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Research Article

Evaluation of learning and memory enhancing activities of protein extract of *Withania somnifera* (Ashwagandha) in Wistar albino rats

Shwetha Shivamurthy[1], Ravi Shankar Manchukonda[1]*, Dinesha Ramadas[2]

[1]Department of Pharmacology Adichunchanagiri Institute of Medical Sciences, BG Nagar, Karnataka 571448, India, [2]Central Research Laboratory, Adichunchanagiri Institute of Medical Sciences, BG Nagar, Karnataka 571448, India

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***Correspondence to:**
Dr. Ravi Shankar Manchukonda, Email: ravipharmac@yahoo.com

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ABSTRACT

Background: Learning may be defined as the ability to alter behavior on the basis of experience. Memory is the process of remembering what has been learnt. Memory loss is the first symptom to manifest in Alzheimer's disease (AD). Currently, the drugs used in Alzheimer's are either associated with side effects or they lack disease modifying effect. Ashwagandha is a rasayana (rejuvenative) and possesses antioxidant and free radical scavenging activity (free radicals are produced during the initiation and progression of AD). Hence, the present study was undertaken to evaluate the learning and memory enhancing effect of protein extract of Withania somnifera in Wistar albino rats.
Methods: In the present study, Wistar albino rats were given 200mg/kg dose of protein extracts of Withania somnifera orally along with scopolamine (i.p) for 14 days. Rats were subjected to elevate plus maze and passive avoidance testing on 14th day to evaluate learning. Same models were repeated on 15th day to evaluate retention (memory). The results were compared with the negative control group treated with scopolamine only and positive control group treated with scopolamine and piracetam.
Results: Significant learning and memory enhancement was observed with the protein extract of Withania somnifera as compared to negative and positive control groups with p value <0.05.
Conclusions: We conclude that the protein extract of Withania somnifera is having learning and memory enhancing activity in Wistar albino rats.

Keywords: Withania somnifera, Learning, Memory, Alzheimer's disease, Elevated plus maze, Passive avoidance test, Wistar albino rats

INTRODUCTION

Learning can be described as a change in behaviour due to experience, which enables to adapt to recent living conditions.[1] It is a process by which brain acquires new information about the events occurring in the given surroundings.[2] Memory is a fundamental mental process, and without memory we are capable of nothing but simple reflexes and stereo type behaviours.[3] It is a faculty by which sensations, impressions, and ideas are stored and recalled.[2] Thus, learning and memory are one of the most intensively studied subjects in the field of neuroscience.

Memory loss is the first symptom to manifest in Alzheimer's disease [AD].[3] AD is a severe progressive neurodegenerative brain disorder that affects approximately 5% of the population older than 65 years.[4] Degeneration of the cortically projecting cholinergic neurons is a pathological hallmark of AD. The presynaptic cholinergic deficits in AD indicate that a cholinergic replacement therapy might be beneficial in alleviating some of the cognitive dysfunctions in this disorder. The accumulation of the protein beta-amyloid (called beta-amyloid plaques) outside neurons and the accumulation of an abnormal form of the protein tau (called tau tangles) inside neurons are two major pathologies contributing for the development of AD. The

accumulation of beta-amyloid is believed to interfere with the neuron-to-neuron communication at synapses and to contribute to cell death. Tau tangles block the transport of nutrients and other essential molecules inside neurons and are also believed to contribute to cell death.[5]

So far, efforts to find a cure for AD have been disappointing, and the drugs currently available to treat the disease address only its symptoms and with limited effectiveness. So, currently the mainstay of management, as the disease progresses, remains symptomatic. Herbal medicine offers several options to modify the progress and symptoms of AD. Ashwagandha is used extensively in Ayurveda as a nervine tonic, aphrodisiac, and 'adaptogen' and helps the body adapt to stress. Ashwagandha is a member of the nightshade (Solanaceae) family, and the root is the part that is widely used. It is categorized as a rasayana (rejuvenative) and is believed to possess antioxidant activity, free radical scavenging activity, and an ability to support a healthy immune system.[6]

Ashwagandha contains steroidal compounds such as the ergostane-type steroidal lactones, including withanolides A to Y, dehydro withanolide R, withasomniferin A, withasomnidienone, withasomniferols A to C, withaferin A, and withanone. Other constituents include the phytosterols sitoindosides VII to X and beta-sitosterol as well as alkaloids (for example, ashwagandhine, cuscohygrine, tropine, pseudotropine, isopelletierine, and anaferine), a variety of amino acids (including tryptophan), and high amounts of iron. A subset of these components (withanamides) has been shown to scavenge free radicals generated during the initiation and progression of AD. Neuronal cell death triggered by amyloid plaques was also blocked by withanamides. Aqueous extracts of this herb have been found to increase cholinergic activity, including increases in the acetylcholine content and cholineacetyl transferase activity in rats.[6]

Treatment with the methanol extract of Ashwagandha has shown neurite outgrowth in a dose and time dependent manner in human neuroblastoma cells. The levels of two dendritic markers, MAP2 and PSD-95, were found to be markedly increased in cells treated with Ashwagandha, suggesting that it stimulates dendrite formation and could posess disease modifying effect.[7,8]

Study on aqueous and methanolic extract of the herb has been done but study on protein extract has not been done. Hence, the present study was designed to investigate the learning and memory enhancing activities of protein extract of Withania somnifera in scopolamine induced dementia in rats.

METHODS

Healthy male Wistar albino rats strain, 150 – 200g were used. The rats were maintained under a reverse photo cycle of 12 h day and 12 h night in temperature and humidity controlled environment with free access to food and water. All experiments were conducted between 9:00 am and 12:00 pm in a noise free environment. The study was approved by Institutional Animal Ethics Committee.

Following drugs and chemicals were used: withania somnifera (vasavambha stores, Mysore), piracetam, scopolamine, 5% dextrose (for suspending test compound).

Methods of protein extract preparation

10g of cleaned withania somnifera powder was mixed with 200 ml of double distilled water. It was vortexed for 6 hours at 20°C. The extract was centrifuged at 6000 rpm for 20 minutes, the supernatant was separated. Further, the supernatant was subjected to 65% ammonium sulphate precipitation and vortexed overnight. The mixture was centrifuged at 10000 rpm. The precipitated Ammonium sulphate protein precipitate was collected and subjected to dialysis using 2.5kDa molecular cutoff biomembrane against water for 76 hours with an interval of 6 hours. The dialyzed protein precipitate was separated and stored at -10°C for further analysis.

Experimental protocol

Learning and memory were assessed with two behavioral paradigms viz. elevated plus maze and passive avoidance. 3 study groups with 6 animals in each were used for the study. Memory impairing dose of scopolamine 1mg/kg i.p was administered for 14 days in group 1 rats. Group 2 received scopolamine 1mg/kg i.p for 14 days and piracetam 200 mg/kg i.p from 8th day to 14th day. Group 3 received scopolamine 1mg/kg i.p for 14 days and protein extract of Withania somnifera 200mg/kg P.O from 8th day to 14th day.

On the 14th day, 90 min after the administration of the last dose of drugs in the respective groups, rats were exposed to elevated plus maze and passive avoidance task for acquisition (learning). Retention (memory) was recorded 24hrs later on the 15th day. Groups were as follows;

Withania somnifera was suspended in 5% dextrose.

Group 1- Treated with scopolamine 1 mg/kg alone i.p. (negative control group).

Group 2- Treated with scopolamine 1 mg/kg+ piracetam 200mg/kg i.p. (standard group/ positive control).

Group 3- Treated with scopolamine 1 mg/kg + protein extract of Withania somnifera 200mg/kg P.O

1. Elevated plus maze:[2]

This consists of a central platform of 10x10 cms connected to two open arms of 50x10cms and two closed arms of 50x40x10 cms in dimension and elevated 50 cms above the floor. Wistar albino rats weighing 150 to 250 g was used.

Procedure

The experiment was performed in 2 stages. On day 14, the day of acquisition testing, each rat was placed at the end of an open arm facing away from the center. The time taken to enter any one of the closed arms was recorded as transfer latency (TL). All four legs inside the closed arm were counted as an entry. Cut off time allotted for each rat was 180 s. Those animals which did not enter the closed arms within the cut off time were excluded from the study. Retention testing was conducted 24 hrs after the first trial and transfer latency was recorded in a similar manner as mentioned before. Shortened transfer latency was considered as an index of improvement of memory.

2. Passive avoidance test:[9]

This is one trial which is fear motivated avoidance task in which rats learn to refrain from stepping through a door, to an apparently safer, but previously punished compartment. The latency to refrain from crossing into the punished compartment serves as an index of the ability to avoid and allows memory to be assessed.

The apparatus consisted of a square box with a grid floor (50x50 cms) and wooden walls of 35 cms height. This box was illuminated with a 7W/12V bulb placed 150 cms above the centre of the compartment. In the centre of one of the walls, there was an opening (6x6 cms) which can be opened or closed using a transparent plexy glass sliding door which leads to a small dark compartment (15x15 cms). This compartment was provided with an electrifiable grid floor which can be connected to a shock source and a removable ceiling.

Procedure

The experiment was conducted in 2 stages. Test animals were given an acquisition trial on 14th day followed by a retention trial 24 hrs later, on 15th day. In acquisition trial, the animal was placed in the illuminated compartment at maximal distance from guillotine door and the latency to enter the dark compartment was measured as step through latency (STL). Rats that did not step through the door within a cut off time 180s were not used. Sliding door between the two compartments was closed as the rat entered dark chamber and unavoidable foot shock (1.5mA, 50HZ, 2 s) was delivered. The ceiling was opened and the rat was returned to home cage. Retention was tested after 24 hrs on 15th day and STL was recorded. Cut off time allotted for retention was 600 s. Increase in STL was considered as an index of improvement of memory.

Statistical analysis

Data entry was done on MS EXCEL and 'SPSS version 17' software was used for data analysis. One way ANOVA test is used to compare the effect of the drugs on different groups. Tukey's HSD test was used for post-hoc analysis of significant overall differences.

For all the tests a 'p' value of 0.05 or less was considered for statistical significance.

RESULTS

Table 1: Effect on transfer latency using elevated plus maze.

Group	Transfer latency in seconds	
	Learning day (day 14)	Retention day (day 15)
1 (Scopolamine only)	77.83 ± 30.04	102.55 ± 37.1
2 (Scopolamine + piracetam)	50.66 ± 4.67	53.98 ± 6.42
3 (Scopolamine + T1)	19.3±10.08*	8.3 ± 4.5**

p < 0.001 [on day 14]
p < 0.05 [on day 15]
*p <0.01 as compared to scopolamine only treated group
*p <0.05 as compared to piracetam treated group
**p <0.01 as compared to scopolamine only treated group
**p <0.01 as compared to piracetam treated group

The rats treated with protein extract of Withania somnifera 200mg/kg showed statistically significant improvement in mean transfer latencies on both learning (p<0.01) and retention day (p<0.01) as compared to scopolamine only treated group indicating learning and memory enhancing effect. There was a significant enhancement in learning and memory in protein extract of Withania somnifera treated group as compared to piracetam treated on both the days.

Table 2: Effect on Step through latency using light and dark apparatus.

Group	Step through latency in seconds	
	Learning day (day 14)	Retention day (day 15)
1 (Scopolamine only)	49.20 ± 35.7	18.78 ± 8.6
2 (Scopolamine + piracetam)	13.71 ± 5.65	29.23 ± 11.3
3 (Scopolamine + T1)	29.3±26.7	*74.4± 35.9

p < 0.01 [on day 15]
*p <0.01 as compared to scopolamine only treated group

The rats treated with protein extracts of Withania somnifera 200mg/kg showed statistically significant improvement in mean step through latency on retention day (P<0.01) as compared to scopolamine only treated group indicating memory enhancing effect.

DISCUSSION

Owing to the complexity of the pathology, management of the neurodegenerative disorder like Alzheimer's

disease is one of the greatest challenges. Herbal medicine offers several options to modify the progression and symptoms of AD. The cholinergic hypothesis of AD postulates that low synaptic levels of acetylcholine resulting from loss of cholinergic neurons in the nucleus basalis magnocellularis lead to cognitive decline.[10] Based on this, strategies for increasing synaptic levels of ACh have been widely explored in the development of antidementia drugs. One such strategy is blockade of synaptic degradation of ACh through the inhibition of acetylcholinesterase by donepezil, rivastigmine and galantamine. Previous studies reveal that aqueous extract of Withania somnifera increases cholinergic activity, including increase in the acetylcholine content and cholineacetyl transferase activity in rats and this might partly explain the cognition-enhancing and memory-improving effects.[6] No study has been conducted so far on the protein extract of the compound which could be beneficial in treatment of AD.

The present study was undertaken to evaluate the effect of protein extract of Withania somnifera on learning and memory performance in rats using two models namely, two compartments passive avoidance test and elevated plus maze test. The latency to enter the dark compartment (STL) and closed arm (TL) was taken as a parameter for assessment in experimental models. The rationale being, if the drug has positive effect on learning, it would be reflected as decrease in latency to enter dark compartment/closed arm. However, interpretation of TL/STL in retention trial is different in both the models, in that, decreased TL in EPM model is inferred as improvement in memory, whereas increased STL after shocking the animal in PA model is interpreted as memory enhancement

Response to Withania somnifera 200mg/kg was compared against negative and positive control in both the models.

In elevated plus maze, on learning day, significant improvement in learning was seen in Withania somnifera treated group as compared to scopolamine only treated group and piracetam treated group. Similarly significant enhancement in memory was seen in Withania somnifera treated group as compared to scopolamine only treated group and piracetam treated group. In a previous study with ferula asafoetida, statistically significant memory enhancement was seen as compared to control group treated with normal saline ($p<0.001$) but though learning improvement was observed in ferula asafetida group, it was not statistically significant in elevated plus maze.[2] In another study, palmatine, a quaternary protoberberine alkaloid, significantly reversed the scopolamine induced amnesia in mice by reducing the transfer latency on retention day though the improvement in learning was insignificant.[11]

In passive avoidance task, on learning day, improvement was evident in group treated with protein extract of Withania somnifera when compared with scopolamine only treated group, which suggests an enhancement of learning though it was not statistically significant. On retention day, significant enhancement in memory was seen in Withania somnifera treated group and piracetam treated group as compared to scopolamine only treated group. Though the memory enhancement in Withania somnifera treated group appeared to be more than the piracetam treated group, it was statistically insignificant. Similar observation was made in the study on ferula asafoetida where there was significant memory enhancement compared to control group but memory enhancement was insignificant when compared with the standard rivastigmine drug.[2] In another study, rats treated with alcoholic and aqueous extract of Tinospora cordifolia significantly reversed the memory impairment induced by cyclosporine.[12]

Results of the study indicate that protein extract of Withania somnifera has learning and memory enhancing effect as noted by changes in the TL/STL in learning and retention trials. Since the amnesic effect of scopolamine which is a muscarinic receptor antagonist has been reversed by Withania somnifera successfully, it indicates that Withania somnifera acts on Ach receptors. Beneficial effect on learing and memory enhancement was superior in Withania somnifera treated group as compared to piracetam treated.

CONCLUSION

To the best of our knowledge, this is the first study of its kind where protein extracts of Withania somnifera has been evaluated for learning and memory enhancing potential in rats. It is evident from our study that there were learning and memory enhancing effects of the test compound. It could be due to facilitation of cholinergic transmission. Further studies would enhance our knowledge regarding the same. Hence, Withania somnifera could be beneficial and could be thought of being used as an adjuvant to existing therapies for the treatment of AD.

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