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Memory and cognition enhancing effects of combination of *Melissa officinalis* and *Panax ginseng*

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Abstract: *Melissa officinalis* and *Panax ginseng* extracts were investigated to determine combinatorial effects on cognitive behaviors' of albino-rats. The study was prospective-experimental; lasted from June-2022 to March-2023. Learning and memory measurements were done by animal-models. Data analyzed by 22nd version of SPSS. In Passive-avoidance-test both doses of *Melissa officinalis* and *Panax ginseng* (100/100mg/kg and 200/200mg/kg) showed significant differences in number of acquisition-trial between groups ($p<0.001$); drug treated groups showed longer latency-period compared to control and scopolamine ($p<0.001$). In time-spent-in-dark-chamber treated groups spent less-time in dark-chamber as compared to control and scopolamine ($p<0.001$). In Morris-water-maze-task treatment groups (100/100mg/kg and 200/200mg/kg) showed significant ($p<0.001$) decrease in escape-latency compared with control and scopolamine. Spatial-memory-probe showed significant interaction between drugs and days ($p<0.001$); time-spent in platform region is significantly increased ($p<0.001$) in both treatment groups compared with control and scopolamine. 8-arm-radial-maze-test showed the significant increase ($p<0.05$) in total number of correct responses in treatment groups (100/100mg/kg and 200/200mg/kg) compared to control and scopolamine. *In-vitro* studies revealed acetyl-choline-esterase inhibition by 36.40% from *Melissa officinalis* and *Panax ginseng* combination. Study concluded that combination of *M. officinalis* and *P. ginseng* extracts may significantly improve the effects on memory and cognition.

Keywords: Cognitive-dysfunction, dementia, *Melissa-officinalis, Panax-ginseng,* memory, cognition.

INTRODUCTION

Development of cognitive abilities is imperative for the intellectuality of an individual (Peng *et al.*, 2020). Mild cognitive dysfunction refers to a decline in the memory and other mental abilities without significant functional impairment. It is assumed that this impairment will worsen over time due to growing elderly population around the world. Mild intellectual impairment in individuals can cause significant strain on the public healthcare system; this impairment is associated with the development of any form of dementia or AD (Alzheimer's disease) (Judge *et al.*, 2019). Dementia cases are expected to be quadrupled every 20 years and may reaching 81.1 million worldwide by 2040 (Srivastav *et al.*, 2023). Delaying the process of cognitive impairment is a solution; several methods are available for doing so e.g. use of the cognitive enhancers (Smart drugs/Nootropics). Rivastigmine, donepezil and galantamine are cholinesterase inhibitors and are licensed for the treatment of the AD (Vecchio *et al.*, 2021).

Traditional medicine (herbs and herbs' ingredients) claims that they can improve cognitive performance and reduce other symptoms of the AD such as dementia, anxiety and depression (Guzman-Martinez *et al.*, 2021). Plant components may not only interact favorably with one another, but may also boost the efficacy of chemicals derived from other plant species; perhaps due to same reason Ayurveda and traditional Chinese medicines (TCM) prescribed in the combination of botanicals (Hickson *et al.*, 2021). The identification of plants and prospective new medications for the treatment of cognitive disorders have been aided by ethnopharmacological approach, this approach may aid in the discovery of a more diverse and effective drugs for the treatment of cognitive neurodegenerative disorders (Süntar, 2020).

Herbal extracts were evaluated for a complicated condition like AD, which consists of a wide variety of ingredients with different pharmacological effects (Singh *et al.*, 2019). As a member of the Labiatae family, *Melissa officinalis* L. is commonly used for anxiety and depression in the Iranian traditional medicine (Naghibi *et al.*, 2022). Alternate names for this plant are balm mint and lemon balm (Waheed *et al.*, 2020). Because of its many beneficial effects on the health, *Melissa officinalis* is commonly used as an anti-oxidant, anti-inflammatory, anti-microbial, anxiolytic, antidepressant and cognitive enhancer through its neuro-protective characteristics (Świąder *et al.*, 2019). The primary chemical components of *M. officinalis* are polyphenols (rosmarinic acid, caffeic

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acid, hydroxyl cinnamic derivatives and chlorogenic acid), triterpenes, flavonoids, monoterpene glycosides and essential oils (Mahboubi, 2019). *Asian ginseng* or *Panax ginseng*, a popular herbal supplement has shown numerous therapeutic and pharmacological effects. Most of the ginseng produce vaso-relaxing, anti-oxidizing, anti-inflammatory, cancer-fighting and cognition enhancing effects, these effects are thought to be originated from ginsenosides; a primary pharmacologically active component (Kim, 2018).

Due to the availability of limited data and potential effects on cognitive functions of these herbs while given in combination; *Melissa officinalis* and *Panax ginseng* were investigated in depth in animal models to determine whether the combination of *Melissa officinalis* and *Panax ginseng* combined extracts have any effect on the CNS (Central Nervous System) and can alter the cognitive behaviors' of albino rats.

MATERIALS AND METHODS

Study design

An experimental study with a prospective design was carried out in the Pharmacology Research Laboratory at the Jinnah University for Women (JUW), Karachi. *In-vivo* and *in-vitro* research were carried out to evaluate the effects of herbs on cognition and memory. The study lasted from June 2022 until March 2023.

Ethical approval

Approved by Institutional Ethical Review Board (Reference No. JUW/IERB/PHARM-ARA-006/2022) and Board of Advanced Studies & Research (Reference No. BASR/Extr./79th/Proc./Feb/2021), Jinnah University for Women, Karachi, Pakistan.

Herbs' collection

Lemon balm extract (Batch # 759578-396595; Expiry: July 2023) was purchased from Biofinest LLC, they produce standardized extract of *Melissa officinalis* L. dry leaves powder for commercial sale (USA). The powder was reconstituted in distilled water after being weighed to the prescribed dose. The roots of *Panax ginseng* were obtained from a reliable local vendor and dried. After determining the appropriate dosage, the roots were grinded in a mechanical grinder into coarse powder.

Extract preparation

A decoction of a commercially available and standardized extract of lemon balm (*Melissa officinalis*) was prepared by adding 1g of sample to 200ml of distilled water; simmered the mixture for a specified amount of time using a heating plate from OM Tope MS300HS; then strained or filtered the resulting liquid. The liquids' volume was reduced by around 25% throughout the extraction process. The purified extract was then given orally to rats for determination of cognitive effects and mice for determination of acute toxicity (Martins *et al.*, 2015).

The roots of 200g of ginseng were finely chopped and extracted three times over the course of 48 hours using 2 liters of 60% concentrated ethanol.(B. Lee *et al.*, 2010) In the next step the solution was filtered through muslin fabric and finally by filter paper no#1 (Whatman). The solvent was evaporated using a rotavapour (BCHI R-200 type) to get a concentrated extract; further it was dried in a drying hood at room temperature. Fraction yield (1.6393 w/v) was calculated using a weighed sample of the dried substance (Lee *et al*., 2010). The resulting extract was stored in the refrigerator in an airtight jar for later use.

Dose preparation

Doses of *Melissa officinalis* and *Panax ginseng* extracts were mixed in the distilled water; the doses for animals were estimated based upon body weight; the mixture was then administered to the animals for 30 days. Weighed 100 mg of dried extract of *Melissa officinalis* and extract of *Panax ginseng* was in weight/volume 200 g/122ml (Ishrat *et al*., 2006). Fraction yield (1.6393 w/v) was calculated using a weighed sample of the dried substance. Dose of 100/100mg/kg (B. Lee *et al*., 2010; E. N. Martins *et al.*, 2012) were prepared by combining 100mg dried *Melissa officinalis* extract and 0.061 ml of *Panax ginseng* which was equivalent to 100 mg of *Panax ginseng.* Similarly, for dose of 200/200 mg/kg (Lee *et al*., 2010; Ozarowski *et al.*, 2016b), 200 mg dried *Melissa officinalis* extract was combined with 0.122 ml of *Panax ginseng* which was equivalent to 200 mg of *Panax ginseng*.

Chemicals and reagents

Analytical quality pharmaceuticals and substances were used. Scopolamine was utilized; a chemical supplied by Sigma-Aldrich®. A total of 10 milliliters of water was used to dilute the scopolamine until it has reached to the desired concentration. The animals received 1mg/Kg of scopolamine through intra-peritoneal (I.P) injection. The memory-impairing drug scopolamine has same effect on humans as it does on animals (Jasira *et al.*, 2014). Donepezil 5mg made by a Pharmaceutical Industry of Pakistan was purchased from the market; which was approved by Drug Regulatory Authority of Pakistan for the indication of Alzheimer's dementia by inhibition of choline-esterase enzyme.

Experimental animals

In the current study mice used to determine acute toxicity and Albino rats to determine cognitive effects. Rats were selected weighed between 150g and 250g. In JUW's animal house rats were housed in the hygienic polypropylene containers. They had access to plenty of food and water; temperatures of 25°C ± 2 (Dehbani *et al.*, 2019), relative humidity of 55 to 60% *and* a light/dark

cycle of 12 hours were provided. Animals in the study spent at least two weeks to acclimate with the laboratory environment before involvement in any experiments (Ozarowski *et al.*, 2016a). Thirty adult male and female wistar albino rats were selected and divided at random in five experimental groups (n=6) and doses were administered using oral syringe to all experimental groups to make sure that all of the doses are consumed by the animals.

Group 1 – Control; received distilled water 5 ml/kg P.O (Per-Oral) once daily for 30 days.
Group 2 – Scopolamine; received 1 mg/kg I.P (Intra-peritoneal) once daily for 30 days.
Group 3 – *Melissa officinalis* & *Panax ginseng*; received combined extract once daily P.O (100/100 mg/kg) for 30 days.
Group 4 – *Melissa officinalis* & *Panax ginseng*; received combined extract once daily P.O (200/200 mg/kg) for 30 days.
Group 5 – Donepezil (Standard); received once daily P.O (5 mg/kg) for 30 days.

Acute toxicity evaluation

Acute toxicity study was conducted as per guideline no. 425 (Up-and-Down method) established by Organization of Economic Corporation Development (OECD) (Sachana *et al.*, 2018). Twenty-four mice(Saleem *et al.*, 2017) were selected for acute toxicity testing (12 male; 12 female). They were separated into four groups and six (n=6) mice in each group. Three groups were referred to treated group, while one group was referred as control group. Combination of *Melissa officinalis* and *Panax ginseng* was administered to all treated groups of mice weighing 20-30g (*n*=6) in the doses of 1000, 3000 and 5000 mg/kg PO, the extract taken for the calculation of these doses were 50% from *Melissa officinalis* and 50% from *Panax ginseng*, that is why we can assume that given dose were 1000mg/kg (500 mg/kg *Melissa officinalis* extract + 500mg/kg *Panax ginseng* extract), 3000 mg/kg (1500 mg/kg *Melissa officinalis* extract + 1500 mg/kg *Panax ginseng* extract) and 5000 mg/kg (2500 mg/kg *Melissa officinalis* extract + 2500mg/kg *Panax ginseng* extract). Initially all groups were observed for thirty minutes, then four hours and then every forty-eight hours after the administration of drugs. Although mortality rate was end-point of the toxicity; however, none of the animal reached to this end-point. The gross behavioral changes were also monitored for the first 2-hour to observe the signs of animals' touch sensitivity, irritability, responsiveness, tremors, salivation, diarrhea, lacrimation and lethargy. The humane end-points (animal ethical end-point) were development of coma symptoms in animals, decrease in body weight ≥15%, immobility, respiratory distress, ataxia and edema (Vilar-Pereira *et al.*, 2021). As per protocol if animal would reach to any of the humane end-point, animal would be euthanized immediately by inhaling 70% CO_2 for 2-3 minutes in the chamber followed by cervical dislocation (Shomer *et al.*, 2020) Further animal would be observed for 30 seconds after cessation of heartbeat.(Boivin *et al.*, 2017) None of the animal reached to humane end-points.

Measurement of learning and memory

Passive avoidance test

Learning and memory were evaluated using the passive avoidance test (Karimi *et al.*, 2019).

Apparatus

Passive avoidance learning (PAL) acquisition test (Dehbani *et al*., 2019) was used to evaluate the memory in rodents. The "shuttle box" was a piece of testing equipment for passive avoidance learning; it consists of a pair of identically sized light- and dark-colored chambers. A guillotine door (5cm × 5cm) isolated the dark area from the light room. There were 6 mm-distance stainless steel rods on the floor in the dark and light chambers.

Passive avoidance acquisition

Each rat underwent this evaluation over the course of four days. On the first and second day of testing, each rat spent 5 minutes for acclimation to the apparatus. The acquisition test was conducted on day third. The rat was facing away from the door and located in the light chamber. The light compartment was opened for it to explore for 20 seconds. After 20 seconds, the guillotine door was opened and a foot shock (50Hz square wave, 1mA current) was delivered through stainless steel rods to the rat. The animal was returned to their cage and given 30 seconds of quiet time. When re-tested, if the animal still did not enter the dark chamber within 120 seconds (2 minutes), the rats were considered to successfully learn the behavior of passive avoidance. The number of attempts was recorded as an indicator of progress in training.

Retention test

A retention test was done 24 hours after the training or PAL acquisition exam on day 5. Similar to PAL acquisition, rats were reintroduced into the light compartment and after 5 seconds guillotine's gate was opened, allowing the rat to move into the compartment, which is dark. At this point STL (step-through latency) and TDC (time spent in the dark chamber) of animals were recorded for 300 seconds. If an animal could not definitively reach its darker container within 300 seconds, test was discontinued (Lee *et al.*, 2017).

Morris water maze task

Apparatus

To perform the Morris water maze (Lee *et al*., 2017) challenge, we used a polypropylene pool with a white interior (2.0 meters in circumference and 0.35 meters in depth). The temperature of the swimming pool water was kept at a constant 25°C. The pool was divided into four

equal parts: the northeast, southeast, southwest *and* northwest. Approximately 50 centimeters from one of the quadrant's margins was a white platform, 10 centimeters in diameter and 30 centimeters in height that has been submerged.

Trial sessions

This phase of experiment lasted for 4 consecutive days (total 16 trials) followed by a probe trial on 5th day. In each session rats were taught to uncover the submerged platform. Time spent by rats searching for hidden platform was recorded for maximum of 120 seconds. The rat was given an extra 10 seconds on the platform if located the submerged stage. If the rat took longer than 120 seconds to find the hidden platform, it was brought to the platform and allowed to stay there for extra 10 seconds (Lee *et al*., 2010).

Probe trial

During the 90-seconds of probe swim trial, platform was removed from the water and from the opposite location rats were released in the water. Time spent by rats searching for the platform to its previous location was used to calculate retention time (Mahboubi *et al.*, 2016).

Eight-arm radial maze task

Apparatus

The 8-arm radial maze task was used for behavioral testing in rats (Kurzina *et al.*, 2020). Each arm of the maze was 12cm wide and 50cm in length. They were attached to a central octagonal chamber that was having 30 cm diameter. At the end of each arm, food pellets were placed that served as bait.

Procedure

The rats were trained with food rewards for 4 consecutive days. Each rat was placed in the center of the maze, timer was started and the rat was allowed to explore the maze in 10 minutes. On day-5 food pellets were removed and rats were allowed to wander all 8- arms of the maze. When the rat went into an arm that it had never been visited previously, a response was considered as correct. Whereas when the animal re-visited any arm, the response was considered as an error. Consequently, our approach assessed working memory for cues encountered during a particular task session.

In-vitro study

Estimation of Acetylcholine inhibitions by sample extracts

The modified Ellman's method was used for the estimation of Acetyl cholinesterase inhibition. 150µl of tested sample solution (various concentrations of *Melissa officinalis and Panax ginseng* such as 4mg, 2mg and 1mg) was taken in 3mL of a 0.1M phosphate buffer of pH=8, 80µL of 0.01M DTNB (3.96 mg of 5:5-dithiobis-2-nitro benzoic acid, 10ml of 0.1M phosphate buffer of pH=7 contained 15mg of sodium bicarbonate) and an enzyme in a quantity of 20µL with concentration of 2U/ml. At 25^{o} C and for 5 minutes mixture was incubated. Following pre-incubation, the substrate in a quantity of 15µL was added and then incubated for 5 minutes. The substrate is ATCI (acetylthiocholine iodide):10.85mg in 5ml of phosphate buffer. The UV-visible spectrophotometer (Shimadzu) was used to determine the absorbance at 412nm.

Mixture of 3ml of phosphate buffer, 50µl enzyme and 1 ml of Ellman's reagent were used as blank control in this type of run. Percentage inhibition was calculated by comparing sample and control rates (Ellman *et al.*, 1961; Vinutha *et al.*, 2007).

The percentage of inhibition was calculated from the following formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance (Control)} - \text{Absorbance (Sample)}}{\text{Absorbance (Control)}} \times 100$$

STATISTICAL ANALYSIS

Data was collected for primary evaluation of cognition and memory enhancing effect of combination of *Melissa officinalis* and *Panax ginseng* extracts. To compare more than one variable at a time, One-way ANOVA (Analysis of variance) was applied to analyze the mean (±standard error) and followed by post-hoc analysis by LSD (Least Significant Difference) through a SPSS (Statistical Package for Social Sciences) version 22. Noted the significant differences if exists in between the treatments (100/100mg/kg, 200/200mg/kg), scopolamine and vehicle group of animals. Difference was considered significant if p-value <0.05

RESULTS

Acute toxicity

Combination extract of *Melissa officinalis* and *Panax ginseng* did not cause any toxicological, behavioral, or physiological abnormalities in mice over a 48-hour observation and 30-days follow-up period. Within the first 48 hours and throughout the next 30 days of follow-up, current investigation revealed no changes in behavioral, neurological, autonomic, or physical profile. The research also showed that the oral lethal dose of the *Melissa officinalis* and *Panax ginseng* combination was greater than 5g/kg.

Effect of Melissa officinalis and Panax ginseng on passive avoidance test

In the PAL test, both doses of drugs (100/100 mg/kg and 200/200 mg/kg) showed the statistically significant ($p<0.001$) difference on the number of acquisition trial between groups. Significant decrease in number of acquisition trial was observed between both treatment groups compared with control and scopolamine

(p<0.001). Significant (p<0.001) increase in number of trial is observed when treatment groups compared with Donepezil (Standard) (table 1).

In Step-through latency (STL), significant effect of drugs was observed among the groups (p<0.001). Drug treated groups showed longer latency period in the comparison with control and scopolamine (p<0.001) (table 2).

In Time spent in dark chamber (TDC), significance of drugs was observed between all groups (p<0.001). Both treated groups spent less time in the dark chamber as compared to control and scopolamine groups (p<0.001) (table 3).

Effect of Melissa officinalis and Panax ginseng on Morris water maze task

In Morris water maze task, data analysis was conducted using a two-way ANOVA. The variation in mean effects of treatments (p<0.001) and variation in mean effects in days (p<0.001) and the interaction between treatments and days (p<0.001) were all statistically significant. Animals of treatment groups (100/100 mg/kg and 200/200 mg/kg) showed significant (p<0.001) decrease in escape latency over the training course of four days in comparison with control and scopolamine groups (table 4).

Spatial memory Probe trial evaluation showed that in between days and drugs significant interaction are noted (p<0.001); drugs had a significant impact (p<0.001) and days had a noteworthy effect (p<0.001). Similarly, Time spent in the platform region is significantly increased (p<0.001) by the animals in both treatment groups compared with control and scopolamine groups; which resulted in a marked enhancement of both memory and cognitive ability (table 5).

Effect of Melissa officinalis and Panax ginseng on 8-arm radial maze task

In 8-arm radial maze test; two-way ANOVA revealed the significant impact of drugs on total number of correct choices (p<0.001) and non-significant impact on days (p=0.460) and interaction between the two components (p=0.803) (table 6). LSD post-hoc test showed the significant increase (p<0.05) in total number of correct responses by the animals in treatment (100/100 mg/kg and 200/200 mg/kg) compared with control and scopolamine groups, treatment resulted in improvement of learning performance over the period of 5 days.

It was found that there was a strong influence of the treatment drugs on number of errors (re-entry) (p<0.001), days (p<0.001) and an interaction of drugs and days (p<0.01) (table 7). Post-hoc test (LSD) showed a statistically significant increase in the overall number of errors (re-entry) (p<0.05), when treatment groups are compared with vehicle and negative control groups. This indicates the improvement of reference memory in the treated-rats because the arms were baited and they learnt their location during the training sessions.

Evaluation of Acetylcholine esterase enzyme inhibition

In-vitro tests have shown inhibitory rates of acetylcholine esterase enzyme of the combination of *M. officinalis* and *P. Ginseng* in three different doses (fig. 1); positive control drug or standard (Donepezil) (fig. 2).

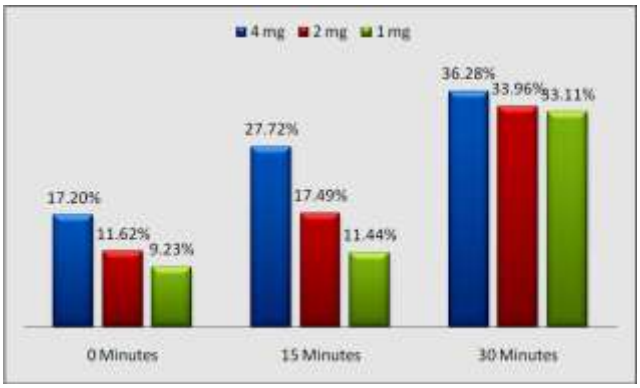

Fig. 1: Percent inhibition of combination of Acetylcholine esterase by combination of *Melissa officinalis* and *Panax ginseng* at different doses.

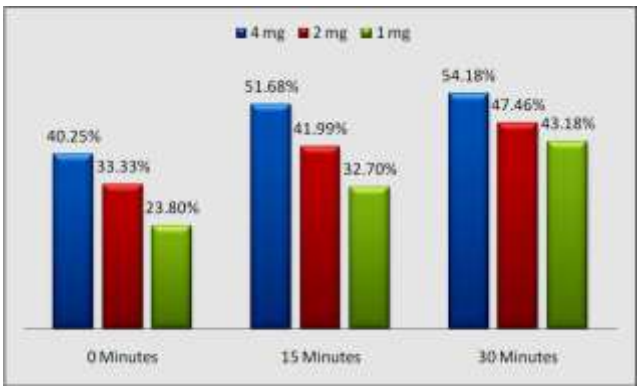

Fig. 2: Percent inhibition of Acetylcholine esterase by Donepezil standard drug at different doses.

DISCUSSION

Globally researches on plants have worked and brought significant outcomes, many compounds are isolated having medicinal values (Salmerón-Manzano *et al.*, 2020). These isolated compounds individually and in combination produce clinical benefits. Similarly; current study has shown synergistic effect of *Melissa officinalis* and *Panax ginseng* and are well tolerated by mice at the highest doses (1,000, 3,000 and 5,000 mg/kg body weight).

Table 1: Number of acquisition trial

Treatment groups	Number of acquisition trial (Mean±S.D)
Group-1 (control)	3.17 ± 0.408
Group-2 (Scopolamine)	4.67 ± 0.516
Group-3 (treated 100/100 mg/kg/day)	2.33 ± 0.516[b,d,e]
Group-4 (treated 200/200 mg/kg/day)	2.33 ± 0.516[b,d,e]
Group-5 (standard)	1.00 ± 0.000[c,d]

Significance are shown in between groups: [b] 3 and 4 with 1 ($p<0.01$), [c] 5 with 1 ($p<0.001$); [d] 3, 4 *and* 5 with 2 ($p<0.001$); [e] 3 and 4 with 5 ($p<0.001$)

Table 2: Step-through latency (STL)

Treatment groups	Mean±S.D (Seconds)
Group-1 (control)	100 ± 3.55
Group-2 (Scopolamine)	2.67 ± 0.516
Group-3 (treated 100/100 mg/kg/day)	299 ± 0.894[b,c]
Group-4 (treated 200/200 mg/kg/day)	300 ± 0.00[b,c]
Group-5 (standard)	300 ± 0.00[b,c]

Significance are shown in between groups: [b] 3, 4 and 5 with 1 ($p<0.001$); [c] 3, 4 and 5 with 2 ($p<0.001$)

Table 3: Time spent in dark chamber

Treatment groups	Mean±S.D (Seconds)
Group-1 (control)	199 ± 2.875
Group-2 (Scopolamine)	300 ± 0.00
Group-3 (treated 100/100 mg/kg/day)	0.83 ± 0.753[b,c]
Group-4 (treated 200/200 mg/kg/day)	0.00 ± 0.00[b,c]
Group-5 (standard)	0.00 ± 0.00[b,c]

Significance are shown in between groups: [b] 3, 4 and 5 with 1 ($p<0.001$); [c] 3, 4 and 5 with 2 ($p<0.001$)

Table 4: Time to find the submerged platform during training sessions

Treatment groups	Day 1	Day 2	Day 3	Day 4
	Escape latency (Seconds Mean±S.D)			
Group-1 (control)	113.17±1.602	110.67±1.211	104.83±1.169	83.17±2.317
Group-2 (Scopolamine)	118.50±1.225[a]	113.00±1.789[i]	111.50±2.258[a,i]	110.17±1.722[a,i]
Group-3 (treated 100/100mg/kg/day)	111.17±1.47[c,f]	109.00±1.673[b,f]	80.67±1.506[a,c,f,i,j]	48.67±1.506[a,c,f,i,j,k]
Group-4 (treated 200/200mg/kg/day)	105.00±2.280[a,c,f,h]	102.50±2.510[a,c,d,h]	70.50±3.834[a,c,f,h,i,j]	44.33±1.633[a,c,e,g,i,j,k]
Group-5 (standard)	100.00±1.673[a,c]	99.33±1.751[a,c]	58.83±4.215[a,c,i,j]	39.83±1.602[a,c,i,j,k]

Significance between the groups is shown as [a] p-value <0.001 in comparison with group-1. [b] shows p-value <0.01, [c] <0.001 in comparison with group-2. [d] shows p-value <0.05, [e] <0.01, [f] <0.001 in comparison with group-5. g shows p-value <0.01, h <0.001 in comparison of group-4 to group-3.
Significance within the same treatment groups shows as [i] p-value < 0.001 in comparison with Day-1. j shows p-value < 0.001 in comparison with Day-2. k shows p-value <0.001, for comparison with Day-3.

Table 5: Time spent in platform quadrant by mouse during Probe Trial

Treatment groups	Day 5 (Seconds Mean±S.D)
Group-1 (control)	60.00 ± 1.414
Group-2 (Scopolamine)	20.00 ± 4.290[a,f,g,h,i]
Group-3 (treated 100/100 mg/kg/day)	72.83 ± 1.722[a,b,d,f,g,h,i]
Group-4 (treated 200/200 mg/kg/day)	75.50 ± 2.168[a,b,c,e,f,g,h,i]
Group-5 (standard)	79.67 ± 1.862[a,b,f,g,h,i]

Significant difference in time spent are shown in between groups: [a] 2, 3, 4 and 5 with 1 ($p<0.001$); [b] 3, 4 and 5 with 2 ($p<0.001$); [c] 4 with 5 ($p<0.01$), [d] 3 with 5 ($p<0.001$); [e] 4 with 3 ($p<0.05$); Significant difference shown in between Days: [f] Day-5 with Day-1 (in group 2, 3, 4 and 5) ($p<0.001$); [g] Day-5 with Day-2 (in group 2, 3, 4 and 5) ($p<0.001$); [h] Day-5 with Day-3 (in group 2, 3, 4 and 5) ($p<0.001$); [i] Day-5 with Day-4 (in group 2, 3, 4 and 5) ($p<0.001$)

Table 6: Effect of combination of *Melissa officinalis* and *Panax ginseng* on total number of correct responses

Treatment groups	Day 1	Day 2	Day 3	Day 4	Day 5
	Correct response Mean±S.D				
Group-1 (control)	6.67±0.516	6.33±0.516	6.67±0.816	6.83±0.753	7.33±0.516
Group-2 (Scopolamine)	4.83±0.753[c]	5.00±1.549[b]	5.00±1.673[b]	4.33±1.862[c]	4.67±2.066[c]
Group-3 (treated 100/100 mg/kg/day)	7.00±0.894[d]	8.00±0.00[b,d,e]	7.83±0.408[a,d]	7.83±0.408[a,d]	7.83±0.408[d]
Group-4 (treated 200/200 mg/kg/day)	7.67±0.516[a,d]	7.50±0.548[a,d]	8.00±0.00[b,d]	7.83±0.408[c,d]	8.00±0.00[d]
Group-5 (standard)	7.50±0.548[d]	8.00±0.00[b,d]	7.67±0.516[a,d]	7.67±0.516[d]	7.83±0.408[d]

Significance are shown in between groups [a] shows p-value <0.05, [b] <0.01, [c] <0.001 in comparison with group 1. [d] shows p-value < 0.001 in comparison with group 2.
Significance within the same treatment groups shows as [e] p-value <0.05 in comparison with Day-1.

Table 7: Effect of combination of *Melissa officinalis* and *Panax ginseng* on number of errors (re-entry)

Treatment groups	Day 1	Day 2	Day 3	Day 4	Day 5
	Number of errors (re-entry) Mean±S.D				
Group-1 (control)	8.00±0.89	8.17±0.753	8.50±0.548	9.83±1.169	10.00±1.414
Group-2 (Scopolamine)	5.00±1.095	2.83±1.169[a]	3.00±1.265[a]	4.00±3.225[a]	4.67±3.615[a]
Group-3 (treated 100/100 mg/kg/day)	9.50±5.468[f,c]	15.00±8.877[a,e,i]	15.17±2.787[a,e,i]	16.33±1.033[a,e,i]	17.33±1.633[b,e,j]
Group-4 (treated 200/200 mg/kg/day)	9.17±5.981[f,c]	9.17±3.920[d,g,h]	16.17±3.189[b,e,i,k]	18.83±1.472[b,e,j,l]	19.33±1.506[b,e,j,l]
Group-5 (standard)	15.50±6.025[b,h,e]	17.67±5.391[b,d]	17.50±1.049[b,e]	18.33±1.862[b,e]	19.17±1.472[b,e]

Significance are shown in between groups [a] p-value <0.01, [b] <0.001 in comparison with group-1. [c] shows p-value <0.05, [d] < 0.01, [e] <0.001 in comparison with group-2. [f] shows p-value <0.01, [g] <0.001 in comparison with group-5. [h] shows p-value <0.01 in comparison of group-4 to group-3.
Significance within the same treatment groups shows [i] p-value <0.01, [j] <0.001 in comparison with Day-1. [k] shows p-value <0.01, l <0.001 in comparison with Day-2 (within the same treatment groups).

The research also showed that the oral lethal dose of the *Melissa officinalis* and *Panax ginseng* combination was greater than 5g/kg, a threshold for fatality set forth by the OECD recommendations (Pandey *et al.,* 2020). The researchers investigated 24 weeks *M. officinalis* extract (500 mg daily dose) for safety and tolerability in moderate dementia patients with Alzheimer's disease; routine blood testing and clinical observations revealed that supplementation with this amount was tolerated for 48 weeks and did not pose any safety issues (Noguchi-Shinohara *et al.,* 2020). Another study conducted by Choi *et.al* found that administration of repeated doses of *Panax ginseng* for 15 days was safe, well tolerated and free of any harmful effects (Choi *et al.*, 2020).

In present experimental investigation memory and cognitive impairments in rats evaluated by passive avoidance paradigm, the Morris water maze and the 8-arm radial maze tests resulted in enhancement of memory and learning by passive avoidance; rats were given combination of *Melissa officinalis* and *Panax ginseng* extract orally on a sub-chronic basis; specially when the number of required trials in rats to get PAL was significantly reduced when *Melissa officinalis* and *Panax ginseng* were used together. Furthermore, time spent in the dark chamber and step-through latency, both showed statistically significant improvements throughout the course of the 24-hour retention assessment. Dehbani *et. al.* (2019) showed similar findings by administering hydro-alcoholic extract of *Melissa officinalis* in rats, where the significant impact of STL and TDC confirmed the improvement in memory and cognition of rats in passive avoidance task (Dehbani *et al*., 2019). *Melissa officinalis* (lemon balm) has been also the subject of numerous clinical researches showing its ability to enhance cognitive function in dementia patients and healthy subjects (Heydari *et al.*, 2019). In addition; the use of *Panax ginseng* enhanced cognitive functions including memory and learning; ginseng for instance significantly enhanced the learning and memory abilities of old and brain-injured rats (Hou *et al.*, 2020). Various clinical studies also have shown that *ginseng* extract greatly enhances psychomotor capabilities and reduces neurologic and neuropsychiatric symptoms in the elderly and Alzheimer's patients (de Oliveira Zanuso *et al.*, 2022).

The additional synergestic outcomes were noted of current investigation on abilities to learn by combining *Melissa officinalis* and *Panax ginseng. Melissa officinalis* and *Panax ginseng* at the dose of 200/200 mg/kg significantly improved memory and learning when tested in a dark chamber (TDC). The research by Parisa H *et al.* is consistent with these findings.(Hasanein *et al.*, 2015) Another study conducted by Bilia *et al.* also demonstrated that oral administration of standardized extract of *ginseng* (30, 100 and 300 mg/kg) showed significant differences in STL and TDC in a dose dependent manner (Bilia *et al.*,

2020). According to the findings of present experimental study, combination of both ginseng and lemon balm was able to boost learning, memory and physical capacities of rats when administered in the optimum amounts (Mirakhori *et al.*, 2022). It is considered that reference memory is thought to reflect learning the trial-independent parts of the task (spatial locations), but working memory depicts the subject's capacity to keep this trial-dependent information (previously visited locations) in mind.

Escape latency is the time an animal takes to find the platform in a Morris water maze, which is used to assess spatial memory and learning capability. Reductions in escape latency and increase duration in target quadrant in the Morris water maze both suggest to enhanced learning and memory (Seo *et al.*, 2023). The results of this study showed that cognitive and memory performance significantly improved in treated rats after they were given a combination of *Melissa officinalis* and *Panax ginseng* (100/100 mg/kg and 200/200 mg/kg doses respectively) for 30 days. One of the study also suggested that naive rats given 200 mg/kg of *M. officinalis* extract had dramatically improved learning and memory (Husain *et al.*, 2021). Further the administration of ginseng at various doses resulted in decrease escape latency and increase time interval in target quadrant; which also confirmed the memory enhancing effect of the drug in dementia-induced rats (Lee *et al*., 2010).

Current study also evaluated Rats' cognitive abilities in an 8-arm radial maze apparatus; the results were compared in three groups; they have given combination of *Melissa officinalis* and *Panax ginseng*, control and scopolamine-induced dementia. Comparison has shown a marked increase in the overall number of correct responses and re-entry to preceding arm (error). *Melissa officinalis* and *Panax ginseng* group findings revealed that at doses of 100/100 mg/kg and 200/200 mg/kg improved short-term memory. Another experimental findings also demonstrated that a low dose (10mg/kg) of *Melissa officinalis* aqueous extract enhanced spatial memory (Al-Snafi, 2021). Another study suggests that experimental rats were given *Panax ginseng* extract and the results showed that the herb prevented the memory loss associated with ageing in the rats as measured by their performance in a learning test (Chen *et al.*, 2019).

By employing Ellman's colorimetric approach in current study, the study found extent to which *Melissa officinalis* and *Panax ginseng* inhibited acetyl cholinesterase (AChE) activity. *In-Vitro* studies revealed that 4mg dose of the combination extracts has shown to have a much greater inhibitory rate (36.4%) than the other doses and a significant effect on AChE inhibition. A commercial AChE inhibitor Donepezil has shown to be more effective than this extract in inhibiting AChE (54.18%). This might be due to changes in sample preparation or chemical characteristics. Dose-dependent inhibition of acetyl cholinesterase activity was observed using *Melissa officinalis* ethanolic extract and essential oils (Mahboubi, 2019). An increase in the levels of the neurotransmitter acetylcholine in the synaptic cleft are attributed to the presence of phenolic chemicals, most notably rosmarinic acid in *Melissa officinalis*(Ghasemzadeh Rahbardar *et al.*, 2020).

Study limitations

The results of current study suggest that a combination of *M. officinalis* and *P. ginseng* might be effective as a neuro-protective supplement in neurodegenerative disorders. The study did not use a neurodegenerative animal model, pre-treated with the combination of both compounds to show improvement in cognitive function recovery. However, its toxicity profile in experimental animals needs to be assessed first. Biogenic amines such as serotonin, dopamine, norepinephrine and acetylcholine plays a vital role in improving cognition and preventing neurodegeneration; so study could be expanded in the future to determine their levels including the mechanisms or pathways they choose to stop the neurodegenerative processes.

CONCLUSION

Findings of current study conclude that combination of *Melissa officinalis* and *Panax ginseng* extract may significantly improve complementary effects on memory and cognition. Hence; study suggested that a mixture of extracts of *Melissa officinalis* and *Panax ginseng* can be used as a memory enhancing drug to boost mental capacity and can also has therapeutic application in dementia in future. The study will have benefit of presenting data collected from a group of animals receving only *Melissa officinalis* and a group receiving only *Panax ginseng* and compared with the combination of both compunds.

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