Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Vernonia anthelmintica (L.) Willd Extract Alleviates Cognitive Deficits and Neurodegeneration Induced by Infusion of Amyloid Beta (1–42) in Rats

Swati Som, Justin Antony[1], S. Palanisamy Dhanabal[2], Sivasankaran Ponnusankar

Departments of Pharmacy Practice, [1]Pharmacology and [2]Pharmacognosy and Phytochemistry, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Ooty, Tamil Nadu, India

Submitted: 02-Dec-2020 Revised: 05-Jan-2021 Accepted: 04-Aug-2021 Published: 15-Sep-2021

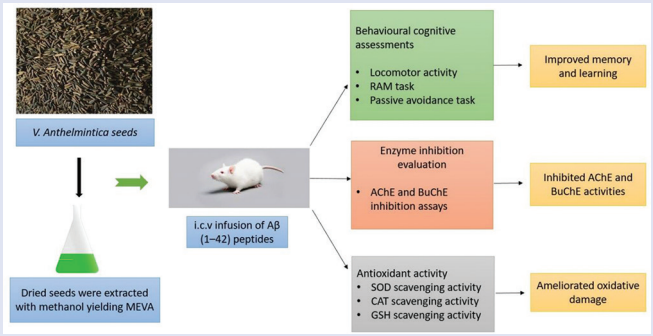
ABSTRACT

Background: There is a constant hunt for the development of new therapeutic alternative to address Alzheimer's disease (AD) due to its failing attempts in the evolution of new therapies and suboptimal results from the existing pharmacological interventions for the treatment of this severe neurodegenerative disease. *Vernonia anthelmintica* is an extensively used medicinal plant in Indian traditional medicine with wide range of therapeutic values. However, there is insufficient scientific documentation available for its protective effect against cognitive disorders. **Objectives:** The objective of the current investigation is to explore the neuroprotective activity of methanolic extract of *V. anthelmintica* (MEVA) in amyloid-beta (Aβ) (1–42) infused sporadic model of AD. **Materials and Methods:** Adult healthy male Wistar rats were treated orally with 250 mg/kg and 500 mg/kg of MEVA for 28 days, after a week from intracerebroventricular (i.c.v) infusion of Aβ (1–42) peptides followed by assessment of neurobehavioral deficits. Subsequently, animals were euthanized and brains were collected for estimation and quantification of neurochemical biomarkers including antioxidant enzymes, neurotransmitters, plaque load, and inflammatory mediators. **Results:** Dose-dependent reversal of cognitive impairment was observed upon MEVA treatment in amyloid intoxicated rats as corroborated by improved learning and memory and diminished oxidative stress, cholinergic hypofunction, and neuroinflammation induced by Aβ (1–42). **Conclusion:** Collectively, evidence-based data suggested the promising neurotherapeutic potential of *V. anthelmintica* and thereby can stand as a novel entity for curbing AD pathology.

Key words: Alzheimer's disease, amyloid-beta (1–42) peptides, neuroinflammation, oxidative stress, *Vernonia anthelmintica*

SUMMARY

- Alzheimer's disease (AD) is a common progressive neurodegenerative disorder characterized by the demolition of intellect
- "One-compound-multi-targets" approach can delay the underlying pathophysiology and can produce needed improvement in clinical signs and symptoms of AD
- Herbal or alternative system of medicine has long been used for treating various ailments due to its wider range of safety
- *Vernonia anthelmintica* has been used as a folk remedy to treat wide spectrum of diseases and thus evaluated for neuroprotective property
- Methanolic extract of *V. anthelmintica* efficiently ameliorated AD-related complication in the amyloid beta (1–42) infused AD model and thus could be the promising lead for the discovery of drug for the management of AD.



Abbreviations used: MEVA: Methanol extract of *V. anthelmintica*; i.c.v: intracerebroventricular; aCSF: Artificial cerebrospinal fluid; Aβ: Amyloid beta; IAEC: Institutional Animal Ethics Committee; AD: Alzheimer's disease; SO: Sham operated; RAM: Radial arm maze; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; ROS: Reactive oxygen species; AChE: Acetylcholinesterase; BuChE: Butyrylcholinesterase; CAT: Catalase; SOD: Superoxide dismutase; GSH: Glutathione; TNF-α: Tumour necrosis factor; IL-6: Interleukin 6; ELISA: Enzyme-linked immune sorbent assay; SE: Standard error of mean; H and E: Hematoxylin and eosin; CV: Cresyl violet; ANOVA: Analysis of variance.

Correspondence:

Dr. Sivasankaran Ponnusankar,
Department of Pharmacy Practice, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Ooty - 643 001, Tamil Nadu, India.
E-mail: ponnusankarsivas@gmail.com

DOI: 10.4103/pm.pm_518_20

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Alzheimer's disease (AD) has become the most familiar progressive neurodegenerative disorder with persistent increase in the number of case incidences, presenting major healthcare challenges and thus possess heavy tolls on public health care systems.[1] This disease is clinically characterized by the demolition of intellect with the appearance of abnormal formation of extracellular deposition of amyloid-beta (Aβ) peptides and intracellular neurofibrillary tangles in the cerebral cortex including the hippocampus which are thought to cause failure in synaptic transmission and neuronal loss.[2,3] Due to the complexity of the AD,

Cite this article as: Som S, Antony J, Dhanabal SP, Ponnusankar S. *Vernonia anthelmintica* (L.) willd extract alleviates cognitive deficits and neurodegeneration induced by infusion of amyloid beta (1–42) in rats. Phcog Mag 2021;17:S172-9.

detailed mechanism behind the disease has not been elucidated yet, but, multiple hypothesis have been put forward regarding AD pathogenesis, including the role of tau protein, amyloid cascade, cholinergic, oxidative stress, neuroinflammation, and so on. It has been found that each one of them has a significant input to the disease progression.[4]

Restoration of acetylcholine (ACh) level by inhibition of acetylcholinesterase (AChE) and few other alternative treatment strategies including N-methyl-D-aspartate glutamate-receptors antagonist, vitamins, antioxidants, and nonsteroid anti-inflammatory drugs are available as current pharmacotherapy for the prevention of AD but these drugs were found to be effective as a short-term therapy in providing partial improvement of memory but not as a putative lifelong treatment.[5] Apart from these, few other candidates, for instance, β-site amyloid precursor protein (APP) cleaving enzyme 1 inhibitors, anti-Aβ immunotherapies, γ-secretase modulators, GSK-3 β inhibitors, 5-HT_6-receptor antagonists, H3-receptor antagonists successfully blocked the production and transportation of molecules contributing to Aβ build up in preclinical studies supporting the strategy of reducing Aβ overproduction but failed in human clinical studies as it was found to be inefficacious.[6,7] Unfortunately, despite extensive studies, recent attempts to invent novel strategies that can effectively prevent or halt the progression of AD have failed, which may be due to the lack of proper understanding of exact initial causative factors and pathogenic mechanisms underlying this disorder. Thereby, no pharmacological interventions for the management of the disease progression have developed to date.[8]

Absence of the effective disease-modifying therapeutics not only imposes poor impact on quality of life but also entails intense suffering to patients, family members, and caregivers. As the etiology of this progressive neuropathological disorder is unclear, hence this uncertainty has persuaded keen interest in developing a novel therapeutic technique that can act as "one-compound-multi-targets" approach which can delay the underlying pathophysiology and can produce needed improvement in clinical signs and symptoms.[9-11]

The herbal or alternative system of medicine has evidently participated in the drug discovery for combating several diseases due to its wider range of safety and thus gained its popularity over synthetic drugs.[12] Notably, extracts and compounds derived from natural sources with promising neuroprotective property show great potential in developing treatments that can reverse the AD related complications.[13,14] *Vernonia anthelmintica* (L.) Willd, commonly known as kalijiri, belonging to the family Asteraceae is one such popular medicinal plant among various ethnic groups since ancient times. Entire plants especially seeds have been used as a folk remedy to treat a wide spectrum of ailments such as influenza, asthma, diarrhea, sinusitis, cardiovascular diseases, convulsion, psoriasis, leukoderma, paralysis, fever, inflammatory swellings, ulcer, and scabies.[15,16] In addition, the seeds have also been credited with versatile health beneficial properties like diuretic, anti-spasmodic, diaphoretic, carminative, stomachic, tonic, depurative, anti-tussive, and purgative.[17] Apart from the aforementioned properties, reports from the earlier studies have also confirmed neuroprotective attribute of *V. anthelmintica*. AChE inhibition is by far the most acceptable therapy available which has proven to alleviate the cognitive impairment in AD individual. Experiments conducted earlier have documented satisfactory inhibitory activity of methanolic and hydroalcoholic extract of *V. anthelmintica* against cholinesterase enzymes.[18,19] Several other experiments were also performed indicating its nootropic and analgesic properties with enhanced cognitive performance.[20]

Evidences of cholinesterase inhibition activities has laid the grounds for the rationale to explore the protective role of *V. anthelmintica* on neuroinflammation, oxidative stress, plaque accumulation and cholinesterase hypofunction against Aβ (1–42) infused rat model of AD. Our findings showed satisfactory results and invigorate new areas of research in AD, thus could be a promising disease-modifying moiety for the treatment or prevention of neurodegenerative diseases such as AD.

MATERIALS AND METHODS

Drugs and chemicals

Aβ (1–42) peptides (≥95%) were acquired from Sigma-Aldrich, USA. Tumor necrosis factor-α (TNF-α) and interleukin-1 β (IL-1 β) Mouse enzyme-linked immunosorbent assay (ELISA) kits were purchased from GenxBio, India. All other chemicals and reagents used for the research work were of analytical grade only.

Animal procurement

Adult healthy male Wistar rats, aged 3–6 months, weighing between 250 and 300 g were provided by the central animal house facility of JSS College of Pharmacy, Ooty, Tamil Nadu, India. The animals were allowed sufficient pellet diet and water *ad libitium*. For the purpose of adaptation to the environmental conditions before commencement of the experiments, all the animals were housed in propylene cages for a week in a climate-controlled laboratory condition (22°C–24°C; 45%–65% humidity) with 12 h light/dark cycles. All the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) (JSSCP/IAEC/Ph.D./Pharmacy Practice/01/2018-19) and the studies were carried out as recommended by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.[21]

Plant extraction and drug preparation

Seeds of *V. anthelmintica* were collected from The Nilgiris district of Tamil Nadu, India during November and was identified and authenticated by authorized field botanist, Dr. S. Rajan, Central Council for Research in Homeopathy, Department of Ayurveda, Yoga, Unani, Siddha and Homeopathy, under the ministry of health and family welfare (Government of India), The Nilgiris, India. The voucher specimen (JSSU-391) was deposited at the Department of Pharmacognosy and Phytopharmacy, JSS College of Pharmacy, Udhagamandalam 643001, India. The seeds were dried under shade, powdered, and passed through 40 mesh sieve. Material (500 g) was extracted with 90% methanol by soaking for 2 days and was then filtered. By applying reduced pressure, the extract was concentrated and the residue was lyophilized, yielding methanolic extract of *V. anthelmintica* (MEVA) to be tested. The extract was kept at 2°C–4°C and dissolved in distilled water prior to administration.

Five μg/μl of Aβ (1–42) was firstly dissolved in artificial cerebrospinal fluid (aCSF) and incubated for 4 days at 37°C before intracerebroventricular (i.c.v) injection.

Surgical procedure

The surgical procedure was performed according to Rahman *et al.* using stereotaxic apparatus, where all the experimental animals were anesthetized with ketamine hydrochloride (91 mg/kg, i.p.) and xylazine (9.1 mg/kg, i.p.).[22] Animals were fixed into the apparatus and by using Hamilton microsyringe, 10 μg of Aβ (1–42) in 2 μl of aCSF was injected in the hippocampus bilaterally as a single dose (anterior-posterior = −3.5 mm, medial-lateral = ±2.0 mm from the bregma and dorsal-ventral = 2.7 mm from the skull surface). To maintain the normal body temperature, animals were then kept in the thermo-regulated chamber as post-operative care and antiseptic were applied over the wound to prevent possible infections.

Experimental protocol

After 10 days of acclimatization period, all animals were allocated to four groups (n = 6). The group I was kept as sham operated (SO) and received cerebral injection of aCSF (2 µl) bilaterally, whereas, rest of the animals from other groups (II, III, and IV) were bilaterally infused with 10 µg of Aβ peptides (2 µl). Group II was designed as negative control and Group III and IV as treatment groups. Oral treatment with MEVA was provided to the Groups III (MEVA 250 mg/kg/day) and IV (MEVA 500 mg/kg/day) animals for 4 weeks after 1 week of Aβ (1–42) peptides infusion [Figure 1].

Behavioural cognitive assessments

Animals were monitored for their locomotor behavior to access the basal activity score of animals using actophotometer.[23] In addition, the learning and memory trails of rats was evaluated by using the passive avoidance test and radial arm maze (RAM) test.[24,25]

Brain homogenate preparation

Rats were euthanized and decapitated immediately after 4 weeks of treatment with MEVA followed by behavioral studies as illustrated in Figure 1. Brains were then extracted and cleaned with ice saline. To perform the histopathological examination, the left cerebral hemisphere was separated and fixed in neutral buffered formalin (10% v/v). For the purpose of biochemical studies, homogenate (10% w/v) was prepared from the right hippocampus with sodium phosphate buffer (0.03 M, pH-7.4) and the supernatant was separated and used after centrifugation at 1000 rpm for 3 min at 4°C. Hippocampal protein was measured using Lowry *et al.* method where bovine serum albumin (1 mg/ml) was used as a reference standard.[26]

Biochemical assessment

Determination of hippocampal acetylcholinesterase and butyrylcholinesterase activities

By using Ellman's modified colorimetric method, both AChE and butyrylcholinesterase (BuChE) inhibitory ability of the MEVA in rat hippocampus was analyzed.[27] Assay mixture containing 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent), sodium phosphate buffer (pH 8), and supernatant was incubated for 20 min at 25°C. Acetylthiocholine iodide/butyrylthiocholine iodide was added to the mixture and was read at 412 nm in ultraviolet spectroscopy.

Estimation of hippocampal amyloid beta (1–42), tumour necrosis factor-α and interleukin-1 β levels

The levels of Aβ (1–42), IL-1 β, and TNF-α in the hippocampal homogenate were determined using commercial ELISA kits as per the manufacturer's instructions.

Determination of hippocampal antioxidants

By using the method suggested by Kakkar *et al.* superoxide dismutase (SOD) activity in brain homogenates were tested. The change in the absorbance of the sample was read at 560 nm.[28] Similarly, catalase (CAT) activity was estimated by using Aebi and the rate of decomposition of hydrogen peroxide was measured at 240 nm.[29] Glutathione (GSH) level in the rat brain was quantified by using the Ellman method, where the absorbance of the mixture was measured spectrophotometrically at 412 nm.[30]

Histopathological analysis of brain

The fixed paraffin-embedded specimens of the hippocampus were sectioned at 5 µL thickness. Sections were processed as per the standard operating procedure and finally stained with cresyl violet (CV) acetate and hematoxylin and eosin (H and E). Neuron morphology of CA zone of rat hippocampus was analyzed and captured under light microscopy at ×40.

Statistical analysis

With the help of Graph Pad Prism 8.0.2 (263) software (San Diego, CA), statistical analysis was performed. To test the group difference in the behavioral cognitive assessment, two-way analysis of variance (ANOVA) was used followed by Bonferroni's *post hoc* test. The statistical comparison between the groups for all other parameters was analyzed using one-way ANOVA followed by Bonferroni's *post hoc* test. All data were expressed as mean ± standard error of the mean (SEM). All the values were considered to be significant when $P < 0.05$.

RESULTS

Effect of methanolic extract of *Vernonia anthelmintica* on locomotor activity

For the purpose of examining the impact of Aβ peptides and MEVA on motor functions, locomotor activity was analyzed on the 1st, 7th, 14th, 21st, and 28th days of the experiment (after MEVA treatment). Interestingly, the absence of ruinous effect of MEVA on motor functions of rats was observed, as none of the doses showed any significant alteration in locomotor activity even after 28 days of treatment [Figure 2].

Effect of methanolic extract of *Vernonia anthelmintica* on radial arm maze task

Learning ability and memory in the rats was evaluated using the RAM task by measuring the number of spatial memory errors made during specified period in Aβ (1–42) infused rats. Like locomotor activity, RAM task was conducted on 1st, 7th, 14th, 21st, and 28th days of experiment protocol and was observed that by the end of the task, on the 28th day, rats of Aβ (1–42) infused group revealed a significant deficit in spatial cognition by making a higher number of reference ($P < 0.001$)

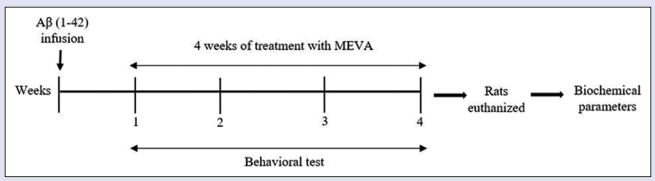


Figure 1: Diagrammatic scheme of experimental procedure

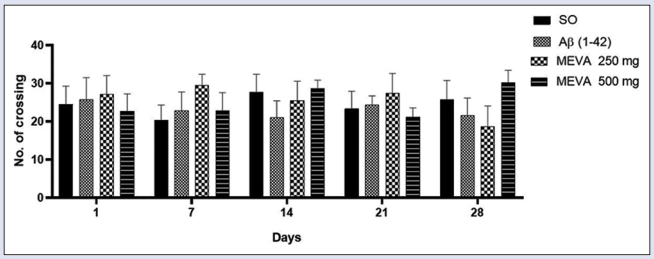


Figure 2: Effect of MEVA on locomotor activity in Aβ (1–42) infused rats. The values were expressed as mean ± SEM (n = 6). SO: Sham operated; Aβ: Amyloid beta; MEVA: Methanol extract of *V. anthelmintica*; SEM: Standard error of mean

and working ($P < 0.001$) memory errors and fewer number of correct choices when compared to that of SO group. Both the doses of MEVA upon oral administration for 28 days showed significant increment in correct choices on comparison with the negative group, however, MEVA 500 mg/kg showed better efficacious outcomes than MEVA 250 mg/kg [Figure 3].

Effect of methanolic extract of *Vernonia anthelmintica* on passive avoidance task

Aβ intoxicated group has significantly decreased the transfer latency time ($P < 0.001$) in both acquisition (F [5, 5] =1.002) and retention trial (F [5, 5] =1.336) when compared with SO, suggesting inability of rats to remember the task [Figure 4]. However, after treatment with MEVA 250 mg/kg, latency to enter into the dark chamber was improved in both acquisition (F [5, 5] =1.702) and retention trial (F [5, 5] =1.565) but fail to show significant results on comparison with the group II. Nevertheless, MEVA 500 mg/kg showed pronounced increase in latency time (acquisition trail [$P = 0.006$, F (5, 5) =1.885] and retention trail [$P < 0.001$, F (5, 5) =1.413]) when compared to negative control [Figure 4].

Effect of methanolic extract of *Vernonia anthelmintica* on hippocampal acetylcholinesterase and butyrylcholinesterase activities

Cholinergic degeneration is highly accepted hypothesis responsible for the cognitive decline in AD patient, which can be mitigated by reducing cholinesterase activity in the brain. In our study, from biochemical estimation of brain tissue, we found enhanced AChE ($P < 0.001$, F [5, 5] =28.38) and BuChE ($P < 0.001$, F [5, 5] =37.2) activities in the hippocampal tissues of negative control when compared to Group I. Both the doses of MEVA showed significant restoration of ACh (250 mg/kg ($P = 0.035$, F [5, 5] =2.74) and 500 mg/kg ($P = 0.001$, F [5, 5] =1.838)) and BuCh (250 mg/kg [$P = 0.0324$, F (5, 5) =20.70]) and 500 mg/kg ($P = 0.001$, F [5, 5] =10.21) activities on comparison with the amyloid challenged group (II) [Table 1].

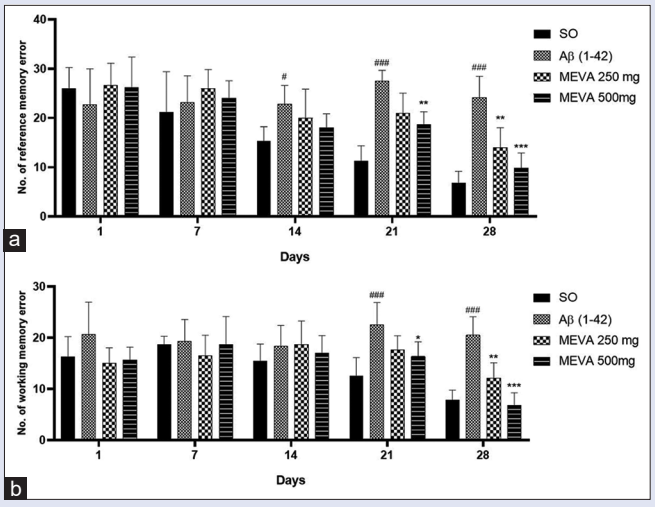


Figure 3: Effect of MEVA on radial arm maze test in Aβ (1–42) infused rats. The values were expressed as mean ± SEM ($n = 6$). $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. SO, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus Aβ (1–42) group. (a) reference memory errors, (b) working memory errors. SO: Sham operated; Aβ: Amyloid beta; MEVA: Methanol extract of *V. anthelmintica*; SEM: Standard error of mean

Effect of methanolic extract of *Vernonia anthelmintica* on hippocampal amyloid beta (1–42) level

Plaque burden due to increased accumulation of Aβ is considered to be the main culprit in AD development. The presence of the significantly high amount of Aβ peptides in the amyloid intoxicated rats brain (Group II) confirms the attenuation of AD in the animals ($P < 0.001$, F [5, 5] =1.198). Unfortunately, both the doses failed to produce a significant effect against Aβ oligomers in the rat hippocampus as tabulated in Table 2.

Effect of methanolic extract of *Vernonia anthelmintica* on hippocampal tumour necrosis factor-α and interleukin-1 β levels

TNF-α and IL-1 β are crucial pro-inflammatory cytokines which plays a major role in AD-associated neuroinflammation. It was observed

Table 1: Effect of Methanol extract of *Vernonia anthelmintica* on hippocampal acetylcholinesterase and butyrylcholinesterase levels in amyloid beta (1-42) infused rats

Groups	AChE (μM/min/g)	BuChE (μM/min/g)
SO	1.86±0.26	14.62±0.55
Aβ (1-42)	19.53±1.41$^{\#\#\#}$	85.51±0.21$^{\#\#\#}$
Aβ (1-42) + MEVA 250	12.88±2.37*	104.09±0.23*
Aβ (1-42) + MEVA 500	11.85±1.04***	31.05±0.39***

$^{\#\#\#}P<0.001$ versus SO, $^{*}P<0.05$, $^{***}P<0.001$ versus Aβ (1-42) group. The values were expressed as mean±SEM ($n=6$). SO: Sham operated; Aβ: Amyloid beta; MEVA: Methanol extract of *Vernonia anthelmintica*; AChE: Acetylcholinesterase; BuChE: Butyrylcholinesterase; SEM: Standard error of the mean

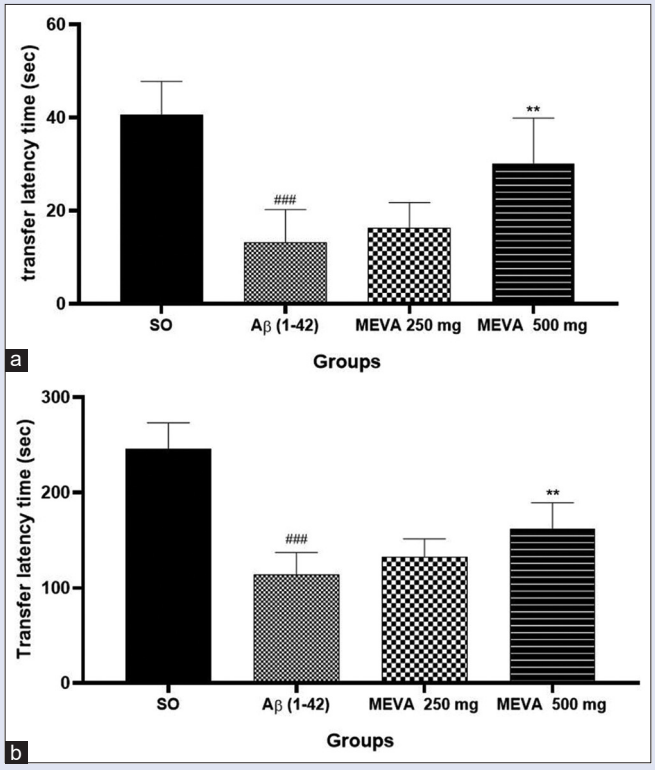


Figure 4: Effect of MEVA on passive avoidance task in Aβ (1–42) infused rats. The values were expressed as mean ± SEM ($n = 6$). $^{\#\#\#}P < 0.001$ versus SO, $^{**}P < 0.01$ versus Aβ (1–42) group. (a) acquisition trial, (b) retention trial. SO: Sham operated; Aβ: Amyloid beta; MEVA: Methanol extract of *V. anthelmintica*; SEM: Standard error of mean

Table 2: Effect of methanol extract of *Vernonia anthelmintica* on hippocampal amyloid beta (1-42), tumour necrosis factor-α and interleukin-1β levels in amyloid beta (1-42) infused rats

Groups	Soluble Aβ (1-42) (pg/ml)	TNF-α (pg/ml)	IL-1β (pg/ml)
SO	66.0±4.54	15.5±3.33	10.66±2.03
Aβ (1-42)	96.67±4.15###	41±3.50###	45±7.09###
Aβ (1-42) + MEVA 250	86.5±6.24	27.33±4.45*	21.66±2.19*
Aβ (1-42) + MEVA 500	81.33±6.71	19±3.56**	15.83±2.41**

###P<0.001 versus SO, *P<0.05, **P<0.01 versus Aβ (1-42) group. The values were expressed as mean±SEM (n=6). SO: Sham operated; Aβ: Amyloid beta; MEVA: Methanol extract of *Vernonia anthelmintica*; TNF-α: Tumor necrosis factor; IL-6: Interleukin 6; SEM: Standard error of mean

that levels of TNF-α (P < 0.001, F [5, 5] =1.103) and IL-1 β (P < 0.001, F [5, 5] =12.21) were significantly high in the hippocampus after Aβ (1–42) infusion when compared to SO animals. Both the doses significantly reduced hippocampal pro-inflammatory markers in animals after completion of the treatment protocol, however, 500 mg/kg exhibited better results with significant reduction in the TNF-α (P = 0.01, F [5, 5] =1.033) and IL-1 β (P = 0.003, F [5, 5] =8.614) levels as compared to Group II [Table 2], thus presenting anti-neuroinflammatory property of MEVA.

Effect of methanolic extract of *Vernonia anthelmintica* on hippocampal superoxide dismutase, catalase, and glutathione levels

Substantial declination in the antioxidant levels such as SOD (P < 0.001, F [5, 5] =8.613), GSH (P < 0.001, F [5, 5] =14.05) and CAT (P < 0.001, F [5, 5] =4.173) was observed in Aβ (1–42) infused group, thus signifies the prominent oxidative damage when compared to the group I. Treatment with MEVA dose dependently improved oxidative stress levels to that of the normal when compared with the negative control. Notably, MEVA at 500 mg/kg counteracted the decline in enzymatic profile of oxidative stress with better significant result than low dose (SOD [P < 0.001, F (5, 5) =1.927], GSH [P < 0.001, F (5, 5) =3.848] and CAT [P < 0.001, F (5, 5) =1.138]). The results are depicted in Table 3.

Histology of hippocampus

H and E and CV stains were utilized in our study to analyze the neuroprotective activity of the MEVA in rat hippocampus. In the negative control group, H and E staining identified significant swelling of neurons, increase in protruding eosinophilic cytoplasm, pyramidal cells shrinkage, pyknotic nuclei, dispersed vacuolization (P < 0.001, F [5, 5] =11.50), whereas, CV staining presented disrupted cell viability (P < 0.001, F [5, 5] =3.602) when compared with SO, clearly indicating extensive neurodegeneration in the rat hippocampus. Treatment with 250 mg/kg MEVA (P = 0.031, F [5, 5] =1.004) and 500 mg/kg MEVA (P = 0.004, F [5, 5] =1.028) manifested efficient reversal of the condition with mild neuronal toxicity against Aβ (1–42) infused group, when assessed by H and E staining. Likewise, MEVA treated groups at 250 mg/kg (P = 0.039, F [5, 5] =2.850) and 500 mg/kg (P = 0.002, F [5, 5] =3.205) displayed significantly high number of CV positive neurons upon comparison with the Aβ (1–42) infused group, thereby boosted neuronal integrity [Figures 5 and 6].

DISCUSSION

AD is one of the major central nervous system disorders and has become a global concern for years now. The elderly population are in persistent risk of acquiring this age-related disorder with its rapid increase in the frequency of cases.[31] Major pitfall with the current treatment is that it only produces symptomatic relief which initially shown to alleviate the destructive effect of neurodegeneration but later on begins to develop tolerance. Not only these drugs are marginally effective but also fail to alter the underlying disease pathology.[32,33] AD is believed to be difficult to treat because of its complexed pathophysiology which has made the development of new curative a tough task. Despite innumerable studies and trials conducted in the past for the hunt of new alternative approaches, no effective treatment is yet discovered for this disorder. The prime reason behind this failure is either due to serious side effects or insignificant therapeutic efficacy.[22]

Since the majority of AD cases are sporadic, rather than inherited (familial), we aimed in our study to employ Aβ (1–42) infused rat model to investigate the neuroprotective effect of *V. anthelmintica*. Wang *et al*. reported that sporadic model of AD in rats can be achieved by i.c.v injection of aggregated Aβ (1–42) in the rat brain.[34] Incubation of Aβ (1–42) in aCSF for 4-6 days results in the conformational transformation which magnifies the neurotoxic potency of Aβ (1–42). Infusion of this aggregated Aβ (1–42) in rodents triggers acute and long-lasting behavioral alterations resembling those occurring in human AD patients. Further, Aβ (1–42) infusion presented memory dysfunction along with long-lasting disruption of both spatial and contextual fear memories, as well as short-term working memory leading to neuronal death in rodents.[21] Further, Bouter *et al*. documented that Aβ (1–42) infusion intensified neuroinflammation, oxidative stress and enhanced the cholinesterase activities, making it a reliable model for accessing the effect of *V. anthelmintica* on AD complications.[35] In our experiment i.c.v injection of aggregated Aβ (1–42) in the rat brain produced similar impairment in cognitive ability.

For the assessment of neurobehavioral deficits in rats, we have chosen three behavioral paradigms: Locomotor activity test, RAM test and passive avoidance task, which are highly depended upon the hippocampus and cortex functions. Deterioration in the learning ability and memory was observed in the amyloid intoxicated rat brain when analyzed using RAM task. Treatment with the MEVA indicated consolidation of memory in the RAM task by demonstrating the higher number of correct choices during the task. Similarly, passive avoidance task was performed to evaluate the short and long-term memory and treatment with MEVA has dose-dependently improved the Aβ (1–42) mediated decline in latency. Notably, MEVA did not produce any impact on locomotor activity in rats, signifying the absence of the deleterious effect of *V. anthelmintica* on motor activity. However, this result of ours is in contradiction to the earlier report documented by Tupe *et al*. 2014, where 250 and 500 mg/kg MEVA significantly raised the locomotor activity.[20] The reason behind the disagreement could be due to the implementation of the different experimental models. Thus MEVA displayed intact learning and memory function in Aβ (1–42) infused rats. To the best of our knowledge, this is the first study to demonstrate the neuroprotective effect of *V. anthelmintica* on the AD-related cognitive behavioral deficits in rats.

Cholinergic hypofunction is the primary clinical observation found in AD. Convincing data from the earlier studies on the neurochemistry of AD indicated decline in ACh and BuCh activities in affected areas of the brain.[36,37] With the growing age, cholinergic neurons were found to become more susceptible to the neurotoxic effects of Aβ fibrils causing cognitive deficits due to neuronal death, thus responsible for learning and memory impairment. This in turn favors the excessive production and accumulation of plaque due to alteration in APP metabolism.[38-40] Cholinergic inhibitors are the widely accepted therapeutic strategy which treat cognitive impairment in AD patients by decreasing the elevated AChE and BuChE activities. Aβ (1–42) infusion resulted in cholinergic hypofunction by elevating the intracellular cholinesterase activity in

Table 3: Effect of methanol extract of *Vernonia anthelmintica* on hippocampal superoxide dismutase, catalase and reduced glutathione levels in amyloid-beta (1-42) infused rats

Groups	GSH (mM/g wet tissue)	CAT (µmol/min/g protein)	SOD (units/min/mg protein)
SO	66.9±4.08	361.83±39.1	10.76±0.12
Aβ (1-42)	11.61±1.08###	109.54±19.1###	1.93±0.35###
Aβ (1-42) + MEVA 250	26.23±1.14***	236.64±34.6**	3.63±0.42**
Aβ (1-42) + MEVA 500	41.44±2.13***	264.96±20.4***	6.03±0.25***

###P<0.001 versus SO, **P<0.01 ***P<0.001 versus Aβ (1-42) group. The values were expressed as mean±SEM (n=6). SO: Sham operated; Aβ: Amyloid beta; MEVA: Methanol extract of *Vernonia anthelmintica*; CAT: Catalase; SOD: Superoxide dismutase; GSH: Reduced glutathione; SEM: Standard error of the mean

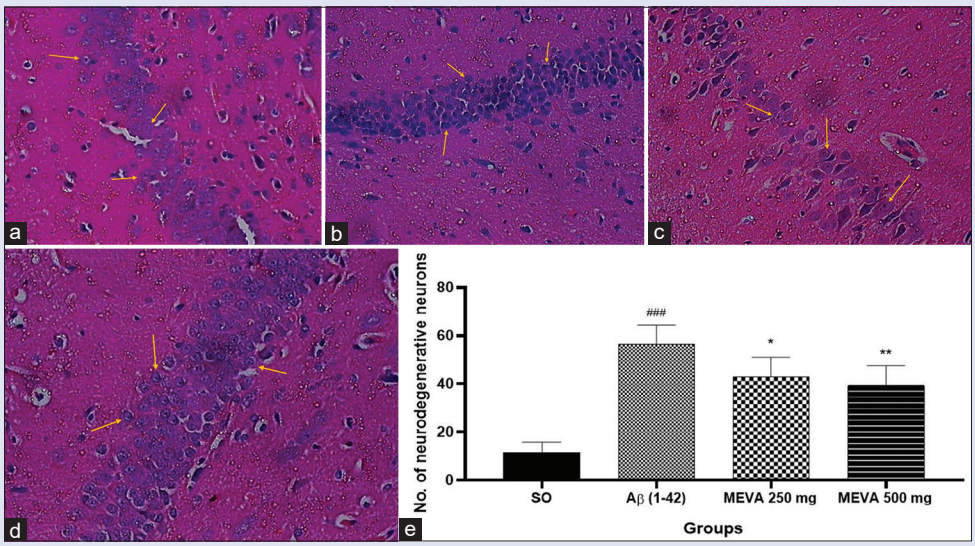


Figure 5: Histology of CA1 region of hippocampus stained with H and E after 28 days of experimental protocol (×40). (a) SO group represents healthy neurons with prominent nuclei, (b) Aβ (1–42) infused group representing neuronal damage, eosinophilic stained cytoplasm, vacuolization and neuronal shrinkage, (c) MEVA (250 mg/kg) and (d) MEVA (500 mg/kg) treated groups representing mild neuronal injury with lesser number of eosiniphilic stained neurons, (e) Quantitative assessment of number of degenerated neurons in CA1 hippocampal section of rat brain. The values were expressed as mean ± SEM ###P < 0.001 versus SO, *P < 0.05 and **P < 0.01 versus Aβ (1–42) group. SO: Sham operated; Aβ: Amyloid beta; MEVA: Methanol extract of *V. anthelmintica*; CA1: Cornu ammonis 1; H and E: Hematoxylin and eosin

the rat brain, which was dose dependently attenuated by the MEVA administration. Previously Som *et al.* 2020 and Kadiyala *et al.* 2014 in their respective studies have documented the *in vitro* anticholinesterase activity of *V. anthelmintica*.[18,19] The present results are in the support of these earlier reports and confirm the anticholinesterase activity of the plant and thereby could be a potential target for the discovery of cholinergic inhibitors.

Overproduction of Aβ and reduction in its clearance results in senile plaque formation which is considered to be the main culprit in AD pathogenesis.[41] The cerebral infusion of Aβ peptides in the rat brain triggers plaque accumulation and caused neuronal death leading to neurodegeneration.[42] In our study, treatment with MEVA produced non-significant reduction in the plaque load suggesting some degree of the preventive effect of MEVA in Aβ (1–42) infused rats. However, to completely understand the anti-amyloidogenic effect of MEVA, its effect on other crucial contributing factors which act as a central player in plaque accumulation such as alteration in the activities of APP, β and γ secretase etc., is needed to be explored.

Plaque load in AD provokes the stress-induced neurodegeneration by hindering the expression of brain-level antioxidant enzymes.[22] This in turn creates the imbalance between the antioxidant defense system and reactive radicle production and thereby aggravates oxidative stress. A similar observation was noticed in amyloid intoxicated group (Group II) which exhibited reduction in the antioxidant levels (GSH, SOD and CAT) indicating neurobiological oxidative damage. Herbal antioxidants have been known for its ability to modulate stress by improving compromised state of the antioxidant defense system.[43] The finding of our investigation supports the antioxidant property of MEVA as it has improved the Aβ (1–42) mediated decline in antioxidant enzymes in rat hippocampus. These results are in agreement with the previous reports which proposed radical scavenging properties of *V. anthelmintica*. Butein, chlorogenic acid, naringinin, isorhamnetin, kaempferol, stigmasterol, and taraxerol were identified in the MEVA seeds previously and reported for antioxidant activities and thus could be accountable for the uplifting antioxidant levels in rat brain.[44,45]

Chronic neuroinflammation plays a prominent role in intensifying the AD complication by providing the neurotoxic environment for plaque formation. Increased plaque load in AD brain activates microglial cells and generates pro-inflammatory cytokines (including IL-1 β and TNF-α) and reactive oxygen species, which ultimately causes neuronal damage and is responsible for up-regulation of APP expression.[46] To the best of our knowledge, no data are available till date on the effect of MEVA on neuroinflammatory markers. In our study, we observed an increase in the pro-inflammatory cytokines (IL-1 β and TNF-α) after Aβ (1–42) infusion in the hippocampus of the rat brain. Treatment with MEVA at both doses significantly reduced the neuroinflammation. This desired anti-inflammatory effect of *V. anthelmintica* might be due to the reported antioxidant properties. Furthermore, the histopathological analysis demonstrated increased neurodegeneration with pyknotic

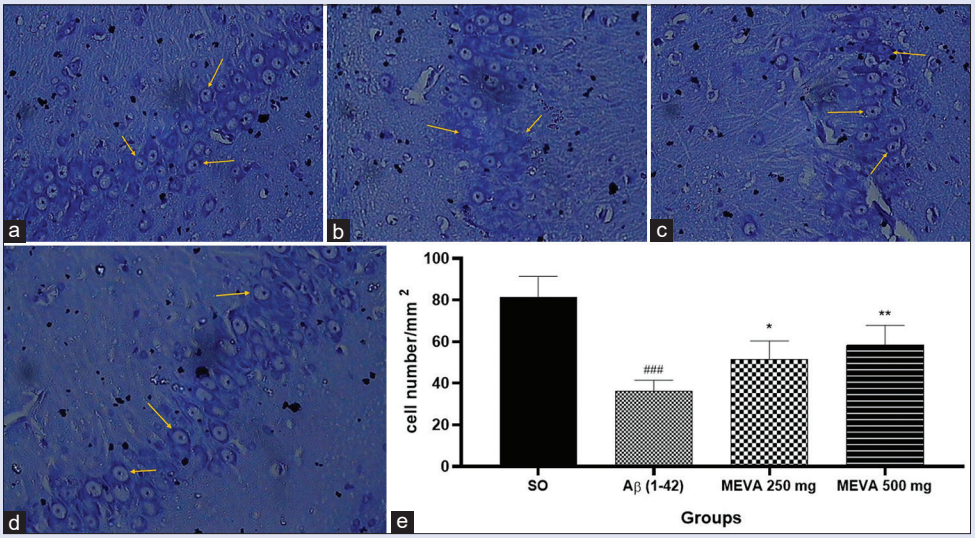


Figure 6: Histology of CA1 region of hippocampus stained with cresyl violet stain after 28 days of the experimental protocol (×40). (a) SO group represents healthy neurons with prominent nuclei and densely stained, (b) Aβ (1–42) infused group representing neuronal damage with lesser number of stained neuron indicating less viable neurons (c) MEVA (250 mg/kg) and (d) MEVA (500 mg/kg) treated groups representing mild neuronal injury with higher number of stained neurons. (e) Quantitative assessment of cell numbers in CA1 hippocampal section of rat brain. The values were expressed as mean ± SEM ###$P < 0.001$ versus SO, *$P < 0.05$ and **$P < 0.01$ versus Aβ (1–42) group. SO: Sham operated; Aβ: Amyloid beta; MEVA: Methanol extract of *V. anthelmintica*; CA1: Cornu ammonis 1

nuclei characterized by nuclei swelling, neuronal shrinking, and disorganization of neurons in H and E staining upon Aβ (1–42) induction, which are in accordant with the previous report by Rahman *et al*. 2019.[22] On the other hand, Aβ (1–42) induced hippocampal cells displayed significant morphological changes in cornu ammonis 1 region of the hippocampus with neuron swelling, vacuolization and apoptotic cells in CV staining, which was found to be clinically similar with the AD patient. MEVA treatment exhibited positive results against this histological alteration by potentially intensifying the number of healthy neurons and protected the neuronal cells from the deteriorating effect of Aβ (1–42) in the rat hippocampus.

CONCLUSION

Collectively, findings from our present investigation demonstrated the neurotherapeutic potential of MEVA against Aβ (1–42) peptides induced cognitive deficits in the rat model. Compromised state of cholinergic activity, cognitive behavioral functions, and antioxidant defense system was achieved after cerebral infusion of Aβ (1–42) peptides, which was successfully ameliorated by oral MEVA treatment. Moreover, based on evidence-based data, biochemical and histopathological evaluation indicated attenuation of AD-related complications by MEVA by inhibiting pro-inflammatory cytokines levels and preventing the loss of neuronal integrity in the hippocampus. However, the present study lacks the insight of bioactive constituents present in the extract responsible for its protective effect against Aβ (1–42) toxicity. Therefore, further investigation on the identification of phytoconstituents and elucidation of underlying mechanism to recognize the neurological characteristics of *V. anthelmintica* is recommended to explore.

Acknowledgements

We sincerely convey our gratitude to the Department of Pharmaceutical Biotechnology, Department of Pharmacology and Department of Pharmacognosy and Phytochemistry, JSS College of Pharmacy, Ooty, for furnishing all necessary facilities and chemicals for successful completion of the research work.

Financial support and sponsorship

This study was financially supported by Lady Tata Memorial Trust, Mumbai, India with senior research fellowship.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Chapman CD, Schiöth HB, Grillo CA, Benedict C. Intranasal insulin in Alzheimer's disease: Food for thought. Neuropharmacology 2018;136:196-201.
2. Souza LC, Jesse CR, Antunes MS, Ruff JR, Espinosa DD, Gomes NS, *et al.* Indoleamine-2, 3-dioxygenase mediates neurobehavioral alterations induced by an intracerebroventricular injection of amyloid-β peptides in mice. Brain Behav Immun 2016;56:363-77.
3. Lian W, Fang J, Xu L, Zhou W, Kang D, Xiong W, *et al.* DL0410 ameliorates memory and cognitive impairments induced by scopolamine via increasing cholinergic neurotransmission in mice. Molecules 2017;22:E410.
4. Uddin S, Kabir T, Rahman M, Mathew B, Shah MA, Ashraf GM. TV 3326 for Alzheimer's dementia: A novel multimodal ChE and MAO inhibitors to mitigate Alzheimer's-like neuropathology. J Pharm Pharmacol 2020;72:1001-12.
5. Zueva I, Dias J, Lushchekina S, Semenov V, Mukhamedyarov M, Pashirova T, *et al.* New evidence for dual binding site inhibitors of acetylcholinesterase as improved drugs for treatment of Alzheimer's disease. Neuropharmacology 2019;155:131-41.
6. Luo X, Yan R. Inhibition of BACE1 for therapeutic use in Alzheimer's disease. Int J Clin Exp Pathol 2010;3:618-28.
7. Wang Y, Yan T, Lu H, Yin W, Lin B, Fan W, *et al.* Lessons from anti-amyloid-β immunotherapies in Alzheimer disease: Aiming at a moving target. Neurodegener Dis 2017;17:242-50.
8. Bharathi MD, Thenmozhi AJ. Attenuation of aluminum-induced neurotoxicity by tannoid principles of *Emblica officinalis* in wistar rats. Int J Nutr Pharmacol Neurol Dis 2018;8:35-40.
9. Kumar A, Aggarwal A, Singh A, Naidu PS. Animal models in drug discovery of Alzheimer's disease: A mini review. EC Pharmacol Toxicol 2016;1:60-79.
10. Pi R, Mao X, Chao X, Cheng Z, Liu M, Duan X, *et al.* Tacrine-6-Ferulic Acid, a novel multifunctional dimer, inhibits amyloid- β-mediated Alzheimer's disease- associated pathogenesis *in vitro* and *in vivo*. PLoS One 2012;7:e31921.
11. Smith LM, Zhu R, Strittmatter SM. Disease-modifying benefit of Fyn blockade persists after washout in mouse Alzheimer's model. Neuropharmacology 2018;130:54-61.

12. Wu Y, Qiu A, Yang Z, Wu J, Li X, Bao K, *et al. Malva sylvestris* extract alleviates the astrogliosis and inflammatory stress in LPS-induced depression mice. J Neuroimmunol 2019;336:577029.
13. Briffa M, Ghio S, Neuner J, Gauci AJ, Cacciottolo R, Marchal C, *et al.* Extracts from two ubiquitous Mediterranean plants ameliorate cellular and animal models of neurodegenerative proteinopathies. Neurosci Lett 2017;638:12-20.
14. Cragg GM, Newman DJ. Natural products: A continuing source of novel drug leads. Biochim Biophys Acta 2013;1830:3670-95.
15. Krithikar KR, Basu BD. Indian Medicinal Plants. Vol. 3. Allahabad, Uttar Pradesh, India: Prabasi Press; 1989. p. 1325-6.
16. Acharya D, Shrivastava A. Indigenous Herbal Medicines. Vol. 1. Jaipur, Rajasthan, India: Aaviskar Publishers; 2008. p. 258-9.
17. The Wealth of India: A Dictionary of Indian Raw Materials and Industrial Products (Industrial Products—Part I). Ind Med Gaz. 1949;84:476-7.
18. Som S, Antony J, Palanisamy DS, Ponnusankar S. A preliminary investigation of anticholinesterase and antioxidant properties of various extracts of *Vernonia anthelmintica* in relation to the treatment of Alzheimer's disease. Int J Res Pharm Sci 2020;11:4511-7.
19. Kadiyala M, Ponnusankar S, Elango K. Screening of siddha medicinal plants for its *in-vitro* acetylcholinesterase and butyrylcholinesterase inhibitory activity. Pharmacogn Mag 2014;10:S294-8.
20. Tupe PN, Dhodi JB, Nagmoti DM, Juvekar AR. Antidepressant potential of *Vernonia anthelmintica* Willd. Extracts in experimental animals. J Nat Remedies 2014;14:199-207.
21. National Research Council (US). Guide for the Care and Use of Laboratory Animals. 8th ed. Washington (DC): National Academies Press; 2011.
22. Rahman SO, Prasad B, Parvez S, Kaundal M, Hussain S, Akhtard M, *et al.* Neuroprotective role of astaxanthin in hippocampal insulin resistance induced by Aβ peptidess in animal model of Alzheimer' s disease. Biomed Pharmacother 2019;110:47-58.
23. Reddy DS, Kulkarni SK. Possible role of nitric oxide in the nootropic and antiamnesic effects of neurosteroids on aging- and dizocilpine-induced learning impairment. Brain Res 1998;799:215-29.
24. Sehgal N, Gupta A, Valli RK, Joshi SD, Mills JT, Hamel E, *et al. Withania somnifera* reverses Alzheimer's disease pathology by enhancing low-density lipoprotein receptor-related protein in liver. Proc Natl Acad Sci U S A 2012;109:3510-5.
25. Barkur RR, Bairy LK. Evaluation of passive avoidance learning and spatial memory in rats exposed to low levels of lead during specific periods of early brain development. Int J Occup Med Environ Health 2015;28:533-44.
26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.
27. Ellman GL, Courtney KD, Andres V Jr., Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-95.
28. Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. Indian J Biochem Biophys 1984;21:130-2.
29. Aebi H. Catalase *in vitro*. Methods Enzymol 1984;105:121-6.
30. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70-7.
31. Cummings J, Lee G, Mortsdorf T, Ritter A, Zhong K. Alzheimer' s disease drug development pipeline: 2017. Alzheimers Dement (NY) 2017;3:367-84.
32. Birks J. Cholinesterase inhibitors for Alzheimer's disease. Cochrane Database Syst Rev 2006;25:CD005593.
33. Chen X, Pan W. The treatment strategies for neurodegenerative diseases by integrative medicine. Integr Med Int 2014;1:223-5.
34. Wang Q, Wang C, Shu Z, Chan K, Huang S, Li Y, *et al. Valeriana amurensis* improves Amyloid-beta 1-42 induced cognitive deficit by enhancing cerebral cholinergic function and protecting the brain neurons from apoptosis in mice. J Ethnopharmacol 2014;153:318-25.
35. Bouter Y, Dietrich K, Wittnam JL, Rezaei N, Pillot T, Papot S, *et al.* N - truncated amyloid β (Aβ) 4 - 42 forms stable aggregates and induces acute and long - lasting behavioral deficits. Acta Neuropathol 2013;126:189-205.
36. Chiu CS, Chiu YJ, Wu LY, Lu TC, Huang TH, Hsieh MT, *et al.* Diosgenin ameliorates cognition deficit and attenuates oxidative damage in senescent mice induced by D-galactose. Am J Chin Med 2011;39:551-63.
37. Son IS, Kim JH, Sohn HY, Son KH, Kim JS, Kwon CS. Antioxidative and hypolipidemic effects of diosgenin, a steroidal saponin of yam (*Dioscorea* spp.), on high-cholesterol fed rats. Biosci Biotechnol Biochem 2007;71:3063-71.
38. Vaucher E, Aumont N, Pearson D, Rowe W, Poirier J, Kar S. Amyloid β peptides levels and its effects on hippocampal acetylcholine release in aged, cognitively-impaired and -unimpaired rats. J Chem Neuroanat 2001;21:323-9.
39. 39. McKinney M, Jacksonville MC. Brain cholinergic vulnerability: Relevance to behavior and disease. Biochem Pharmacol 2005;70:1115-24.
40. Masilamoni JG, Jesudason EP, Dhandayuthapani S, Ashok BS, Vignesh S, Jebaraj WC, *et al.* The neuroprotective role of melatonin against amyloid β peptides injected mice. Free Radic Res 2008;42:661-73.
41. Andreeva TV, Lukiw WJ, Rogaev EI. Biological basis for amyloidogenesis in Alzheimer's disease. Biochemistry (Mosc) 2017;82:122-39.
42. Miloso M, Scuteri A, Foudah D, Tredici G. MAPKs as mediators of cell fate determination: An approach to neurodegenerative diseases. Curr Med Chem 2008;15:538-48.
43. Xie H, Hou S, Jiang J, Sekutowicz M, Kelly J, Bacskai BJ. Rapid cell death is preceded by amyloid plaque-mediated oxidative stress. Proc Natl Acad Sci U S A 2013;110:7904-9.
44. Arya A, Cheah SC, Looi CY, Taha H, Mustafa MR, Mohd MA. The methanolic fraction of *Centratherum anthelminticum* seed downregulates pro-inflammatory cytokines, oxidative stress, and hyperglycemia in STZ-nicotinamide-induced type 2 diabetic rats. Food Chem Toxicol 2012;50:4209-20.
45. Wang JL, Quan Q, Ji R, Guo XY, Zhang JM, Li X, *et al.* Isorhamnetin suppresses PANC-1 pancreatic cancer cell proliferation through S phase arrest. Biomed Pharmacother 2018;108:925-33.
46. Krstic D, Madhusudan A, Doehner J, Vogel P, Notter T, Imhof C, *et al.* Systemic immune challenges trigger and drive Alzheimer-like neuropathology in mice. J Neuroinflammation 2012;9:1-23.