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BEHAVIORAL AND BIOCHEMICAL EVALUATION OF ANTI-AMNESIC ACTIVITY OF *ACACIA AURICULIFORMIS* LEAF EXTRACT IN RATS

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ABSTRACT

Background: Alzheimer's disease (AD), the most common cause of progressive dementia in the elderly, is a chronic neurodegenerative disorder that leads to disturbances of cognitive functions. The effects and benefits of *Acacia auriculiformis* (AA) on health are not well established. **Aim of the study**: This study was planned to evaluate the protective effect of ethanolic extract of *Acacia auriculiformis* leaves against scopolamine-induced memory loss using learning and memory model in rats. **Materials and Methods:** Twenty four adult male albino rats were divided into 4 equal groups. Memory retention performance of rats was evaluated using Morris' water maze after oral administration of two different doses (200 mg/kg and 400 mg/kg) of ethanolic extract of *Acacia auriculiformis* with rivastigmine 5 mg/kg as positive control and 1% Tween-80 p.o. to the normal control group for 45 successive days. On the last day of treatment, a single i.p injection of scopolamine 3 mg/kg was administered one hour after the test agent administration and 20 minutes prior to water maze test. Post behavioral testing, the animals were sacrificed and brain cholinesterase activity was estimated to substantiate the findings of behavioral test. Data was analyzed using one way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test using GraphPad Prism software, version 6.01. **Results:** The AA extract exhibited a dose-dependent improvement in memory retention performance in rats with scopolamine-induced dementia tested on water maze model. Dose-dependent inhibition of brain cholinesterase activity ($P < 0.001$) was also noted

in extract-treated groups. 400 mg/kg AAE group showed much superior results than rivastigmine in all four parameters of water maze like target quadrant entry ($P < 0.001$), island entry, escape latency and total time spent in target quadrant ($P< 0.05$), and also in AChE inhibitory activity ($P< 0.001$).There was no significant difference between 200 mg/kg AAE and rivastigmine groups in both behavioral and biochemical tests. **Conclusion:** It is evident that acetylcholinesterase inhibiting property of *Acacia auriculiformis* is the most likely contributor for its anti-amnesic activity. Further well-designed large scale studies are required to clearly elucidate its benefits as well as safety in humans. In future this may offer a promising novel option for the treatment of dementia disorders.

KEYWORDS: *Acacia auriculiformis*, acetylcholinesterase, water maze, Alzheimer's disease

INTRODUCTION

Dementia is a neurological condition characterized by impairment of memory, general intellectual functions including cognition and loss of skill in performing the daily routine activities. Alzheimer's disease (AD) is the most common cause of progressive dementia in the elderly population world-wide.[1,2]Although the primary cause of AD remains unclear, brain acetylcholine deficiency and oxidative stress are principal pathogenic factors. The occurrence of neuropsychiatric symptoms is common in AD which leads to lowering of quality of life in patients posing a challenge to their family, caretakers, health services and the general public. Since its first description about 100 years ago, AD continues to be one of the most taxing and disabling diseases despite tireless efforts to cure the condition. It is estimated that about 81.1million people world-wide would acquire AD by the year 2040.[3]*Acacia auriculiformis* is a common Indian plant also called Black Wattle. This plant is known for its medical properties notably anthelminthic, antifilarial, virucidal, microbicidal etc.[1,4,5] Its stems are reported to have antioxidant, anti-inflammatory, anti-mutagenic, anti-carcinogenic and wound healing activities.[2,3,6,7] The study conducted by Crowch *et al* demonstrated acetylcholinesterase inhibitory property of *Acacia nilotica* in *in vitro* models.[8]One recent study on Acacia auriculiformis has shown dose-dependent brain AChE inbition *in vitro*.[9] This property is said to contribute significantly for its memory enhancing potential.[10]Scopolamine is an antimuscarinic agent which produces transient anterograde amnesia both experimentally and clinically, very much resembles Alzheimer's memory deficit pattern. However, the neuropathology of dementia of the AD is not confined to the cholinergic system. With this background information, we planned to investigate the anti-

amnesic effect of *Acacia auriculiformis* leaf extract on scopolamine-induced behavioral changes in rats using experimental model for spatial learning and memory retention and also biochemically estimate its potential to inhibit brain AChE enzyme, which could possibly justify its putative ability of memory retention and enhancement.

MATERIALS AND METHODS

Animals

The study was conducted from November 2014 to January 2015 (3 months) in Department of Pharmacology, Melaka Manipal Medical College. Twenty four healthy male inbred albino rats of Wistar strain, weighing 150-250g selected from Animal House, Kasturba Medical College, Manipal University, Manipal, Karnataka, India were used for the study. The rats were maintained under a reverse photo cycle of 12h day-12h 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 (IAEC/KMC/84/2014).

Drugs and chemicals

Following drugs and chemicals were used in the study: rivastigmine (Sun Pharma, Ind. Ltd), DTNB [(5,5'-dithiobis-2-nitrobenzoic acid), Sigma chemicals, St Louis,MO, USA], 10% Tween 80, acetyl-thiocholine [(ATC), Sigma chemicals, St Louis, MO, USA]. Buffers and other reagents used were of analytical grade.

Method of preparation of ethanolic extract

Acacia auriculiformis leaves were obtained locally from Udupi, Karnataka State. Dr. K. Gopal Krishna Bhat, Professor of Botany, Poorna Prajna College, Udupi confirmed its authenticity. Fresh leaves were collected in the morning in the month of November and care was taken to collect leaves of uniform growth (i.e. of around 15 to 20 days old plants). The shade dried and subsequently powdered leaves were loaded into Soxhlet extractor in batches of 200g each and subjected to extraction for 30 to 40h with 95% ethanol. After extraction, the solvent was distilled off. The extract was then concentrated under reduced pressure on a water bath at temperature below 50ºC to yield consistency of a syrup which was then dried in a dessicator. The final yield of extract was 80g.

Acute toxicity study

The acute toxicity studies were performed in accordance with the Organization for Economic Co-operation and Development (OECD) Test Guidelines 425 (Up-and-Down Procedure).[11] Adult female rats were used. A limit test was done using a limit dose of 2000 mg/kg. Animals were observed individually, for mortality and general behavior, at least once during the first 30 min after dosing, periodically during the first 24h (with special attention being given during first 4h), and daily thereafter for a total of14 days. No mortality was observed till the end of the study. The test samples were safe up to dose of 2000 mg/kg, and from the results obtained, 400mg/kg dose was chosen as the maximum dose for further experimentation, along with a dose of 200mg/kg.

Experimental protocol

Learning and memory was assessed using a behavioral paradigm i.e. Water maze test. Four study groups with six animals in each were tested on this model at the end of treatment period of 45 days. Groups were as follows:

• Group I (control): Equivolume of 1% Tween-80 p.o.

• Group II (200mg ACA): Ethanolic extract of *Acacia auriculiformis*200mg/kg p.o.

• Group III (400mg ACA): Ethanolic extract of *Acacia auriculiformis* 400mg/kg p.o.

• Group IV (RIVA): Suspension of rivastigmine 5mg/kg p.o.

I]. Evaluation of memory retention in rats with scopolamine-induced memory loss using Morris' water maze paradigm

Rats of groups 200 ACA, 400 ACA and RIVA were treated with 200 mg/kg and 400 mg/kg *Acacia auriculiformis extract*, and rivastigmine 5 mg/kg per oral respectively for 45 successive days. The rats in normal control group were administered 1% Tween-80 per oral. From treatment-day 43 onwards, rats were subjected to one trial of training on water maze on each day for 3consecutive days one hour after administration of test agents. Water maze paradigm is an aversively-motivated rodent model first designed by Morris in 1984 mainly for evaluation of spatial learning and memory. [12] It consists of a circular tank with four equal quadrants: north-east, north-west, south-east and south-west. A wooden platform which acted as a "cue" was placed in north-west quadrant. The position in the target quadrant wherein the cue was placed was referred to as "island". In each trial, rats were released one at a time, into the water once in every quadrant and allowed for 60 seconds to escape onto the platform, failing which we would guide them with a piece of twig until they reached the cue. At the end

of 45 days treatment period, dementia was induced in all the rats by a single intra-peritoneal injection of scopolamine 3 mg/kg, one hour after the last dose of test agents and animals were exposed to water maze paradigm about 20 minutes after scopolamine injection. Unlike training period, this time no cue was placed in the target quadrant, i.e. north-west. We tested their spatial learning retention performance despite inducing short term amnesia with scopolamine. They were released into water and in the quadrant opposite to target quadrant and were observed for following responses: number of entries to target quadrant, escape latency (i.e. the time from when the rat is placed in water in the opposite-to-target quadrant to the time it finds the platform), number of island entries and total time spent in the target quadrant. Escape latency is the index of its memory retention, i.e. lesser the escape latency better is its memory retention.

II]. Collection of brain samples for biochemical estimation of brain cholinesterase

After behavioral testing, the animals were sacrificed by cervical decapitation under excessive ether anaesthesia. Immediately after decapitation, whole brain was isolated and carefully removed from the skull, weighed and then kept in cold normal saline.

***In vitro*Acetylcholinesterase (AChE) assay**

The activity of brain AChE was measured according to a method which is modification of Ellman *et al.*[13] Rat brain was homogenized under 37°C in 0.1M KH2PO4 buffer (30 mg/ml rat brain wt/ml of buffer) at pH = 8 and was kept frozen in an ice chest. The homogenate was then centrifuged at 3000 rpm for 10 min and the resultant cloudy supernatant was used for estimation of brain AChE activity. A volume of 3 ml of phosphate buffer (pH = 8) was being added to test tubes labelled as sample blank and test to which 200µL of supernatant was added and the mixture was vortexed. Subsequently DTNB was added to both the test tubes and incubated for 5 min at room temperature and absorbance was read at 412nm using spectrophotometer and was set at zero-absorbance when the enzyme activity stopped increasing. Finally 20µL of ATC was added to the test sample, and 20µL of phosphate buffer (pH = 8) to sample blank. Mixture was vortexed and absorbance was read at 412nm for 10 min at 37°C at 1 min interval. The enzyme activity was calculated based on the changes in absorbance/min.

III]. Statistical analysis

The data were analyzed using GraphPad Prism software version 6.01. Data were expressed as Mean ±S.E.M. and comparisons between means were carried out using ordinary one-way

analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. A probability level of less than 0.05 was accepted as being significant.

RESULTS

I]. Evaluation of retention performance in short-term amnesia model using spatial learning paradigm

The results of retention performance are mentioned in Table 1. The number of entries to target quadrant and to the island by all four groups are depicted in Fig 1 and Fig 2 respectively. The escape latency and total time spent in target quadrant by the rats of various groups is depicted in Fig 3.

1. Number of target quadrant entries– ANOVA test applied for means of all groups was statistically significant ($P< 0.0001$), and multiple comparison showed that the mean values of 400 ACA and RIVA are statistically significantly higher than that of control group ($P< 0.0001$ and $P < 0.05$ respectively), also 400 ACA showed significantly higher entries than 200 ACA ($P< 0.0001$) and RIVA groups ($P< 0.001$) whereas200 ACA was not significantly different from control and RIVA groups.

2. Number of Island entries – Statistical analysis using ANOVA reflected that mean differences among the groups were statistically significant ($P< 0.0001$). Post hoc analysis using Tukey's test showed that mean of the island entries in 200 ACA, 400 ACA and RIVA were significantly higher as compared to control. Mean value of 200 ACA was not significantly different from RIVA. Though 400 AAE was not significantly different than 200 ACA, yet it had a significantly higher number of entries than RIVA.

3. Escape latency – Escape latency was statistically significantly shorter with all three treated groups than control (200 ACA – $P< 0.05$, 400 ACA – $P< 0.0001$ and RIVA – $P< 0.01$). Comparing the mean values, rats in 400 ACA took significantly lesser time to reach target quadrant than those in 200 ACA and RIVA ($P< 0.05$). The latter groups did not significantly differ in this aspect.

4. Total time spent in target quadrant –Besides shorter escape latency, duration of stay in target quadrant was also significantly higher with treated groups than control (200 ACA – $P< 0.01$, 400 ACA – $P< 0.0001$ and RIVA – $P< 0.001$). On an average, the rats in 400 ACA not

only reached target quadrant earlier but also stayed for longer timespan when compared to 200 ACA and RIVA. The latter groups did not show a significant difference in this regard.

Table 1.

GROUPS (n = 6)	RESPONSES OBSERVED IN MORRIS' WATER MAZE PARADIGM (Mean ± SEM)			
	No. of Target quadrant (NW) entry	No. of Island Entry	Escape Latency (time in seconds)	Total time spent in target quadrant (time in seconds)
Control	2.33 ± 0.333	0.67 ± 0.210	5.77 ± 0.595	5.933 ± 0.815
AAE 200 mg/kg	4.00 ± 0.816*	2.17 ± 0.307$^{\$\$}$	4.02 ± 0.375$^{\&}$	11.65 ± 0.769$^{\&\&}$
AAE 400 mg/kg	7.83 ± 0.477**	3.17 ± 0.307$^{\#\#}$	2.28 ± 0.117$^{@@}$	16.60 ± 1.290$^{\%\%}$
Rivastigmine	4.67 ± 0.333$^{\#\$}$	1.83 ± 0.307$^{@}$	3.77 ± 0.141$^{\%!}$	12.78 ± 0.796$^{\wedge\$}$

*P<0.001 (vs.400 AAE); **P<0.0001 (vs. control); #P<0.05 (vs. control); $$P$<0.01 (vs. 400 AAE)

$^{\$\$}P$<0.01 (vs. control); $^{\#\#}$ P<0.0001 (vs. control); $^{@}$ P<0.05 (vs. control, and vs. 400 AAE)

$^{\&}P$<0.05 (vs. control, and vs. 400 AAE$^{);}$ $^{@@}P$<0.0001 (vs. control); $^{\%}P$< 0.01(vs. control);$^{!}P$ < 0.05 (vs. 400 AAE)

$^{\&\&}$P<0.01 (vs. control, and vs. 400 AAE); $^{\%\%}$P<0.0001 (vs. control); ^P<0.001 (vs. control);$^{\$}$P<0.05 (vs. 400 AAE)

SEM- Standard error of mean; NW- north west; AAE=*Acacia auriculiformis* extract.

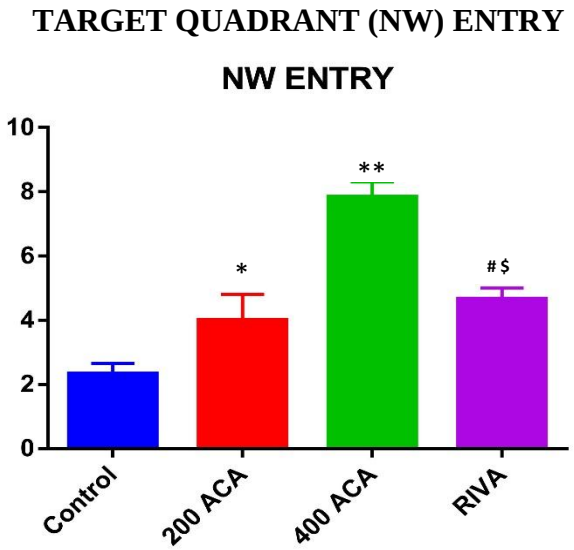

Figure 1. Number of Target quadrant entries by rats of various groups

***P*<0.001 (vs.400 AAE); ***P*<0.0001 (vs. control); #*P*<0.05 (vs. control); $*P*<0.01 (vs. 400 AAE)

200 ACA - *Acacia auriculiformis* extract 200 mg/kg; 400 ACA – *Acacia auriculiformis* extract 400 mg/kg; RIVA- Rivastigmine

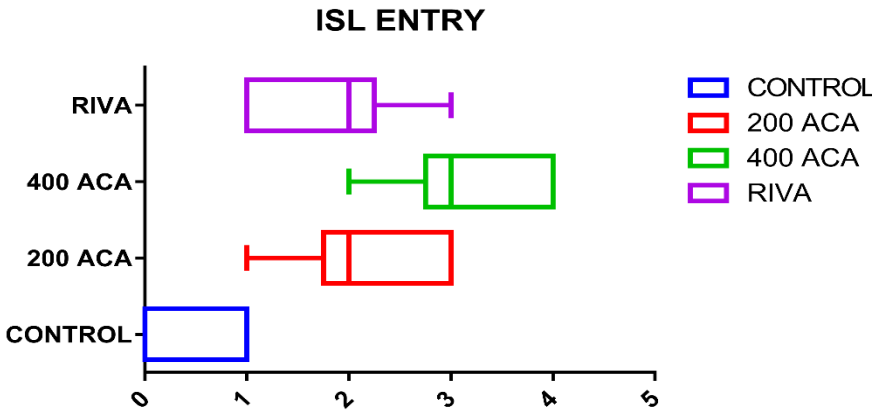

Figure 2. Number of Island entries by rats in various groups

$^{*}P$<0.01 (vs. control); $^{**}P$<0.0001 (vs. control); $^{\#}P$<0.05 (vs. control, and vs. 400 AAE)

200 ACA - *Acacia auriculiformis* extract 200 mg/kg; 400 ACA – *Acacia auriculiformis* extract 400 mg/kg; RIVA- Rivastigmine

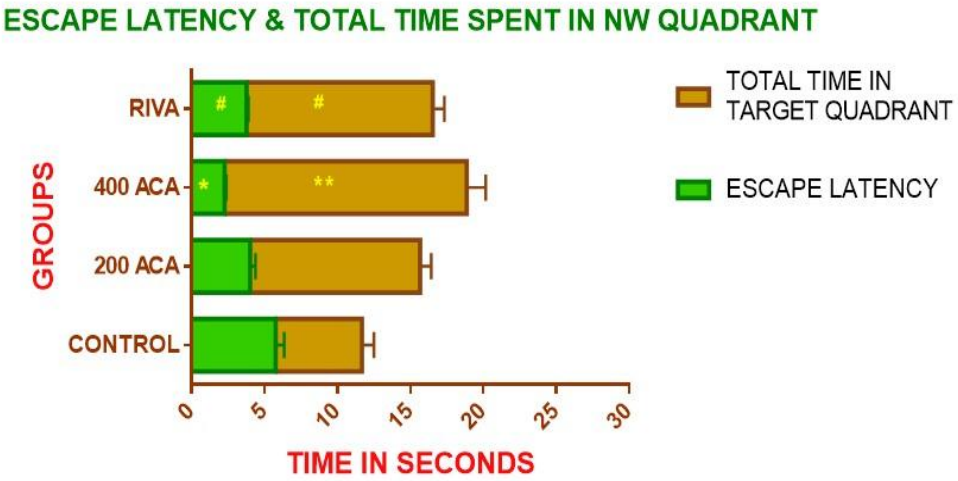

Figure 3. Escape Latency and Total time spent in Target quadrant (NW)

*****- P<0.05 (vs. 200 AAE) and P<0.0001 (vs. control);$^{**}P$<0.01 (vs. 200 AAE) and P<0.0001 (vs. control); #- P<0.05 (vs. 400 AAE) and P<0.01(vs. control)

200 ACA - *Acacia auriculiformis* extract 200 mg/kg; 400 ACA – *Acacia auriculiformis* extract 400 mg/kg; RIVA- Rivastigmine

II]. Effect on Acetylcholinesterase enzyme activity (Unit - Moles of substrate hydrolyzed ×10^6/min/g of brain tissue)

All the study groups had significantly lower brain AChE enzyme activity as compared to control (P < 0.001). Figure 4 depicts the effect of drugs over their brain AChE activity. A dose-dependent inhibition of AChE was seen in the extract treated groups (P < 0.001). A

highly significant difference was also observed between 400 mg/kg dose of AAE and rivastigmine group ($P < 0.001$).

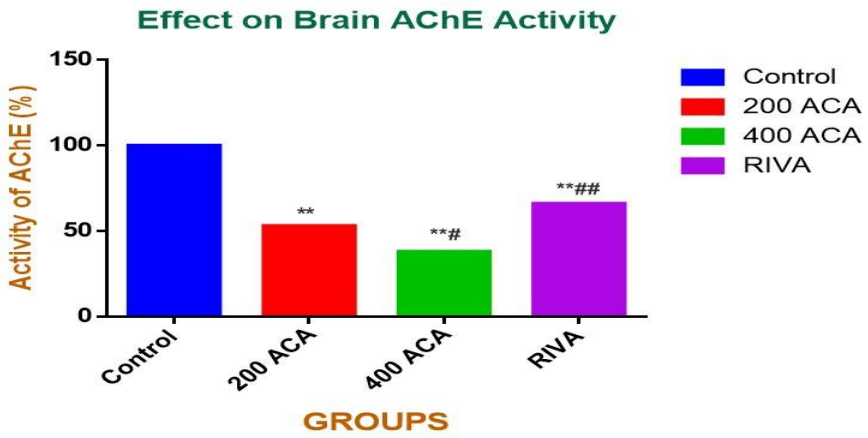

Figure 4: Effect of Study drugs on brain Acetylcholinesterase (AChE) Activity

**P< 0.001 (vs. control); # P< 0.01 (vs. 200mg/kg AAE); ## P< 0.001 (vs. 400mg AAE)
200 ACA - *Acacia auriculiformis* extract 200 mg/kg; 400 ACA – *Acacia auriculiformis* extract 400 mg/kg; RIVA- Rivastigmine

DISCUSSION

Alzheimer's disease is a neurodegenerative disease of global concern which has been posing a great challenge to health care system, because of the complexities in the underlying pathological mechanisms and availability of only a handful of clinically effective drugs that enable slowing of progression but do not modify the course of disease. The cholinergic hypothesis of AD proposes that low synaptic levels of ACh resulting from loss of cholinergic neurons lead to cognitive decline. [14,15] This hypothesis resulted in the exploration of various strategies to increase the brain levels of ACh. The AChE inhibitors like donepezil, rivastigmine and galantamine have emerged as successful treatment options to this end. However, they produce nasty adverse effects like vomiting, diarrhoea, sweating, bradycardia and headache owing to its ACh potentiating action.[16]Study conducted by Mukherjee *et al.* has demonstrated the usefulness of phytotherapy in treatment of AD.[17]Various phytochemicals provide protection against wide range of etiopatholological factors for neuropsychiatric diseases.[8,18,19]In the current study, the extract treated groups showed a dose-dependent increase in memory retention as compared to control in rats with scopolamine-induced amnesia in spatial learning model. 200 mg/kg of AAE showed to possess memory enhancement potential that is almost equivalent to rivastigmine 5 mg/kg p.o.

dose, whereas rats treated with 400 mg/kg AAE showed a much superior memory retention than 200 mg/kg AAE and rivastigmine treated group. *In vitro*, the AAE treated groups demonstrated dose-dependent inhibition of brain AChE in an ascending fashion and 400 mg/kg AAE group exhibited a significantly greater inhibition than rivastigmine group. This AChE enzyme inhibiting activity in the brain was consistently found on measuring at two separate occasions. This also provides an indirect evidence of its blood brain barrier penetrating ability. These biochemical and behavioral findings further strengthen the claim of dose-dependent improvement in memory with AAE. A possible explanation for the memory retention property of AA in rats could be the potentiation of central cholinergic activity due to inhibition of synaptic AChE, which enables to eventually surmount the antagonism by scopolamine and facilitate further cholinergic transmission. Scopolamine-induced dementia has been used extensively to evaluate potential therapeutic agents for treating AD.[19,20]Scopolamine influences the expression of several genes associated with muscarinic receptor signaling pathways, apoptosis, and cell differentiation in rat brain.[21]Hence it causes transient yet profound memory impairment in animals and humans as degeneration and dysfunction of the cortical cholinergic neurons is closely associated with cognitive deficits in AD.[22]Moreover in one of the recent study, scopolamine administration *per se* has shown to increase brain AChE activity by 73.72% as compared to the non-treated normal control group rats (1% Tween-80).[23]Hence the drug/extract treated groups would also further decrease this scopolamine induced elevation in AChE activity.

CONCLUSION

To the best of our knowledge, this study is one among the maiden attempts to evaluate neuropharmacological property of *Acacia auriculiformis* in rats using memory and learning experimental paradigm. The loss of cholinergic innervation and transmission correlates well with the degree of dementia and the severity of the neuropathological hallmarks of AD. Owing to the inhibitory effect on AChE activity of *Acacia auriculiformis* as observed in the present study and reported by other investigators[9,24], it could emerge as an adjuvant to the existing therapies or an alternative treatment option for AD and other dementia disorders in near future. In this regard, well-designed and well-equipped preclinical and clinical studies are necessary to characterize the clinically useful active principles in *Acacia auriculiformis*, assimilate its PK/PD features and to establish the underlying mechanisms at molecular and genetic level, so as to prove its benefits conclusively.

Conflict of interest

None.

Source of support

Nil.

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