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Original Article

Neuroprotective Effects of Withaferin-A Nanoparticles on Scopolamine Rat Model of Alzheimer's Disease

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ABSTRACT

Background: Alzheimer's Disease (AD) suffers from dementia more often in 65-year or older patients. Symptoms of AD's are linked to disease-causing neurons, and current pharmacological therapies inadequately regulate deadly outcomes. *Withania somnifera* (L*)*, has been contributing as a traditional multifunctional herb with a wide variety of health benefits. **Materials and Methods:** This research examined the importance of Withaferin A nanoparticles against scopolamine induced neuron loss (Impair on memory). Five groups of 6 rats weighing 150-200 g were randomly split. Negative and positive control animals received 2mL/kg saline solution in groups 1 and 2. 3rd and 4th group received 5mg/kg of pure Withaferin-A and Withaferin-A nanoformulation. Group 5 received 10 mg/kg of tacrine as a normal medication. Except for group 1, all other groups were induced 30 min after drug administration with 1mg/kg scopolamine. EPM was used to understand the behavioral parameters. **Results:** Inflexion ratios of 1.1 and 1.3 were seen in groups treated with Withaferin-A nanoparticles and the conventional medication. AchE, MDA, GSH reductase were also measured. Compared to the pure drug, Withaferin-A nanoparticles exhibited substantial activity. Withaferin-A exhibits an anti-amnesic effect similar to tacrine at a specific level. **Conclusion:** Withaferin-A nanoparticles may help neurodegenerative disease. To establish the formulation as a standard AD's treatment, pharmacokinetic aspects should be explored.

Keywords: Withaferin-A, EPM, Brain enzymes, Inflexion ratio, Nano formulations.

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INTRODUCTION

Dementia is a general term for loss of memory, language, problem-solving and other thinking abilities that are severe enough to interfere with daily life. Alzheimer's is the most common cause of dementia. Millions of people are afflicted by this age-related illness, and it is predicted that 1 in 85 people worldwide will have AD by the year 2050. AD is neurodegenerative disorder that causes widespread loss on cognition, orientation, judgment and memory. Alzheimer's disease results from the accumulation of beta-amyloid (plaques) and twisted tau fibres (tangles) inside and outside neurons in the brain, respectively, which are the main pathogenesis of the disease.

Currently available pharmaceutical treatments for AD are ineffective in controlling the disease-causing neurons associated with the disease's symptoms and fatal consequences. Consequently, most of the medications on the market today suppress the enzyme Acetylcholinesterase (AChE) to enhance memory. When developing a drug, it is important to keep in mind that AD is a multifactorial condition rather than being caused by a single factor like AChE. The cognitive deficits associated with AD are significantly influenced by additional factors like oxidative stress and synaptic dysfunction. In pathological conditions, natural products may provide neuroprotective medications by keeping brain cells communicating normally and preventing loss of neuronal function. Natural products are currently being investigated as neuroprotective agents by many AD research teams.

As well as being known as Indian ginseng and Ashwagandha (ASH) Sanskrit, *Withania somnifera* (L.) has been studied in recent years for its wide range of pharmacological activities and capable of enhancing nerve function, enhancing memory, reducing stress, and enhancing immunity. There has seen a substantial increase of its pharmacological studies in recent years. *Withania somnifera* (L.) root extract reduces neuronal degeneration in the hippocampus of stressed rat brains and it was a neuroprotective agent in Parkinson's disease animal models.[1-6] The development of deficits in behavioral skills and plaque pathology was reversed in recent studies of withanolides and withanosides taken by orally from *Withania somnifera's* root. The amyloid peptides

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and oligomers were found in the brains of mid-age elderly mice expressing memory loss on rodents.[7]

MATERIALS AND METHODS

Formulation and Characterization

As the isolated Withaferin-A was confirmed by 1H and $_{13}$C-NMR on the ashwagandha extract, it was obtained from *Withania somnifera* L.[8] A solution evaporation method was used to encapsulate Withaferin-A into PLGA nanoparticle size, zeta potential, and surface morphology measurements.[9]

Animal Care

The animal facility bearing the C.L. Baid Metha College of Pharmacy was home to albino Wistar rats that were domestically grown, six to eight weeks old; they weighed 180-200 grams. Animal husbandry standards were followed when housing and maintaining the rats (Light/dark cycle of 12:12 hr, controlled room temperature (23±2°C)), The environment must be stress-free, with unlimited water, a standard diet, and hygienic conditions.). In order to lessen stress, the rats were given a week to acclimate before the experiment started.

Experimental Design

Animals Used

The study was carried out on both male and female Wistar rats aged between seven and eight weeks (200-250 gm weight), supplied by Mass Biotech, Chennai. Protocol was approved by Institutional Animal Ethics Committee (IAEC) of CLBM. Approval number: 07/321/PO/Re/S/01/CPCSEA dated 04/02/2021. Animals were kept for one week to get acclimatized to laboratory conditions. All the animals were healthy and were maintained at 22 ± 2°C and 50–60% RH in room which was well ventilated with 100% fresh air and under 12 h dark/light cycle. Animals were randomly allocated to control and treatments groups and were fed with standard pellet diet and water *ad libitum*.

Acute Toxicity Study

Acute toxicity test and dose Optimization (OECD/OCDE 423 Acute Toxic Class Method). Six Female Wistar rats weighing 200-250 gm were selected for the study. Rats were fasted overnight prior to study but had free access to water. The acute toxicity class method is a stepwise procedure with 3 animals of single sex per step. Depending on the mortality and morbidity status of the animals, an average of 2-4 steps may be necessary to allow judgement on the acute toxicity of the test substance.

Neuroprotective Activity of Withaferin-A Nanoparticles

In this study, Withaferin-A Nanoparticles (WANP) were tested for their reversibility of memory impairment caused by scopolamine administration at 1mg/kg/i.p for 10 days. 5 groups of 6 rats weighing between 150-200g in each group were divided randomly. Group 1 was taken as negative and group 2 was considered as positive controls and the animals were treated with saline solution 2mk/kg/p.o and scopolamine as inducing agent at 1mg/kg/i.p Group 3rd and 4th received pure Withaferin-A and Withaferin-A nanoformulation in the dose of 5mg/kg/i.v. Group 5 animals were treated with Tacrine in the dose of 10mg/kg/i.v and is considered as standard drug. The drugs treatment was continued for about 10 days and except group 1 all the remaining groups were induced using scopolamine as inducing agent at 1mg/kg/i.p 30 min after administration of drugs. Further assessments were made after the administration of the induction agent.

Elevated Plus Maze (EPM)

Elevated plus maze was adopted to investigate behavioral parameters of rats after induction of disease and drug treatment.[10] The rats were evaluated between 9-6 pm under dim red illuminated light. The maze was sanitized using 70% alcohol and the memory retention was evaluated 24 hr after each training session. Memory impairment index was calculated by measuring the drop in transfer latency during each session in seconds.

Biochemical Assays

After 10 days the rats were decapitated under anaesthesia and brains of each animal were removed rinsed and homogenized using 50mM Tris-HCl buffer. The brain enzymes like Malondialdehyde (MDA), Glutathione (GSH) levels and Acetylcholine Esterase (AchE) activity were determined using biochemical methods. The results are expressed in Table 3.

Histopathologic Examination of Brain Tissues

Two to three rats from each group were randomly selected for histopathological examination. Five-mm longitudinal sections of the brains were taken after formalin fixation 10% phosphate buffered formaldehyde, embedding paraffin, preserving them in 10% formalin and paraffin. H&E was used to staining the sections and the same was examined microscopically. Results are shown in (Figure 1).

Statistics

The findings had denoted as mean ± Standard Error of the Mean (SEM). A statistical analysis was performed on these data ANOVA followed by Dunnett's test. We determined statistical findings using p^*value of 0.05, 0.01, and 0.001.

RESULTS

Acute Toxicity Studies

No sign of toxicity was observed in any of the treated rats at 2000 mg/kg with withaferin-A nanoparticles. There was no mortality and the animals displayed normal behaviour and did not reveal any abnormality or pathological significance. Depending on the

acute toxicity studies the LD_{50} value of withaferin-A nanoparticles were determined to be greater than 2000mg/kg. The changes in the body weights were recorded in the Table 1.

The Inflexion Ratio (IR) was calculated by using the formula as follows and the results were tabulated in (Table 2).

$$\text{Inflexion ratio (IR)} = \frac{L_0 - L_1}{L_0}$$

Where L_0 is the initial TL (s) on first day and L_1 is the TL (s) on the second day.

The negative control group showed a ratio of 1.16, positive control group showed a negative value and the pure drug withaferin-A treated group showed a value that is higher than the negative control group. The animals treated with withaferin-A nanoparticles and the standard drug showed similar results of inflexion ratio of 1.1 and 1.3 respectively. The brain enzymes like acetyl choline esterase, malondialdehyde, glutathione reductase were estimated and tabulated (Table 3). Results of Withaferin-A nano particles showed a significant neuroprotective activity as compared with standard drug Tacrine.

Brain sections are photographed under a microscope. NC; Meninges, cerebral cortex, and hippocampus showed their normal histological structure, PC; The hippocampus of scopolamine-induced demented rats shows edema and encephalomalacia with severe congestion and perivascular edema, WA treated; Demented scopolamine-induced rats treated with Withaferin-A (5 mg/kg) demonstrating focal gliosis in the cerebral cortex and pyramidal cells separated with irregular outlines in the hippocampus, WANP treated; Scopolamine-induced demented rat treated with Withaferin-A nanoparticle (5 mg/kg) displaying normal neuropathology while some pyramidal cells exhibit pyknotic nuclei and the hippocampus exhibits vacuolated cytoplasm. STD treated; Scopolamine-induced demented rat treated with Tacrine (10mg/kg) showing diffuse gliosis in brain cortex and shrinkage of pyramidal neurons in hippocampus with pyknotic nuclei; (H&E×40, 60, 150, 600) (Figure 1).

DISCUSSION

In this study, withaferin-A nanoparticles are examined to determine if they modulate the cholinergic pathway in a way that prevents amnesia. Withaferin-A nanoparticles were evaluated during the learning and memory processes by combining multiple doses of scopolamine injection with multiple withaferin-A nanoparticle exposures. Prior to this trial, Withaferin-A dose was determined. According to a previous literature review, Withaferin-A dosage ranges of 100mg/kg and 300 mg/kg was observed in animal models related to the central nervous system.[11-14] Preliminary research, found that Withaferin-A 5mg/kg/i.v doses had a neurobehavioral impact on motor nerve activity and motor nerve coordination and causing improvements in coordination and motor skills in rats.[15]

In this investigation, the EPM approach was used to comprehend the behavioral models used to assess memory and learning. As part of Alzheimer's disease research, the EPM test is particularly useful because it allows assessing visual recognition memory early in the illness's course, using brain areas similar to those afflicted by the fatal and crippling disease.[16] However, EPM is a behavioural

Table 1: Results of acute toxicity studies after administering Withaferin-A nanoparticles.

Rat number	Body weight (g)			Mortality
	Day 1	Day 7	Day 14	
1	160	171	177	
2	163	169	175	NIL
3	159	166	172	
4	158	168	174	
5	177	184	189	
6	171	176	190	

Table 2: Effect of Withaferin-A nano particles on the behavioural parameters in scopolamine induced Alzheimer's disease.

Groups	Treatments	Inflexion ratio
Group 1: Negative control	Saline	1.16±0.04
Group 2: Positive control	Scopolamine	-0.89±0.06[a]
Group 3: Withaferin A	Scopolamine + WA	3.98±0.15*
Group 4: Test	Scopolamine + WANP	1.14±0.09*
Group 5: Standard (Tacrine)	Scopolamine + (Tacrine)	1.32±0.22*

Values were expressed in the form of mean ± SEM (n=6). * indicates the p<0.001 in comparison to positive control. [a] indicates the p<0.001 in comparison to negative control.

Table 3: Effect of Withaferin-A nanoparticles on the brain enzymes.

Groups	Treatments	AchE (µmol/min/mg protein)	MDA (µ mol/mg protien)	GSH (µmol/mg protein)
Group 1: Negative control	Saline	10.76±1.2	0.12±0.1	19.0±1.2
Group 2: Positive control	Saline + Scopolamine	27.28±5.18	0.59±0.12	13.28±1.8 [a]
Group 3: Withaferin A	Scopolamine + WA	21.12±2.16	0.49±0.04	14.12±1.02
Group 4: Test	Scopolamine + WANP	12.25±1.82	0.24±0.12	20.02±0.4 *
Group 5: Standard (Tacrine)	Scopolamine + (Tacrine)	12.51±6.58	0.20±0.08	22.6±0.2 *

Values were expressed in the form of mean±SEM (n=6). *indicates the p<0.001 in comparison to positive control. [a] indicates the p<0.001in comparison to negative control.

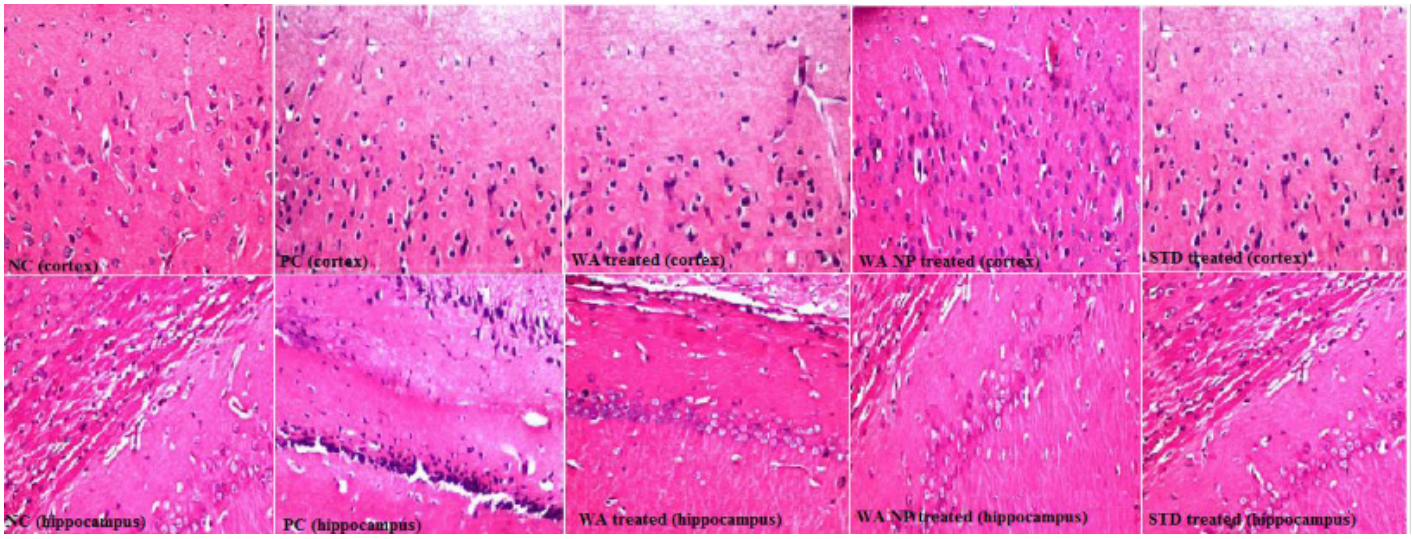

Figure 1: Brain sections.

test used assess memory for long-term spatial information.[17] Retention transfer latency and other EPM measures are used to assess memory. An improvement in memory, as well as a decrease in transfer latency, occurs after 24 hr.[18]

In addition to impairing learning and memory, scopolamine acts as a nonselective antagonist of muscarinic cholinergic receptors.[19] In this study, mice were given inducer drug for one week to cause cholinergic neurotoxicity as well as cognitive impairments. The scopolamine received animals has fewer than 20% of other groups' recognition index after 6 days against scopolamine treatment. In a dose-dependent way, pre-treatment with Withaferin-A reduced the impairments of scopolamine on cognition, with recognition index being two times higher than in the scopolamine negative control group (Figure 1). Additionally, the results demonstrated that Withaferin-A therapy reduced amnesic behaviour in EPM, although it was not significantly different. Scopolamine was given to rats for one week as part of this study. In contrast, perivascular edema and severe congestion of blood capillaries were found in the brain sections of scopolamine-induced demented rats; a demyelination and neuronal degeneration were present in the hippocampus, with encephalomalacia in the tissue matrix and edema in the tissue matrix (Figure 1). The cerebral cortex of tacrine-treated rats showed diffuse gliosis, and some neurons, associated with cytoplasmic vacuolation, exhibited pathological changes in the hippocampi. Brain samples pre-treated Withaferin-A (5 mg per kg) reveals that the cortex region had focal gliosis as well as the hippocampus from each brain has pyramidal cells with irregular outline. Despite the normal histological structure of the cerebral cortex in rats treated Withaferin-A nanoparticles (5 mg/kg), there was shrinkage in some pyramidal cells and vacuolation of some cytoplasm in the hippocampus. The results presented here indicate that Withaferin-A prevented amnesia in the scopolamine model.

In contrast to prior research that used Withaferin-A, our findings in this scopolamine model are uncommon because Withaferin-A causes distinct dosage dependencies in the behavioural model and neurotransmitters, notably Ach. It was found that visual recognition memory was impaired after scopolamine-induced amnesia, but long-term spatial memory remained intact.

The results of histopathological studies demonstrated that 10 day's administration of scopolamine resulted in neurodegenerative

processes in the dentate gyrus when compared to WANP treated rats. This cell death in the hippocampus dentate gyrus was significantly prevented by a pretreatment with Withaferin-A nanoparticle. The dentate gyrus is the part of the brain where adult neurogenesis takes place and it is also implicated in hippocampal neurogenesis and plasticity.[20]

CONCLUSION

Withaferin-A nanoparticles have been shown to be neuroprotective in rats with scopolamine-induced amnesia. Withaferin-A also has an anti-amnesic action equivalent to Tacrine at a certain dosage, albeit occasionally this effect is not dose-dependent. As a result, Withaferin-A nanoparticles can be a promising therapeutical option in patients suffering from neurodegenerative illnesses a viable therapy. To establish the formulation as a standard therapy for Alzheimer's disease, pharmacokinetic factors can also be investigated.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ABBREVIATIONS

AD: Alzheimer's disease; **EPM:** Elevate plus maze; **AchE:** Acetylcholinesterase; **MDA:** Malondialdehyde; **GSH:** Glutathione reductase; **ASH:** Ashwagandha; **^{13}C-NMR:** Carbon-13-nuclear magnetic resonance; **PLGA:** Poly (lactic-co-glycolic acid); **DMSO:** Dimethyl sulfoxide; **i.p:** Intraperitoneal; **OECD:** Organisation for Economic Co-operation and Development; **PDB:** Protein Data Bank; **LD_{50}:** Lethal Dose; **ANOVA:** Analysis of variance; **H&E:** Hematoxylin and Eosin; **WA:** Withaferin-A; **WANP:** Withaferin-A Nanoparticles.

SUMMARY

In summary, the neuroprotective effect of Withaferin-A Nanoparticles was tested by Scopolamine Rat Model of Alzheimer's Disease. Nanodelivery of Withaferin-A not only decreases surrogate marker levels of acetylcholinesterase, malondialdehyde, glutathione reductase, but also increases outcome in the experimental scopolamine rat model of Alzheimer's disease. Withaferin-A was delivered as such in its pure form and in its nanoformulation. Out of the various assays performed, the group treated with Withaferin-A although reversed back a little but not significantly when compared to Withaferin-A nanoparticle treated group. This adds an evidence to prove, Withaferin-A when administered as such, does not cross the Blood Brain Barrier (BBB) to delivery the medicament in the target site, where a nanocarrier is needed and the developed nanocarrier to deliver Withaferin-A across the BBB had explored successfully in deliverying the same. The Withaferin-A nanoformulation used has been shown to have neuroprotection as a proof of improvement in the acetyl cholinesterase, malondialdehyde and glutathione reductase.

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