
		Mean Cell viability (%)		
1.	25	98.0	109.7	103.7
2.	50	92.2	108.9	101.2
3.	100	88.8	107.0	102.3
4.	200	89.0	112.5	107.4

Effect of transfer latency using elevated plus maze

Transfer latency was defined as the time (in seconds) taken by the animal to move from the open arm into one of the covered arms with all its four legs. With little modification, an opposite gender of the rat was placed in any one of the covered places to observe retentive memory of test rat to come faster toward that area. Significant decrease in TL value of retention indicated improving memory. Test FM27 formulation at different doses showed a decrease in TL on the second day (after treatment) in the rat when compared to control groups indicating significant (p <0.001) memory improvement (Fig. 1). $FM27^{O}$ at 50 mg and 100 mg dose showed better result compare to other groups, have a significant effect (p <0.001) for memory improvement.

Effect of transfer latency using water maze test

The transfer latency on a water maze test of the rat was studied using a circular pool (diameter 70 cm; height 28 cm) and a platform (diameter 3.8 cm) was placed 1.5 cm below the water level in the middle of a fixed quadrant. The differences in the transfer latency were noted in control, standard, and test group. The results indicated that test group, $FM27^{O}$ at 50 and 100 mg po showed less TL (seconds) and found to be an extremely significant decrease in transfer latency (p <0.001) compared to respective control groups and comparatively better than standard (*Bacopa monnieri*) (p <0.05) on po. The test formulation $FM27^{T}$ also found to be significant (p <0.001) on brain targeted transcranial application (Fig. 2) compared to respective control groups.

Effect of escape latency using pole climbing test

Rat placed inside the Pole climbing apparatus- to study the EL (seconds); a shock for controlled duration of 200V AC 50 Hz single phase - 0.2 mA was applied. The test group, $FM27^{O}$ (at 50 and 100 mg/kg po) doses revealed a statistically significant (p <0.01), whereas on Transcranial application was highly significant (p <0.001) decrease in escape latency in pole climbing test as compared to the control groups and comparatively similar effect like standard group3 (Fig. 3).

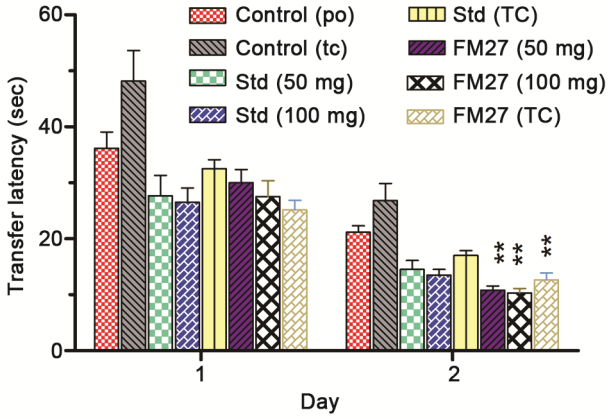

Fig. 1 — Mean Transfer Latency in elevated plus maze using rat *p <0.05 vs positive and negative control.

Discussion

Poor memory, lower retention, and slow recall are common problems in today's stressful and competitive world. The herbal drug has shown a promising effect in the treatment of memory loss. The herbs acting on the brain are called Nootropic herbs (Nootropic is derived from Greek and means acting on the mind) and their isolated constituents referred to as smart drugs. Memory enhancer herbs like *Zingiber officinale*, enhance the memory and increase blood circulation in the brain, drugs like *Mentha piperita* enhance memory alertness and vasodilation. *Cinnamomum zeylanicum*, *Aloe vera*, *Zingiber officinalis*, *Glycine max*, *Avena sativa* and *Ribes nigrum* are useful for the treatment of memory loss and to support the poor brain function.

Dose selection for the *in vivo* study was made on the basis of acute toxicity studies (300, 1000 and 2000 mg/kg body weight) result and in consideration of estimating the human equivalent dose (HED) for treating the patient. The dose 50 mg/kg in rats, means 8 mg/kg in humans calculated by division method:

Human dose= mg/kg animal dose χ [Km human/Km animal]

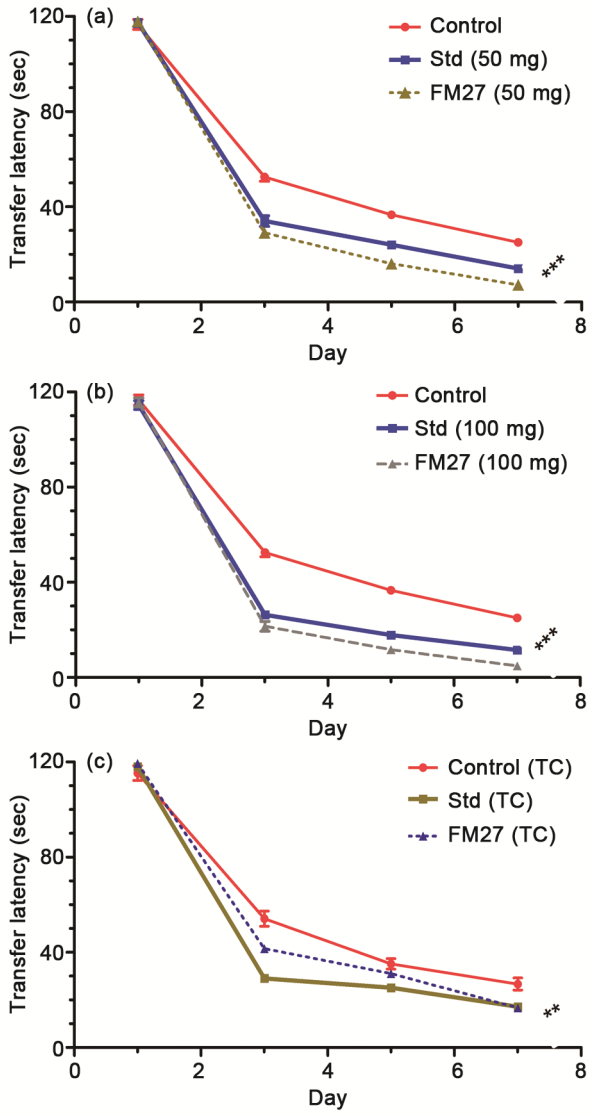

Fig. 2 — The transfer latency of polyphyto formulations FM27 in rat in secs using MWM *p <0.05 vs control

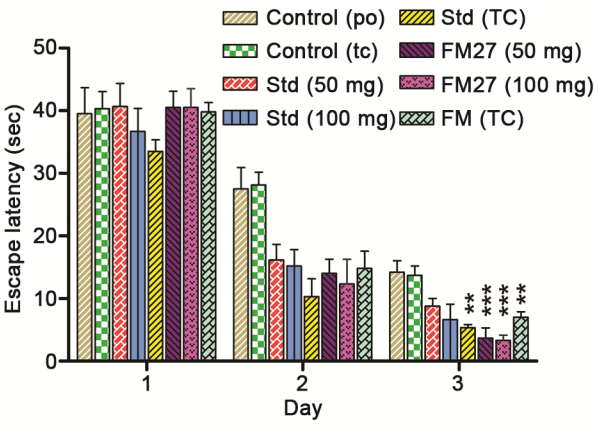

Fig. 3 — Bar Graph of escape latency of rat n=6 in seconds using Pole Climbing Apparatus; *p <0.05 vs positive and negative control

The drug dose 50 or 100 mg/kg was found to be safe as well as effective for FM27^{O} drug, so it can use clinically at a dose range of 500 mg to 1000 mg to a 60 kg adult human[38].

The standard herbal drug; Memorin capsule, is prescribing at a dose of 300 mg one capsule for twice a day for the memory boosting or memory support[39]. The test formulation having nutrient and home remedies substances and it was proven for its learning and memory enhancing properties by oral and transcranial route of drug delivery can be used in a dose of 300 mg, twice a day[39].

The medhya formulation; FM27 (*Mentha piperita* 10%, *Ribes nigrum* 20%, *Cinnamomum zeylanicum* 20%, *Aloe vera* 10%, *Zingiber officinalis* 5%, *Glycine max* 5% and *Avena sativa* 30%), on memory acquisition and retention was studied using elevated plus maze, morris water maze (MWM) and pole climbing apparatus (PCA) in rat.

In an Elevated plus maze study, the percentage of decrease in transfer latency time was calculated and it was found to be 41.5, 44.3, 47.6, 49, 47.7, 63.9, 62.4 and 49.6 for control (po), control (tc), standard (50mg po), standard (100 mg po) standard (tc), FM27 (50 mg po), FM27 (100mg po) and FM27 (tc) respectively; in MWM test observed that, the percentage of decrease in transfer latency time was calculated and it was found to be 78.5, 76.87, 88, 90, 85.4, 93.9, 95.8 and 86 for control (po), control (tc), standard (50mg po), standard (100 mg po), standard (tc), FM27 (50 mg po), FM27 (100mg po) and FM27 (tc) respectively whereas in a pole climbing test the percentage of decrease in escape latency time was calculated and it was found to be 64, 66.1, 78.2, 81.81, 84.1, 90.9, 91.7 and 82.4 for control (po), control (tc), standard (50 mg po), standard (100 mg po), standard (tc), FM27 (50 mg po), FM27 (100mg po) and FM27 (tc) respectively.

The FM27^{O} (po) has been shown better efficacy compare to FM27^{T} (tc) oil, the reason may be as the formulation having *Aloe vera, Avena sativa* and *Ribes nigrum* contained 60% of composition which is mostly water soluble but other parts; *Cinnamomum zeylanicum, Mentha piperita, Zingiber officinalis* and *Glycine max* having 40% of formulation impart action for oil preparation. The oil preparation action can be improved by increasing the concentration of *Cinnamomum zeylanicum, Mentha piperita, Zingiber officinalis* and *Glycine max.* The test drug FM27^{O} (po) also showing better action compare to standard drug;

the transfer latency decreases by 90 and 95.8 seconds for test drug and standard at 100 mg dose in the water maze test. MWM test is a traditional tool in assessing learning and memory performance in laboratory animals. The difference suggested that test drug $FM27^{O}$ showed better response and it was observed a significant effect of memory improvement comparing standard group containing *Bacopa monnieri*, whereas transcranially applied $FM27^{T}$ showed a significant effect of memory improvement comparing control group and observed similar effect like a standard drug.

Conclusion

The cell line toxicity study confirmed that formulation FM27 was non toxic in SH-SY-5Y human neuroblastoma cell line, Vero monkey normal kidney epithelial cells, and Chang liver cell line. In conclusion polyherbal formulations FM27 composing (*Mentha piperita* 10%, *Ribes nigrum* 20%, *Cinnamomum zeylanicum* 20%, *Aloe vera* 10%, *Zingiber officinalis* 5%, *Glycine max* 5% and *Avena sativa* 30%) was found to be safe and effective for treating dementia as well as prophylactically can use for improving memory loss.

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