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COMPARATIVE STUDY OF POLYHERBAL COMBINATIONS ON OXIDATIVE STERSS ASSOCIATED DEMENTIA

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ABSTRACT: The term dementia refers to a severe loss of thinking abilities; especially memory. The most common kind of dementia is Alzheimer's disease (AD). *Bacopa monnieri* and *Withania somnifera* are the most potent herbs for the treatment of loss of memory. *Piper nigrum* Linn. is essential ingredients of numerous prescriptions and formulations. It is reported that Black pepper increases the blood levels of drugs more than 100%. In this study, alcoholic extract of *B. monnieri*, methanolic extract of *W. somnifera* and powder of *Piper nigrum* and their combinations were subjected to young mice. Elevated plus Maze and Passive Avoidance apparatus served for the exteroceptive behavioural model for testing memory. Piracetam was taken as standard drug and Scopolamine was used to induce amnesia. *In-vitro* antioxidant activity was carried out by using DPPH free radical scavenging model and Gallic acid as standard drug. This study demonstrates that Alcoholic extract of *Bacopa monnieri* alone and Alcoholic extract of *B. monnieri* in combination with methanolic extract of *Withania somnifera* and powder of *Piper nigrum* diminishes neuronal death induced by AChE activity. Alcoholic extract of *Bacopa monnieri* also exhibited *in-vitro* antioxidant properties.

INTRODUCTION: The term dementia refers to a severe loss of thinking abilities; especially memory. The most common kind of dementia is Alzheimer's disease (AD). AD is a neurodegenerative disease causes gradual death of brain tissues.

The neuropathologic features of this disease are neurofibrillary tangles and amyloid plaques and it is also characterized by the loss of cholinergic neurons in the basal forebrain.

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Currently, there is no drug therapy that provides definite solution for curing Alzheimer's disease. The pharmacological treatment conventionally used to maintain cognitive functions of patients consists of two classes of drugs, the acetylcholinesterase inhibitors (AChEI) and the glutamate modulators [1].

One of the most accepted strategies in Alzheimer's disease treatment is the use of cholinesterase inhibitors. In addition, several alternative approaches for controlling the symptoms of this disease have been displayed such as anti-inflammatory drugs and antioxidants. Extracts of several medicinal plants have been reported to show AChE inhibitory activity like *Ginkgo biloba*, *Bacopa monnieri* , *Piper nigrum*, *Withania somnifera*, (also called as Indian Ginseng and Ashwagandha) [2].

This study, therefore, is focused on the effect of the extracts of *Bacopa monnieri*, *Withania somnifera* and their combinations along with powder of *Piper nigrum*.

Bacopa monnieri is one of the most honoured medicinal plants in Ayurveda .It is cold, bitter, digestive, carminative, laxative, bronchodilator, anticonvulsant and tonic for heart and nerves [3]. Antioxidant property [4] of it is responsible for its antistress, immunomodulatory, and cognition fascilatatory effects. Main chemical constituents of *Bacopa monnieri* include alkaloids brahmine, herpestine and nicotine, saponin monierin, hersaponin , bacoside A1, A2, A3 and B and four saponin bacogenin A1 to A4 [5].

In Ayurveda, The roots of *Withania somnifera* are categorised as rasayanas, a group of plant-derived drugs that are reputed to promote health and longevity by augmenting defence against disease, arresting the aging process, revitalising the body, increasing the capability of the individual to resist adverse environmental factors and creating a sense of mental well-being [6]. The root contains two acyl steryl glucoside viz. sitoindoside VII and sitoindoside VIII, two glycowithanoloids viz. sitoindoside IX or sitoindoside X. It is reported that two glycowithanoloids (sitoindoside IX or sitoindoside X) possessed augmented learning acquisition and memory retention in both young and old rats.[7]

In Ayurveda, some drugs such as black pepper *Piper nigrum* Linn. long pepper *(Piper longurn* Linn.) and ginger ***(****Zingiber officinalis* Rose.) are essential ingredients of numerous prescriptions and formulations, used for a wide range of diseases. It is reported that Black pepper and Long pepper increases the blood levels of drugs more than 100%. Piperine, the main constituent of *Piper nigrum* and *Piper longum,* plays an important role in increasing drug bioavailability when given oral [8].

Formation of free radicals and reactive oxygen species (ROS) is a normal consequence of a variety of biochemical reactions. The free radicals produced in the body are toxic, and if not removed or neutralized, they react with lipids, proteins, and nucleic acids and damage cellular functions. Generally, oxidative damage to the cellular components results in alteration of the membrane properties such as fluidity, ion transport, enzyme activities, and protein cross-linking [9]. Here the extracts of individual drugs and the combination of two *Bacopa monnieri* and *Withania somnifera* are screened for *In vitro* antioxidant activity study using DPPH (1, 1-diphenyl-2-picryl-hydrazyl) assay methods. Gallic acid is used as a standard.

MATERIALS AND METHODS:

Collection of plant Material and Extraction: The plant *Bacopa monnieri* was collected from Arista Agro Pvt. Ltd. Bangalore and the crude drugs *Withania somnifera* and *Piper nigrum* were collected from Khari-baoli, local market of New Delhi identified. The crude drugs were authenticated by Dr.Anjula Pandey (Principal Scientist), National Bureau of Plant Genetic Resources (NBPGR), Pusa Campus, New Delhi, With Specimen No: NHCP/NBPGR 2694, 2633, and 3634 respectively. After authentication the crude drugs were powdered to obtain coarse powder. The coarse powder of *B. monnieri* was extracted with ethanol and coarse powder of *W.somnifera* was extracted with methanol by using soxhlet apparatus. *Piper nigrum* was taken in powder form .The extracts were concentrated *in vacuo* and kept in vacuum desiccators for complete removal of solvent.

Nootropic Activity: The studies were carried out in our Institute (Animal house Reg. No. 385/CPCSEA) after approval form IACE (File No- RIT. M. Pharm/2011-2012/005, 24 Nov 2011.

Animals: Swiss albino young mice of either sex, weighing between 25-40 g were used in this study. They were acclimatized to the laboratory conditions for 5 days before behavioural studies. Mice had free access to food and water and were maintained under 12 h light/12 h dark cycles.

Drugs: Piracetam, Scopolamine, extracts of *B. monnieri and W. somnifera* and powder *of P. nigrum* were used in this study. Drugs were dissolved in 1% Carboxy Methyl Cellulose (Thomas Baker Chem. Pvt. Ltd.) and given orally to the mice.

Acute toxicity studies: Acute toxicity studies were performed according to OECD guidelines [10]. The animals were for 4 hrs with free access of water only. Alcoholic extract of *B. monnieri*, methanolic extract of *W. somnifera* and powder of *P. nigrum*, were

administered orally at dose of 5 mg/kg and mortality if any was observed for 3 days. If no mortality was observed the higher dose like 50, 300 and 2000 mg/kg were administered.

Development of Polyherbal combinations:

Drugs:	**Dose**
Alcoholic extract of *B. monnieri*:	1000 mg/kg
Methanolic extract of *W. somnifera*:	1000 mg/kg
Powder of *P. nigrum*:	250mg/kg

Polyherbal formulation was prepared by mixing drugs in following combinations

1) BM : WS :: 1 : 0.4
2) BM : PN :: 1 : 0.4
3) WS : PN :: 1 : 0.4
4) BM : WS : PN :: 1 : 1 : 0.4

***BM:** *B. monnieri*; **WS:** *W .somnifera*; **PN:** *P. nigrum*

Exteroceptive Behavioural Models:

Elevated Plus Maze: The elevated plus maze served as the exteroceptive behavioural model (wherein the stimulus existed outside the body) to evaluate learning and memory in mice. The apparatus consisted of two open arms (16 cm · 5 cm) and two covered 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 taken as the time taken by the mouse to move into any one of the covered arms with all its four legs. TL was recorded on the first day. If the mouse did not enter into one of the covered arms within 90 s, it was gently pushed into one of the two covered arms and the TL was assigned as 90 s. The mouse was allowed to explore the maze for 10 s and then was returned to its home cage. Memory retention was examined 24 h after the first day trial on the second day.

Passive Shock avoidance Paradigm: Passive-avoidance behaviour based on negative reinforcement was recorded to examine long-term memory. The apparatus consisted of a box (27 · 27 · 27 cm3) having three walls of wood and one wall of Plexiglas, featuring a grid floor (3 mm stainless steel rods set 8 mm apart), with a wooden platform (10 · 7 · 1.7 cm^3) in the centre of the grid floor. The box was illuminated with a 15 W bulb during the experimental period. Electric shock (20 V AC) was delivered to the grid floor. Training was carried out in two similar sessions. Each mouse was gently placed on the grid floor and pressed the start button till the animal reaches centrally located shock free zone.

Note down the latency in seconds as displayed in the timer. The second session was carried out 90 min after the first test. When mice stepped down before 60 s, electric shocks were delivered for 15 s. During the second test, animals were removed from shock free zone if they did not step down for a period of 60 s. Retention was tested after 24 h in a similar manner. Each mouse was again placed on the platform, and the step down latency was recorded [11].

Experimental Design: Mice were divided into 9 groups and each group consisted of a minimum of five animals. Separate animals were used for each experiment.

Grouping of Animals:

Group I: (n=6), Control animals (normal saline treated).

Group II: (n=6), Animals treated with Scopolamine (0.4 mg/kg i.p.).

Groups III: (n=6), Animals treated with standard drug Piracetam (200 mg/kg i.p.).

Group IV: (n=6), Animals treated with Alcoholic extract of *Bacopa monnieri* (1000 mg/kg BW, p.o.).

Group V: (n=6), Animals treated with combination of Alcoholic extract *Bacopa monnieri* and powder of *Piper nigrum* in ratio of 1:0.4 BW, p.o.

Group VI: (n=6), Animals treated with Methanolic extract of *Withania somnifera* (1000 mg/kg BW, p.o.)

Group VII: (n=6) Animals treated with combination of Methanolic extract *Withania somnifera* and powder of *Piper nigrum* in ratio of 1: 0.4 BW, p.o.

Group VIII: (n=6), Animals treated with Alcoholic extract of *Bacopa monnieri* and Methanolic extract of *Withania somnifera* in ratio of 1:1 BW, p.o.

GroupIX: (n=6), Animals treated with combination of Alcoholic extract of *Bacopa monnieri*, methanolic extract of *Withania somnifera* and powder of *Piper nigrum* in ratio of 1:1: 0.4 BW, p.o.

Dose Treatment: The different groups were treated orally with various combinations of drugs for 8 days. The last dose was given 45 min before subjecting the animals to Exteroceptive Behavioural Models (Elevated plus maze and Passive avoidance Test) Transfer latency (TL) and Step Down Latency (SDL) were noted on the eighth day and again after 24 h. Scopolamine (0.4 mg/ kg i.p.) was administered to drug treated animals (Group II to IX) and Transfer latancy (TL) and Step Down latency (SDL) were noted after 45 min of injection on the eighth day and again after 24 h, i.e. on the ninth day.

***In-vitro* Antioxidant activity:**

Reagents: TRIS [2-amino-2 (hydroxy methyl) propane 1-3di-ol] buffer (pH 7.4), DPPH (1, 1-diphenyl-2-picryl-hydrazyl), Gallic acid.

Determination of Diphenyl -2-Picryl Hydrazyl (DPPH) Radical Scavenging activity: The antioxidant activity of alcoholic extract of *Bacopa monnieri*, methanolic extract of *Withania somnifera*, and the combination of both was determined by using a method based on the reduction of methanolic solution of coloured-free radical 1, 1 di phenyl-1-2 picryl hydrazyl (DPPH). Gallic acid was used as reference standard.

In 5 ml volumetric flasks, added 1 ml of DPPH solution, 1 ml of TRIS Buffer and 0.5 ml of final dilutions of different concentrations range prepared from stock solutions of alcoholic extract of *Bacopa monnieri*, methanolic extract of *Withania somnifera* and their combination and made up the volume to 5 ml with methanol. In same way, we prepared the control dilutions of DPPH, replacing 0.5 ml of prepared dilutions (the drug solution under investigation) with methanol.

The absorbance of all the dilutions was taken after 30 minutes at λ max 517nm using methanol as blank.

Statistical analysis: The percentage inhibition was calculated using:

$$\text{Percent Inhibition} = \frac{A_C - A_S}{A_C}$$

Where, A_C is absorbance of control and A_S is the absorbance of sample.

RESULTS:

Nootropic activity:

Acute toxicity studies: All the doses (50, 300 and 2000 mg/kg, p.o.) of Extracts of *B. monnieri, W. somnifera* and powder of *P. nigrum* were found to be non-toxic. They did not produce mortality even at the highest dose (2000 mg/kg, p.o.) employed.

Effect on Transfer Latency (Using Elevated plus Maze): Transfer Latency (TL) was defined as the time (in seconds) taken by the animal to move from one open arm into one of the covered arms with all its legs. Significant reduction in TL value of retention indicated improvement in memory. Alcoholic extract of *Bacopa monnieri* (1000 mg/kg, p.o.) and its combination with powder of *Piper nigrum* (250 mg/kg) showed reduction in TL of 8th day (10.99 ± 0.448 sec and 8.35 ± 0.657 sec respectively) in animals, when compared to control group (15.27 ± 2.092 sec) indicating significant improvement in memory.

While methanolic extract of *Withania somnifera* (1000 mg/kg, p.o.) alone and in combination with powder of *Piper nigrum* with TL (26.40 ± 5.340 sec and 17.92 ± 4.565 sec) showed no improvement in memory of animals as compared to the control group (**Fig. 1**). However, the combination of Alcoholic extract of *B. monnieri* and methanolic extract of *W. somnifera* showed reduction in TL (10.45 ± 1.465 sec) of animals while when this combination was given with powder of *Piper nigrum* showed more reduction in TL (8.16 ± 0.196 sec) of animals. This indicates that the combination of Alcoholic extract of *B. monnieri*, methanolic extract of *W. somnifera* and powder of *Piper nigrum* shows significant decrease in TL of animals as compared to control group.

Scopolamine (0.4 mg/kg) administered animals showed significantly increased TL (50.05 ± 14.15 sec) of 9th day indicating impairment in memory of animals. *B. monnieri* alone and in combination with *P. nigrum* successfully reversed memory deficits induced by scopolamine similarly, combination of *W. somnifera*, *B. monnieri* and *P. nigrum* reversed memory deficits induced by scopolamine. The positive control Piracetam (200mg/kg) improved memory of mice and reversed the amnesia induced by scopolamine (**Fig. 2**).

Effect on Step down Latency (using Passive Avoidance Paradigm): Step down Latency (SDL) was defined as the taken time by the mouse to step down from the wooden platform to grid floor with all its paws on the grid floor. Increase in SDL value indicated improvement in memory. SDL of 8th day reflected the long term memory of mice Alcoholic extract of *Bacopa monnieri* (1000 mg/kg, p.o.) and its combination with powder of *Piper nigrum* (250 mg/kg) showed reduction in SDL of 8th day (40.3 ± 1.577 sec and 70.786 ± 12.601 sec) in animals, when compared to control group (80.12 ± 8.458 sec) indicating no improvement in long term memory.

Similarly, methanolic extract of *W. somnifera* (1000 mg/kg) and its combination with powder of *Piper nigrum* (250 mg/kg) showed reduction in SDL of 8th day (20.37 ± 2.234 sec and 60.83 ± 7.265 sec) in animals, when compared to control group (80.12 ± 8.458 sec) indicating no improvement in long term memory (**Fig. 3**).

However the combination of *B. monnieri* and *W. somnifera* did not show any improvement in long term memory but the combination of *B. monnieri*, *W. somnifera* and *P.nigrum* showed a significant improvement in SDL (116.42 ± 15.569) of 8th day in mice when compared to control group. Scopolamine (0.4 mg/kg) administered showed significant decreased SDL (10.44 ± 0.143 sec) of 9th day indicating impairment in memory of mice.

All the drugs used alone and in combination did not reverse the amnesia induced by scopolamine. The positive control Piracetam (200 mg/kg, p.o.) showed improvement in memory and reversed amnesia induced by scopolamine.

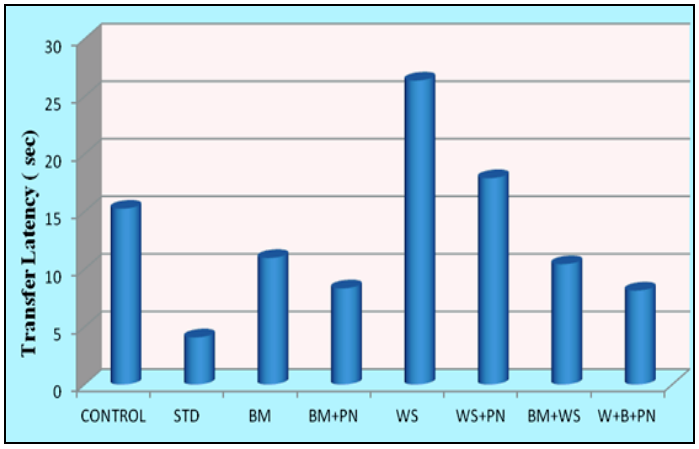

FIG. 1: EFFECT DRUGS AND THEIR COMBINATIONS ADMINISTERED ORALLY FOR SUCCESSIVE 8 DAYS IN TRANSFER LATENCY OF MICE USING ELEVATED PLUS MAZE

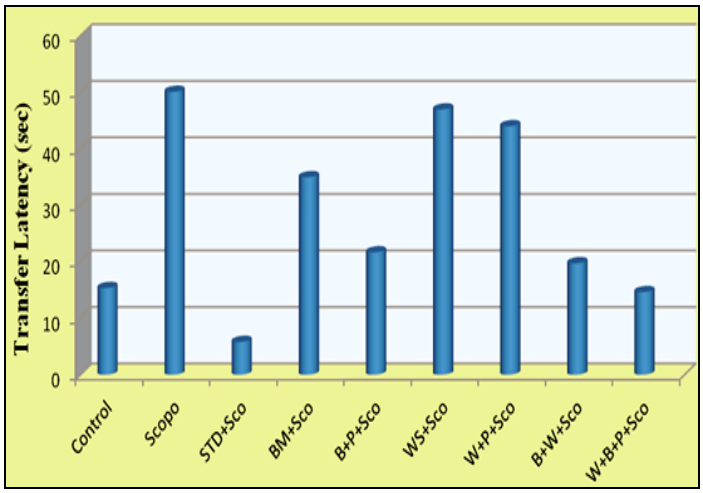

FIG. 2: REVERSAL OF SCOPOLAMINE INDUCED AMNESIA BY SINGLE DRUGS AND THEIR COMBINATIONS ADMINISTERED ORALLY FOR 8 SUCCESSIVE DAYS IN TRANSFER LATANCY IN MICE USING ELEVATED PLUS MAZE.

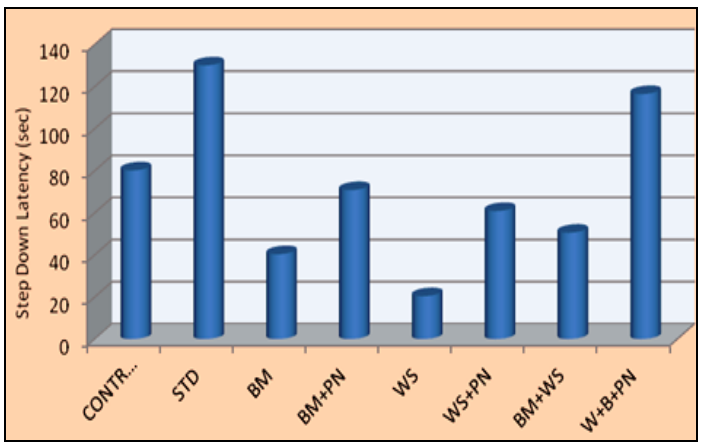

FIG. 3: EFFECT OF DRUGS AND THEIR COMBINATIONS ADMINISTERED ORALLY FOR 8 SUCCESSIVE DAYS IN STEP DOWN LATENCY OF MICE USING PASSIVE AVOIDENCE PARADIGM

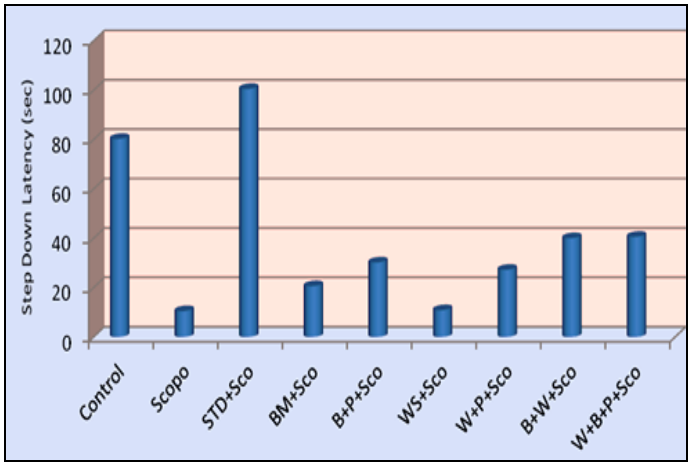

FIG. 4: REVERSAL OF SCOPOLAMINE INDUCED AMNESIA BY SINGLE DRUGS AND THEIR COMBINATIONS IN MICE USING PASSIVE AVOIDENCE PARADIGM.

Antioxidant activity: The present study was undertaken to evaluate the antioxidant activity of alcoholic extract of *Bacopa monnieri*, methanolic extract of *Withania somnifera* and their combination. In the free radical scavenging (DPPH) assay lower IC_{50} value indicates higher free redical scavenging activity. Here we used Gallic Acid as standard. The IC_{50} value of Gallic Acid was found to be 6.03µg/ml **(Fig. 5).** The IC_{50} value of alcoholic extract of *B. monnieri*, methanolic extract of *Withania somnifera* and their combination was found to be 56 µg/ml, 7.6 mg/ml and 0.812 mg/ml respectively **(Fig. 6, 7** and **8** respectively**).** These results indicate that *B. monnieri* has a noticeable effect on scavenging free radicals, but lower than Gallic acid and higher than methanolic extract of *W. somnifera* and the combination of both extracts.

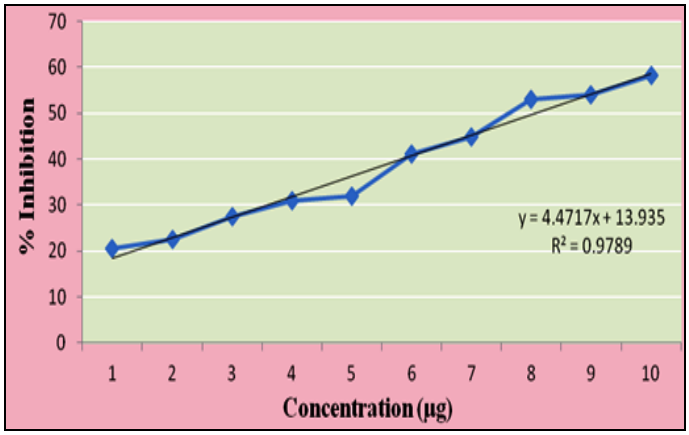

FIG. 5: FREE RADICAL (DPPH) SCAVENGING ACTIVITY OF METHANOLIC SOLUTION OF GALLIC ACID IN *IN VITRO* SYSTEMS. Graphical representation of the concentration required inhibiting 50 percent of free radicals. (Regression coefficient =0.9789, IC_{50} = 6.03µg/ml), from the equation, When Y= 50, X=8.066

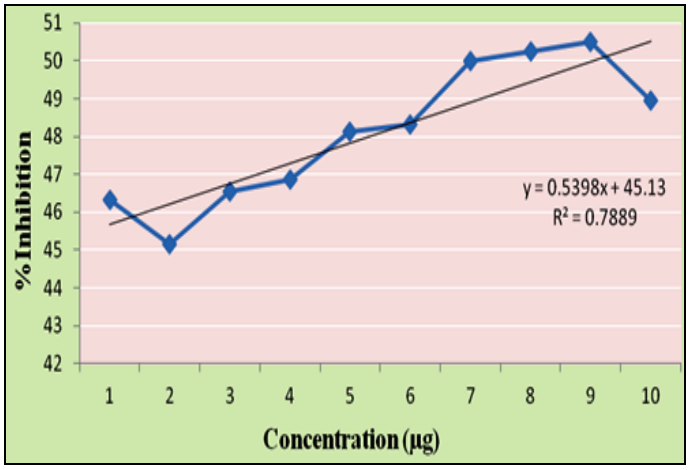

FIG. 6: FREE RADICAL (DPPH) SCAVENGING ACTIVITY OF ALCOHOLIC EXTRACT OF *BACOPA MONNIERI* IN *IN-VITRO* SYSTEMS. Graphical representation of the concentration required inhibiting 50 percent of free radicals. (Regression coefficient =0.7889, IC_{50} =56µg/ml), When Y= 50, X=9.021

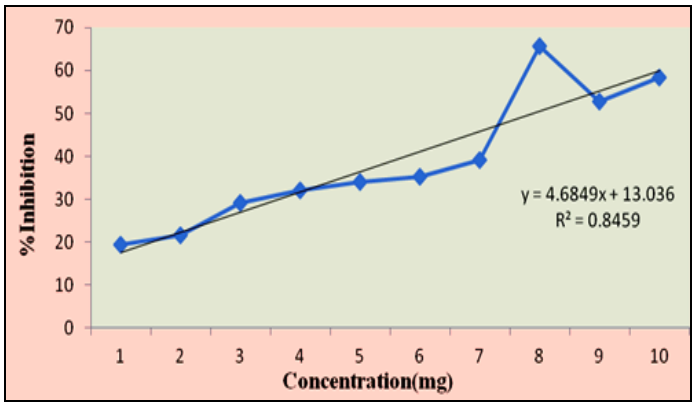

FIG. 7: FREE RADICAL (DPPH) SCAVENGING ACTIVITY OF METHANOLIC EXTRACT OF *WITHANIA SOMNIFERA* IN *IN-VITRO* SYSTEMS. Graphical representation of the concentration required inhibiting 50 percent of free radicals. (Regression coefficient =0.8459, IC_{50} =7.6mg/ml)

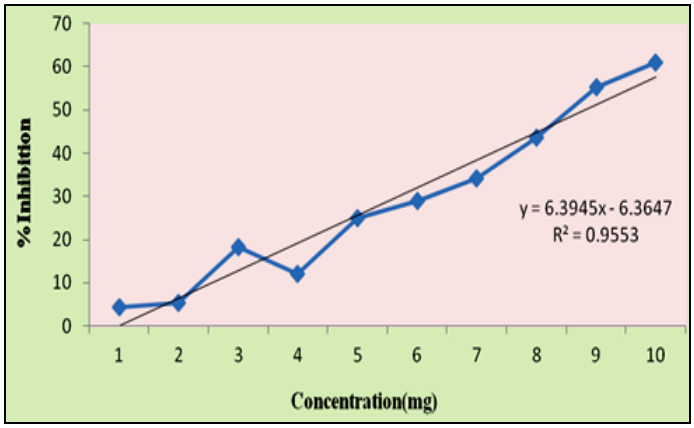

FIG. 8: FREE RADICAL (DPPH) SCAVENGING ACTIVITY OF COMBINATION OF ALCOHOLIC EXTRACT OF *BACOPA MONNIERI* AND METHANOLIC EXTRACT OF *WITHANIA SOMNIFERA* IN *IN-VITRO* SYSTEMS. Graphical representation of the concentration required inhibiting 50 percent of free radicals. (Regression coefficient =0.9553, IC_{50} =0.812mg/ml).

DISCUSSION: Several research studies have identified natural compounds that serve as nootropic agents. Extracts and compounds have been selected and isolated from medicinal plants based on their value in traditional medicinal system. To, date pharmaceutical companies have been investing enormous resources in the identification of agents that could possibly alleviate debilitating disorders and slow the onset of mental retardation. There is a focus on phytochemicals, which seem to have these properties, through their full potential is yet to be determined. *B. monnieri* and *W. somnifera* are such plants with wide medicinal properties that are being used as treatment for memory related disorders [13].

The present study was designed to determine whether the alcoholic extract of *B. monnieri* and methanolic extract of *W. somnifera* and in combination with *P. nigrum* administered at fixed dose (*B. monnieri*: 1000mg/kg, *W. somnifera*: 1000 mg/kg and *P. nigrum*: 250 mg/kg) would bring about behaviour changes especially in learning and memory.

The results of Elevated Plus maze test showed that the combination of *B. monnieri, W. somnifera* and *P. nigrum* has significant reduction in TL as compared *B. monnieri,* combination with *P. nigrum. W. somnifera* alone and in combination with P. nigrum and *B. monnieri* did not show any positive effect and these combinations were not found to be beneficial. These results clearly indicate that the oral administration of the combination of *B. monnieri, W. somnifera* and *P. nigrum* improved learning and memory in mice. In the Passive Avoidance Paradigm, there was no significant change in behaviour during exploration.

In view of the importance of these plants, groups of researchers have carried out systematic chemical examinations and reported the major chemical constituents responsible for memory facilitating action are steroidal saponins and bacosides A and B which are present in alcoholic extract of *B. monnieri*[14], and sitoindiside VII – X and withaferin - A present in methanolic extract of *W. somnifera*. These compounds may be responsible for improved learning and memory in mice.The main constituent of *P. nigrum*, Piperene is responsible for increasing the bioavailability of drugs more than 100%.

When the extracts of *B. monnieri* and *W. somnifera* were combined with powder of *P. nigrum* there is reduction in TL it may be due to increment in bioavailability of drugs.

The DPPH redical scavenging method is a standard procedure applied to evaluate the general antioxidant activity of plants extracts, especially which assigned to phenolic compounds, i.e. Flavonoids [13]. The antioxidant activity of *B. monnieri* and *W. somnifera* might be attributed to their polyphenolic contents and flavonoids [15, 16]. Earlier phytochemical studies of *B. monnieri* and *W. somnifera* have shown the presence of polyphenolic compounds and flavonoids. Thus, the polyphenolic compounds and flavonoids may be responsible for Antioxidant Activity of *B. monnieri* and *W. somnifera.*

CONCLUSION: *Bacopa monnieri* and *Withania somnifera* have been used as traditional medicine due to its neurotonic and memory enhancing property. This study demonstrates that Alcoholic extract of *Bacopa monnieri* alone and its combination with methanolic extract of *Withania somnifera* and powder of *Piper nigrum* diminishes neuronal death induced by AChE activity. Alcoholic extract of *Bacopa monnieri* also exhibited in-vitro antioxidant property which may be responsible for the reduction in oxidative stress. It is reported that neuroprotective effects of *Bacopa monnieri* appeared to be the results of its antioxidant to suppress neuronal oxidative stress and the acetyl cholinesterase inhibitory activities [17].

Overall results from the present study shows the potential of *Bacopa monnieri* extract and its combination with extract of *Withania somnifera* and powder of *Piper nigrum* as a remedy to prevent Dementia in natural aging as well as an alternative remedy for neurodegenerative disorders associated with oxidative stress and AchE induced memory loss.

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