



Vasicinone, a pyrroloquinazoline alkaloid from *Adhatoda vasica* Nees enhances memory and cognition by inhibiting cholinesterases in Alzheimer's disease

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ABSTRACT

Background: Alzheimer's disease (AD) is a neurodegenerative disease with cognitive and memory decline. Due to the lack of effective treatment for AD, there is an interest in finding novel compounds to treat AD. The leaves of *Adhatoda vasica* Nees (AV) exhibited anti-AD effects and known to be rich in pyrroloquinazoline alkaloids. However, the role of pyrroloquinazoline alkaloids as an anti-AD was not explored. Therefore, the present study aimed to isolate active pyrroloquinazoline alkaloids through bioactivity guided fractionation from AV to explore their anti-AD effect.

Method: Column chromatography of dichloromethane (DCM) fraction of methanolic extract was used to isolate the alkaloids from AV. The isolated compounds were characterized by TLC, HPLC, ATR, HRMS, and NMR techniques. *In-silico*, studies were performed to investigate the interaction of the isolated compounds with cholinesterase enzymes (AChE and BuChE). Further, *in-vitro* assays were carried out to evaluate the AChE, BuChE, and Aβ inhibition properties. Propidium iodide displacement assay was performed to know the selectivity of isolated compounds at AChE PAS site. Furthermore, *in-vivo* studies were performed to evaluate memory and cognitive improvement against scopolamine-induced amnesia and Aβ induced neurotoxicity in rats.

Results: A couple of leads were identified through bioactivity guided fractionation, such as vasicinone (VAS) and vasicine (VA) were responsible for the cholinesterase inhibition in DCM fraction. *In-silico* studies identified that both compounds exhibited stable interactions at AChE and BuChE active sites. VAS and VA inhibited the AChE, BuChE, and Aβ aggregation *in-vitro* studies and effectively displaced the propidium iodide at AChE PAS site. Further, *in-vivo* studies showed that administration of VAS and VA significantly improved the memory and cognitive dysfunction in scopolamine and Aβ induced cognitive and memory impairments in rats. Besides, both VAS and VA recovered the hippocampal cell density in AD rats. Moreover, VAS and VA did not show any sign of toxicity.

Conclusions: The present study revealed that VAS acts as potent anti-AD agent from *Adhatoda vasica* Nees and it showed similar potency profile to VA against memory and cognitive impairment in AD. Both compounds VAS and VA possess strong potential in the preclinical development as natural drugs for anti-AD.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease, which impairs memory and cognitive functions (Kumar et al., 2015). Acetylcholine (ACh) is a major neurotransmitter that plays important role in

learning and memory. The loss of memory and learning in AD is due to exaggerated activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) which cleaves the ACh in the brain. These enzyme intensities drastically increase AD. Few therapeutic drugs are available for the management of AD, namely donepezil, galantamine,

Abbreviations: AD, Alzheimer's disease; AVME, *Adhatoda vasica* Nees methanolic extract; DCM, dichloromethane; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; ACh, acetylcholine; BBB, blood-brain barrier; Aβ, amyloid-beta; PAMPA, parallel artificial membrane permeability assay; PDB, protein data bank; PBS, phosphate buffer solution; VAS, vasicinone; VA, vasicine; DPZ, donepezil.

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rivastigmine, and memantine (Breijyeh and Karaman, 2020), which majorly act by improving memory in AD patients. These drugs are prescribed to overcome AD symptoms and can slow down the progression of the disease. Currently available drugs are enhancing acetylcholine in brain by inhibiting acetylcholinesterase (AChE). More recently, BChE inhibitors have been developed and studied for their anti-AD effects (Singh et al., 2020). The most common side effects of donepezil, rivastigmine, and galantamine are loss of appetite, nausea, vomiting, and diarrhea. However, due to the lack of effective treatment, there is a need for novel drugs to treat AD.

Naturally derived plant-based drugs like galantamine, physostigmine, and rivastigmine act as cholinesterase inhibitors (Akıncioğlu and Gülçin, 2020) and are used to improve memory function in AD patients. Several studies are ongoing for the identification of plant-based drugs for the management of AD. Medicinal plant, *Adhatoda vasica* Nees (AV), commonly known as vasaka has well-known medicinal benefits in the Ayurveda and Unani systems of medicine. Moreover, vasaka leaves are used in the Indian system of medicine Ayurveda in the treatment of vomiting, leprosy, diarrhea, tuberculosis, and skin diseases (Sharma et al., 2021). Numerous scientific reports acknowledged that vasaka is the most considerable medicinal plant for the treatment of various disorders and diseases (Hossain et al., 2016). Vasaka has been reported to have various pharmacological activities viz. antioxidant, antimalarial, antibacterial, antidiabetic, anti-inflammatory, anti-Alzheimer's disease (Ali et al., 2013; Liu et al., 2015), and anti-cancer, etc. (Claeson et al., 2000). Vasaka plant reported to have pyrroloquinazoline alkaloids compounds such as, vasicine, vasicinone, vasicinol, adhatodine, adha-vasicinone, anisotine, deoxy vasicinone, vasicol, aniflorine, peganidine, vasicinolone, deoxypeganine, and vasicolinone (Soni et al., 2008). *Adhatoda vasica* Nees leaves showed anticonvulsant activity in pentylenetetrazol model at 200 mg/kg (Sumitha et al., 2019). Huang et al. (2020) vasicinone protects the neuronal cells by upregulating autophagy and PINK-1/parkin-mediated mitophagy in SH-SY5Y cells. Vasicine (VA) an alkaloid from AV has been documented to improve the cognitive and memory function in AD (Zhu et al., 2018). There may be other chemical constituents of AV which involved in the protection against AD.

Considering the disease complexity and currently available treatment drugs mode of action and treatment challenges, we have interested in naturally obtained leads from medicinal plants. Further, numerous reports on vasaka and its pharmacological effects as discussed above taking into consideration of vasaka plant for current study. Therefore, present study was aimed to extract and isolate the active alkaloidal metabolites i.e., pyrroloquinazoline alkaloids which may responsible for anti-Alzheimer's disease potency. To achieve promising compounds from AV, bioactivity guided fractionation approach was incorporated, as this approach was reported to have successful lead identification strategy from natural products. Further, isolated compounds were characterized and evaluated through *in-silico*, *in-vitro*, and *in-vivo* studies.

2. Materials and methods

2.1. Chemicals

Amyloid beta (A β), scopolamine, donepezil, acetylthiocholine iodide, butyrylthiocholine iodide, DTNB, AChE, and BuChE were purchased from Sigma-Aldrich, USA. DPPH (2,2-diphenylpicrylhydrazyl) was procured from TCI chemicals, India. Rat ACh ELISA kit was obtained from Krishgen biosystems, India. All other chemicals and solvents were purchased from local suppliers.

2.2. Extraction and fractionation

The shade-dried and hand-crushed powdered leaves of *Adhatoda vasica* Nees (dehydrated vasa/vasaka/Malabar nut) were purchased (Batch no. MC/ADA/2/21) from Vedic vatica private limited,

Chhattisgarh, India. A simple maceration technique was executed to get *Adhatoda vasica* Nees methanolic extract (AVME) by adding 1 kg leaves power into 3 L of methanol. After 72 h, extract was filtered and concentrated under reduced pressure (rotary evaporator). Further, AVME (100 g) was dissolved into 200 ml water and transferred into a separating funnel for solvent-solvent extraction process. These fractions were prepared using organic solvents (hexane, toluene, ethyl acetate, dichloromethane (DCM), and butanol) which are selected based on polarity index i.e., non-polar to polar organic solvents to get all nature of compounds. The obtained fractions from each solvent were concentrated and dried under reduced pressure using rotary evaporator. The resulting crude samples (semi-solid) were transferred into a petri dish and calculated the yields of each extract/fraction using following formula [Percentage yield = (weight of the plant material grams – weight of obtained crude extract or fraction in grams) / 100].

2.3. Cholinesterase inhibition assay

Cholinesterase (AChE and BuChE) inhibitory activity of AVME and fractions, were evaluated by *in-vitro* enzyme assay method (Gutti et al., 2019). In this assay, stock solutions (1 mg/ml) of AVME/fractions were prepared by dissolving samples into biological grade DMSO. Further, six different concentrations (200, 100, 50, 30, 20, and 10 μ g) were prepared using phosphate buffer 7.4. to assess IC₅₀. The test samples with different concentrations were incubated with 100 μ L of DTNB (0.0005 M) for 10 min, followed by addition of enzyme 50 μ L of AChE/BuChE (0.5 U mL⁻¹) and incubated for another 30 min. Further, 20 μ L of substrate ATCI (0.00375 M) was added for AChE test and 20 μ L of substrate BTCI was added for BuChE assay. The absorbance was measured at 415 nm, by SpectraMax M5 multi-mode microplate reader, US. In this assay, blank readings were measured using 2 μ L biological grade DMSO instead of test samples. The standard drug donepezil was served as a reference compound.

2.4. Isolation of active constituents

DCM fraction was selected for isolation of phytoconstituents on basis of cholinesterase inhibition and DPPH antioxidant activity results. The bioactive compounds were isolated using column chromatography technique. The sample was prepared by dissolving DCM fraction (10 g) into 20 ml of DCM. Followed by addition of sufficient amount of silica gel (60–120 mesh) and evaporated under reduced pressure (rotary evaporator) to get dried powder (extract bound with silica gel). Further, column was packed with silica gel (60–120 mesh) by preparing slurry in hexane; then, slurry was poured into column by tapping column to avoid column silica breaks and air gaps. The mobile phase solvents were used as hexane, ethyl acetate, dichloromethane, and methanol. The concentrated DCM fraction was loaded into column and mobile phase flow started with low polar solvent hexane (100 % hexane). Followed by increasing polarity using ethyl acetate, then dichloromethane, and finally 10 % methanol was run by gradually increasing polarity (gradient method). The obtained elutes from column were collected into 100 ml conical flasks and dried under reduced pressure (rotary evaporator). Each elutes was evaluated by thin-layer chromatography (TLC) using precoated TLC silica gel 60 F₂₅₄ and mobile phase polarity set to DCM: methanol (90:10). Resulting TLC plates were observed under the iodine chamber, UV light (254 nm), and Dragendorff's reagent solution for detection of alkaloids.

2.5. Structural characterization of constituents

The isolated compounds (VAS and VA) from DCM fraction were characterized by ATR, ¹H NMR, ¹³C NMR, HRMS, and HPLC techniques. The ATR (Bruker Alpha, Germany) fingerprints were taken and interpreted in functional groups present in the compounds. Further, Nuclear magnetic resonance (¹H, ¹³C NMR) studies were performed using Bruker

advance (Bruker Bio spin International AG, Germany), 500 MHz (^1H NMR), and 125 MHz (^{13}C NMR), in this experiment TMS served as an internal standard. Preparation of NMR samples, CDCl_3 solvent was used. Furthermore, the isolated compounds purity was estimated with help of HPLC (waters 1500 series system). The working concentration of samples (100 μg) was prepared in methanol; then, passed through a 0.45 μm membrane filter. The following HPLC conditions were maintained: Lichro CART 250–4 C_{18} column, injection volume (10 μL), mobile phase uses as methanol and water (9:1) (pH 2–3 adjusted with acetic acid), flow rate (0.8 ml/min), PDA detector 2998 (280 nm). The chromatogram data was analyzed, using waters breeze software. Further, high-resolution mass spectrometry (HRMS) studies were carried out by SCIEX, X500R QTOF, US at the Department of chemistry, Banaras Hindu University, Varanasi to estimate molecular mass of compounds.

2.6. Drug-likeness, ADME, and toxicity predictions

The drug-likeness, ADME, and toxicity properties of VAS and VA were predicted using online software tools swissADME (<http://www.swissadme.ch/>) (Daina et al., 2017) and preADMET (Seoul, 2004) (<https://preadmet.qsarhub.com/>). Drug-likeness properties of compounds were analyzed through Lipinski rule of 5. Further, ADME properties of the isolated compounds were predicted by using Swiss ADME software.

2.7. Molecular docking studies

The crystal structure of AChE co-crystallized with donepezil (PDB ID: 4EY7) (Cheung et al., 2012) and BuChE complexed with tacrine (PDB ID: 4BDS) (Nachon et al., 2013) was downloaded from the protein data bank (PDB) (<https://www.rcsb.org/>) (Berman et al., 2000). These proteins were prepared by removing unwanted chains, water molecules, and other heteroatoms. In addition, quality of protein was evaluated by analyzing the results from Ramachandran plots using precheck software (<https://saves.mbi.ucla.edu/>). Further, structure of ligands (VAS and VA) was drawn on Perkin Elmer Chem 3D 20.1.1 software and ligand energies were minimized.

After completion of above sequential steps, Auto dock 4.2 (Morris et al., 2009) software was used for further docking studies. The prepared protein structures and ligand structures were separately saved into auto dock supported format i.e., *pdbqt* file format. Further, grid box was fixed and saved with the help of reference compound donepezil/tacrine interaction with active site amino acids. Followed by docking files were created by following parameters i.e., search parameters set to genetic algorithms (GA runs set to 100 ns), docking parameters set to default parameters, and output file saved into Lamarckian GA (4.2). The docking results were analyzed under analyze window by clicking play rank by energy. Moreover, ligands have interacted with amino acids profile (2D and 3D) images were generated using BIOVIA Discovery studio visualizer (BioVIA, 2021).

2.8. Molecular dynamic simulations

The molecular dynamics studies were performed on VAS/VA using Desmond (Maestro-Desmond) software tool (Release, 2020). The binding interaction profile and stability of the docked ligands under physiological conditions, 100 ns MD simulations were carried out at AChE active site. In these studies, TIP3P was selected as a solvent model, 10 $\text{\AA}$ cubic box, and minimized the volume. Further protein energy minimization was done by 300 ns. The system was prepared by system builder and it was neutralized by adding salt 0.15 M of Na^+ and Cl^- ions. Further, molecular dynamics simulations were carried out up to 1000 frames with NTP enabled system, and temperature was set to 310 K with 1 bar pressure. The processed results were analyzed under simulation interaction diagram panel of Desmond.

2.9. DPPH antioxidant assay

2,2-diphenylpicrylhydrazyl (DPPH) assay was performed to study the antioxidant potential of AVME/fraction and isolated bioactive compounds. Various concentrations of AVME/fraction (500, 200, 100, 50, 30, 20, and 10 μg) and VAS/VA/ascorbic acid (200, 100, 50, 30, 20, and 10 μM) were made in methanol (Singh et al., 2020). Further, test samples (75 μL) of various concentrations were added to 96-well plates, and to this 75 μL of DPPH (100 μM) was added. Then, samples were incubated at 37 $^\circ\text{C}$ for 25 min. and absorbance was measured at 520 nm wavelength using a microplate reader (SpectraMax M5, US). In this study, Ascorbic acid (AA) served as a reference compound and blank readings were taken by using methanol in the place of test samples. The reducing capacity of DPPH was calculated using following equation, reducing percentage = [(absorbance of control) - (absorbance of the test)] / (absorbance of control) $\times$ 100.

2.10. Cholinesterase inhibition assay

Cholinesterase (AChE and BuChE) inhibitory activity of isolated compounds was evaluated using above described procedure (Gutti et al., 2019). Six different concentrations (0.01, 0.1, 1.0, 10, 20, and 40 μM) were prepared for each compound (VAS/VA) to assess IC_{50} . The donepezil was used as a reference compound. The absorbance was measured at 415 nm, by multi mode microplate reader (SpectraMax M5, US).

2.11. Propidium iodide displacement assay

Propidium iodide displacement assay was carried out to evaluate binding mode of test compounds at PAS site of AChE (Sharma et al., 2019; Singh et al., 2020). A solution of eeAChE 5.0 U/ml of 150 μL was incubated with or without test compounds with various concentrations of VAS (10, 20, and 30 μM), VA (30 μM), and DPZ (20 μM) for 6 h at room temperature (24 $^\circ\text{C}$). Followed by, addition of propidium iodide (final volume consists of 20 μM) and incubated for 20 min. The fluorescence intensity was measured at 535 nm (excitation) and 595 nm (emission) using a microplate reader (SpectraMax M5, US). The blank was measured using DMSO instead of test samples. The percentage inhibition results were calculated using the formula: $100 - (\text{IF}_i / \text{IF}_0 \times 100)$; where IF_i and IF_0 are fluorescence intensities with and without inhibitors respectively.

2.12. PAMPA-BBB assay

The blood-brain barrier (BBB) permeation is a prerequisite condition for the drugs acting on CNS. The BBB permeability of test compounds (VAS and VA) was tested through a parallel artificial membrane permeability assay (PAMPA) (Gutti et al., 2019; Kumar et al., 2018). Porcine brain lipid (PBL) 4 μL dissolved in dodecane (20 mg/ml of PBL in dodecane) was coated on the bottom of the porous filter disks of receptor plates. The test compounds were prepared in DMSO and further diluted with PBS buffer (pH 7.4) to get a final concentration of 25 $\mu\text{g}/\text{mL}$. Further, The donor plates (pore size 0.45 μm) were filled with 200 μL of test solution. The acceptor plate was placed on a donor plate like sandwich and incubated for 18 h at room temperature. After, test compounds absorbance was measured at 280 nm by microplate reader (SpectraMax M5, US). The concentration of 30 μM of VAS and VA was used in this study.

2.13. $\text{A}\beta$ inhibition activity

The assessment of $\text{A}\beta$ aggregation inhibition activity was evaluated through thioflavin T assay (Gutti et al., 2019). The following concentrations were used viz., 10, 20, and 30 μM for VAS, 30 μM for VA, and 20 μM for DPZ. The solution of $\text{A}\beta_{1-42}$ (10 μM) with 50 μM PBS pH 7.4 was used. The $\text{A}\beta_{1-42}$ 10 μL was incubated with or without test compounds at

37 °C for 48 h. After incubation period, 20 µM of 178 µl thioflavin T was added to previously incubated 96-well plate. The fluorescence intensities were measured at 450 nm (excitation) and 485 nm (emission) on microplate reader (SpectraMax M5, US). The Aβ aggregation assay samples were further used for confocal microscopic imaging (Carl Zeiss Microscopy GMBH, Germany) studies. After, 15 days of incubation period, all samples were mounted on glass slides using 1,4-diazabicyclo [2.2.2] octane (DABCO) as a fixing agent. Further, images were captured at 20X using fluorescence filter cube at 494 and 518 nm.

2.14. In-vitro neuroprotection assay

The neuroprotective property of VAS was evaluated against Aβ₁₋₄₂ induced neurotoxicity on neuroblastoma cell line (SH-SY5Y) (Gutti et al., 2019). The cultured SH-SY5Y cells (density 1×10^5 cells/wells) were transferred into 96 well plates. Then, the cells were incubated (24 h at 37 °C) in a humidified atmosphere under 5 % CO₂ condition. Further, cells were treated with 10 µM of Aβ₁₋₄₂ for the next 24 h. The test compound VAS (10, 20, 30 µM) cytotoxicity was evaluated against a VA (30 µM), DPZ (20 µM) was added, and cells were incubated for another 72 h. After that, 20 µl of MTT reagent was added into wells and incubated for additional 2 h. As it results, purple-coloured formazan crystals were solubilized into 100 µl of DMSO to evaluate absorbance at 570 nm, and % cell viability was calculated.

2.15. Experimental animals and study design

The male Wistar rats (220 ± 15 g weight) were obtained from the Institute of Medical Science, Banaras Hindu University, Varanasi. The animals were housed in the environmentally controlled conditions of a 12 h. day/night cycle, temperature (25 ± 2 °C), and humidity (55 ± 10 RH) at Animal House, Department of Pharmaceutical Engineering and Technology, Indian Institute of Technology (BHU), Varanasi. Animals were allowed to acclimatize for 7 days. All the experimental protocols were approved (approval no. Dean/2019/IAEC/1643) by the Institutional Animal Ethical Committee, Banaras Hindu University, Varanasi. The animal experiments are further divided into three different sets of experiments. The first set of experiments was designed to assess of acute oral toxicity of isolated compounds. The second set of experiments involved the evaluation of memory enhancement by isolated compounds against scopolamine-induced memory deficits. The third set of experiments was carried out to assess neuroprotection, cognitive, and memory enhancement activity of isolated compounds against Aβ induced AD.

2.16. Acute oral toxicity

Acute toxicity was evaluated by following OECD 423 guidelines (OECD, 2002). The acute toxicity studies were performed on female rats ($n = 6$) by administering the VAS and VA (300 mg/kg) orally. After 14 days of test compound administration, animals were killed, and organs were isolated (brain, liver, kidney, and heart). The transverse sections (10 µm) were cut using cryostat (SLEE MEV, Germany) and mounted on glass slides, and stained with hematoxylin and eosin. The slides were observed under microscope (Magnus MLX plus, India) at 10X resolution.

2.17. Scopolamine induced amnesia

The Y-maze test was conducted to assess working memory in rats. The animals were divided into seven groups and each group consists of six rats ($n = 6$) i.e., group 1 normal control, group 2 disease control, group 3 VAS (5 mg/kg p.o.), group 4 VAS (10 mg/kg p.o.), group 5 VAS (20 mg/kg p.o.), group 6 VA (20 mg/kg p.o.), group 7 DPZ (5 mg/kg p.o.). Groups 1 and 2 received 0.5 % carboxy methyl cellulose (vehicle alone) for 7 days. Groups 3, 4, and 5, were treated with VAS, and group 6 was treated with VA for up to 7 days. Group 7 received DPZ orally for 7

days. On the 7th day, after 30 min of final dose administration, scopolamine (0.5 mg/kg, i.p.) was injected into respective groups. Further, 30 min post administration of scopolamine-induced animals were tested on Y-maze apparatus for 5 min. The animals were placed at the center of the arm and allowed to explore for 5 min. When rat passes through all four paws into each arm was considered arm entry. The improvement in learning and memory is directly proportional to the increased spontaneous alterations (three consecutive arm entries). The percentage of spontaneous alternations was noted and calculated by using following formula percentage of spontaneous alternations: [number of alterations/ (total arm entries-2) × 100] (Ramakrishna et al., 2021).

2.18. Morris water maze test

The Morris water maze (MWM) test was performed to assess spatial learning and memory in rats (Fischer et al., 2008). The rats were randomly divided into five groups ($n = 6$): normal, Aβ control, VAS (20 mg/kg p.o.), VA (20 mg/kg p.o.), and DPZ (5 mg/kg p.o.). Animals were anesthetized using i.p. injection with a mixture of ketamine and xylene (dose of 90 and 9 mg/kg). Then, rats were placed into stereotaxic apparatus, ear bars were fixed and the scalp was incised and retracted. The head position was adjusted to locate the bregma and lambda region. The stereotaxic co-ordinates were set to bregma (-0.5 mm anteroposterior, +1.2 mm mediolateral, -3.2 dorsoventral and incision bar was set at -3.3 mm). Next, previously prepared Aβ₁₋₄₂ sterile saline injection (4 µM, 5 µl) was infused into all groups except sham group through a Hamilton microsyringe at a flow rate of 2 µl/min. The syringe was kept for another 5 min to prevent efflux of infused solution. In sham group, saline was infused in the place of Aβ₁₋₄₂. All the animals were kept under special care after surgery was done.

After seven days of stereotaxic injection, test drugs VAS and VA treatment was given for next seven days. Further, the spatial memory in rats were investigated using MWM test. The MWM test apparatus (62 cm height, 32 cm depth, and 121 cm diameter) was equally divided into four quadrants filled with water (25 ± 2 °C) and titanium dioxide was used to make water opaque. MWM test was divided into two trials, the first trial is a training trial, where animals find a hidden platform in one of the quadrants (5 days, 4 trials per day, intra-trial interval of about 10 min), and on the 6th day, a probe trial, where the platform was removed and animals are allowed to find the location of the platform as a spatial memory recognition (Fig. 1). The duration for both trials were conducted for 2 min.

2.19. AChE, ACh estimation, and histopathological studies

All groups of animals were euthanized, after completion of behavioral studies, and whole brain was isolated from each animal. The brain samples were homogenized with 10 mM phosphate buffer solution (pH 7.4) and centrifuged (4 °C) at 4350 g for 15 min. The obtained supernatants were separated and used for AChE estimation as per Ellman method (ELLMAN et al., 1961). Further, Acetylcholine (ACh) levels were measured using a rat acetylcholine estimation ELISA kit (Krishgen Biosystems, India). Estimation of AChE, 100 µl of supernatant was incubated with 15 mM of 100 µl ATCI (prepared in PBS buffer 7.4) for 5 min. Followed by addition of 100 µl of 1.5 mM DTNB and absorbance was measured at 415 nm. The rate of hydrolysis was calculated as µM of substrate hydrolyzed/min/mg of protein.

The whole brain of all animals was isolated for evaluating neuronal cell density in the hippocampus. The brains were washed with saline and fixed in 4 % paraformaldehyde solution at 4 °C overnight. Then, kept in 15 % of sucrose solution (in PBS) and 30 % sucrose (in PBS). Next, embedding was done in an optimum cutting temperature (OCT) solution 10 µm thick transverse sections were cut by a cryostat (SLEE MEV) and mounted on poly-L-lysine coated slides. These sections were hydrated in 95 % ethyl alcohol solution at room temperature for 5 h, rinsed in 75 %

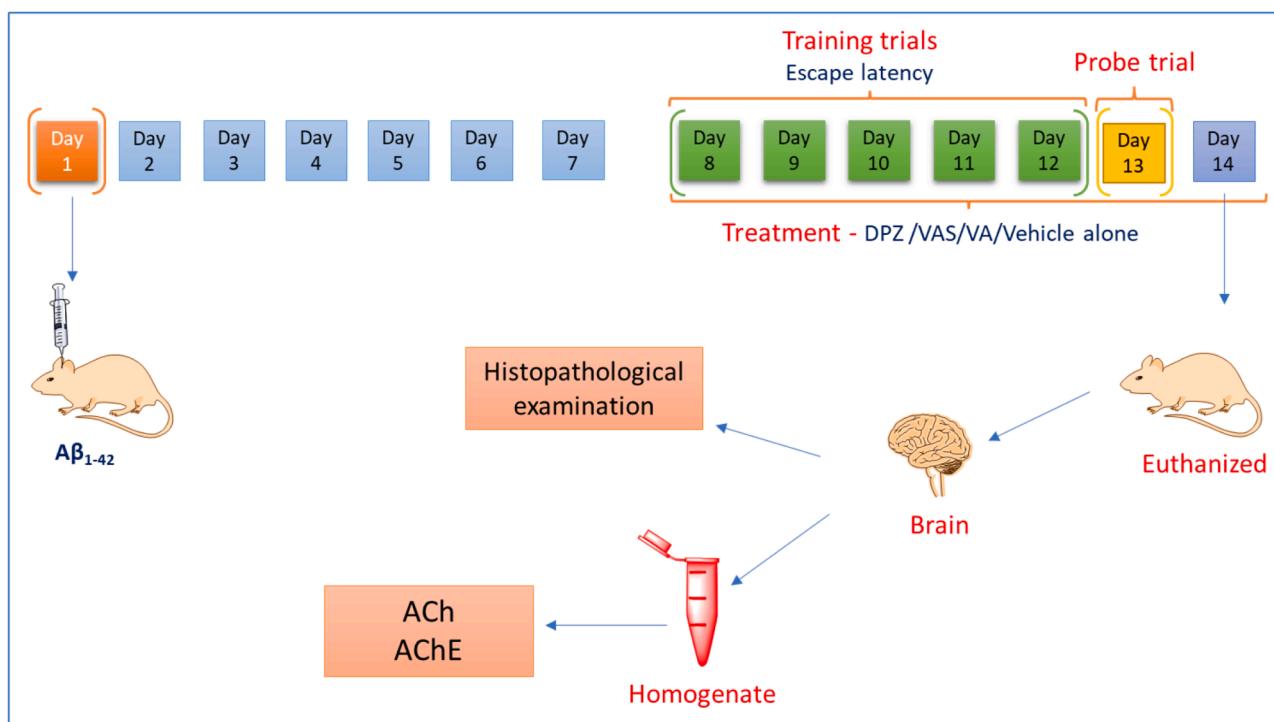


Fig. 1. The schematic representation of $A\beta$ induced Alzheimer's model study design.

of ethyl alcohol for 5 min, and then distilled water for another 5 min. After, incubated in 1 % cresyl violet acetate (warmed at 50–60 °C) for 20 min, rinsed in water for 5 min, and dehydrated at 75 % alcohol for a few seconds, 95 % ethyl alcohol for 2 min, and then absolute alcohol for 2 min. Finally, they were cleared in xylene for 2–3 min and mounted with DPX and slides were observed under a microscope (Magnus MLX plus, India) at 4X and 10X magnification. The cell density and cell count of hippocampus regions (DG, CA3, and CA1) were calculated using ImageJ software (NIH, USA) (Mishra and Thakur, 2022).

2.20. Statistical analysis

The statistical data was expressed in mean $\pm$ standard deviation (S. D). Graph pad prism 5 software was used to analyze the experimental data. All the results were analyzed by one-way ANOVA followed by Tukey's post hoc test, except escape latency in MWM, which was analyzed by two-way ANOVA followed by Bonferroni's post hoc test. A P-value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Chemical and structural properties of isolated compounds

The AVME (green, semi-solid) yield was found to be 12.35 % w/w from dry leaves of AV. The DCM fraction of AVME extraction produced high yield, i.e., 13.54 % w/w than, hexane (10.17 % w/w), butanol (9.33 % w/w), toluene (6.72 % w/w), and ethyl acetate (4.56 % w/w). In DCM fraction, two phytoconstituents were isolated using column chromatography and were identified as VAS (yield, 0.451 % w/w) and VA (yield, 4.37 % w/w). The compound VAS was eluted from fraction numbers 62–75 at mobile phase polarity of DCM: methanol (99:1). While VA was eluted from fractions 102–134 at polarity of DCM: methanol (96:4).

VAS (Fig. 2) is characterized by a solid, pale yellow color, characteristic odor, and a melting point of 199 °C. The ATR (Fig. S1): The absorption peak (broad) at 3143.65 cm^{-1} represents O-H group. The medium intensity peaks at 2977.02 and 2919.48 cm^{-1} indicate C-H and C-H₂ (aliphatic stretching groups). The strong peaks at 1665.89 cm^{-1}

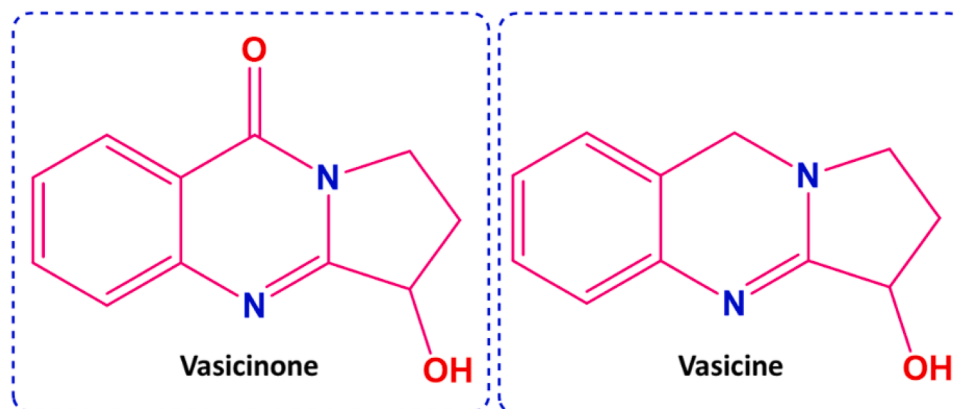


Fig. 2. The structures of pyrroloquinazoline alkaloids isolated from *Adhatoda vasica* Nees.

results C=O (carbonyl) group. The aromatic ring C=C groups were observed at 1466.33 cm^{-1} . The peak at 1466.33 cm^{-1} conforms to C-N aliphatic amine present in VAS. The wavenumber at 1389.17 and 1324.67 cm^{-1} conforms to CH_2 bending groups. The low intensity and sharp peaks at 1075.27 and 1025.37 cm^{-1} are indicates C-O group. The C=N group results in absorption at 982.88 and 893.41 cm^{-1} . The absorbance at 771.97 and 693.06 cm^{-1} medium intensity with sharp peak clears C-H groups. ^1H NMR (Fig. S2.): (500 MHz, CDCl_3) δ 8.31 (dd, $J = 6.8, 5.8\text{ Hz}$, 1H), 7.90–7.62 (m, 2H), 7.53–7.46 (m, 1H), 5.35–5.20 (m, 1H), 4.48–4.26 (m, 1H), 4.06–4.01 (m, 1H), 2.76–2.62 (m, 1H), 2.40–2.28 (m, 1H), 1.98 (s, 1H); (s= singlet, dd= doublet of doublet, m= multiplet). ^{13}C NMR (Fig. S3.): (125 MHz, CDCl_3) δ 160.6, 148.4, 142.9, 134.4, 126.9, 126.6, 126.5, 71.7, 55.8, 43.4, 29.3. The HRMS (Fig. S4.) of $[\text{M}+\text{H}]^+$ was calculated as 203.0821 and found to be 203.0797, for $\text{C}_{11}\text{H}_{11}\text{N}_2\text{O}_2$. HPLC purity of VAS was estimated as 98 % (Fig. S5).

VA (Fig. 2) was identified as dull brown color, solid, and characteristic odor, and a melting point of 209°C . The ATR (Fig. S6): The

absorption peak at 3392 cm^{-1} confirms the presence of O-H group. The highest intensity peak at 2977.41 cm^{-1} corresponds to C-H and C-H_2 (aliphatic stretching groups) to alkane groups. The wavenumbers at 1680.04 and 1630.27 cm^{-1} represents C=C alkene groups (aromatic ring). The spectra peak at 1455.50 cm^{-1} declares C-N aliphatic amine is present. The absorption at 1304.72 cm^{-1} shows C-H_2 bending groups present in VA. The spectra peak at 1108.08 cm^{-1} depicts the C-O group is present. The group C=N results absorption peak at 959.14 cm^{-1} . The observed value at 757.81 cm^{-1} indicates the C-H group is present in the ring. ^1H NMR (Fig. S7): (500 MHz, CDCl_3) δ 7.21–7.09 (m, 2H), 7.05–6.97 (m, 1H), 6.89 (d, $J = 7.5\text{ Hz}$, 1H), 4.96 (s, 1H), 4.83–4.80 (m, 1H), 4.68 – 4.55 (m, 2H), 3.48–3.42 (m, 1H), 3.30–3.25 (m, 1H), 2.45–2.36 (m, 1H), 2.16–2.08 (m, 1H); (d=doublet, dt= doublet of triplet, m= multiplet). ^{13}C NMR (Fig. S8): (125 MHz, CDCl_3) δ 163.8, 141.3, 128.5, 125.9, 124.5, 123.2, 118.7, 70.2, 48.6, 47.1, 28.7. The HRMS (Fig. S9) of $[\text{M}+\text{H}]^+$ was calculated as 189.1028 and found to be 189.1000, for $\text{C}_{11}\text{H}_{13}\text{N}_2\text{O}$. HPLC purity of VA was estimated as 99 %

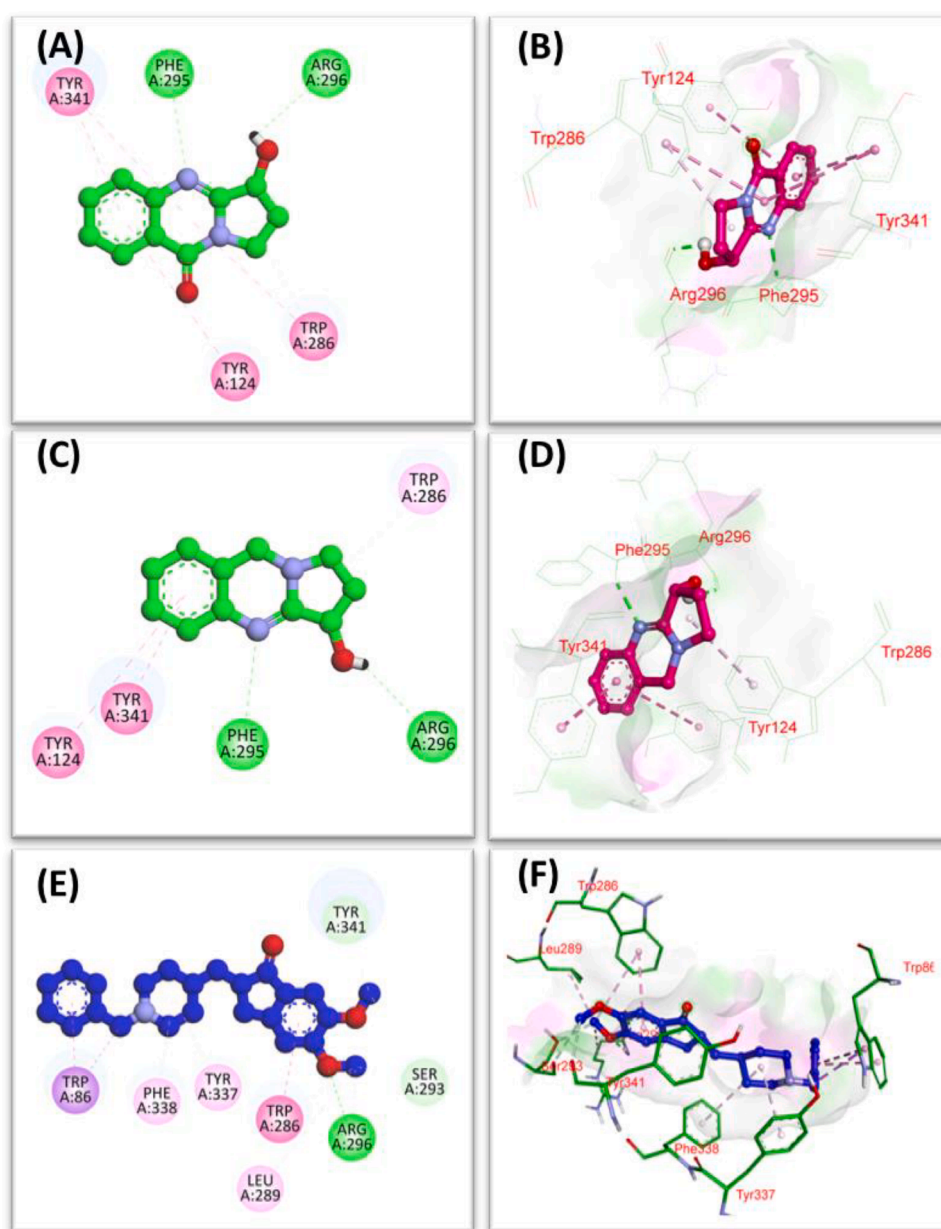


Fig. 3. The binding interactions of VAS, VA, and DPZ with AChE active site. Fig. 3A and B, depicts the interaction of VAS with AChE, Fig. 3C and D depicts the interaction of VA with AChE, and Fig. 3E and F depicts the interaction of DPZ with AChE.

(Fig. S10).

3.2. Drug-likeness, ADME, and toxicity parameters

ADME prediction of screened compounds revealed that VAS and VA are obeying Lipinski's rule of five. Further, we found that VAS and VA displayed better human intestinal absorption (HIA) and exhibits low skin permeability. The results showed, no predicted toxicity in mice and rats. The cardiotoxicity (hERG) prediction indicates that low risk against hERG channel. The obtained results were listed in supplementary information (Table S1).

3.3. VAS and VA exhibited low binding energy to AChE

The binding interaction of VAS and VA at active site of AChE was depicted in Fig. 3. The docking parameters were validated by superimposed position with its crystalized conformer donepezil at the active site of AChE (Fig. S11A). The compound VAS (Fig. 3A, 3B) and VA (Fig. 3C, 3D) formed interactions with AChE active site and showed low binding energies (Table S2). The quality of protein was evaluated through Ramachandran plot (Fig. S11B). It was observed that VAS and VA interacted with several key amino acid residues present in the active site of AChE by forming multiple chemical bonds like hydrogen bonds, π - π stacked, and π -alkyl. These interactions were similar to reference compound DPZ (Fig. 3E, 3F).

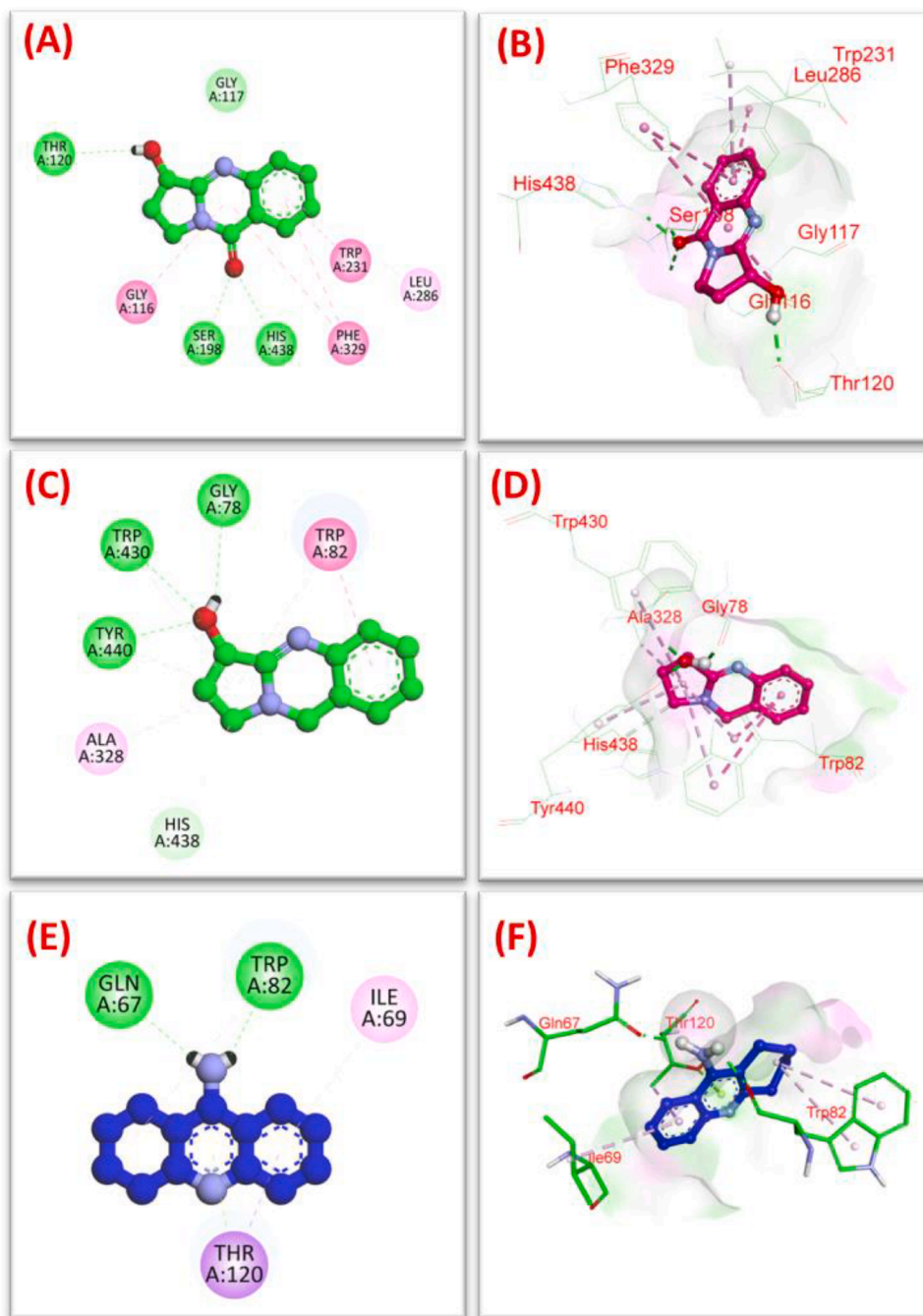


Fig. 4. The binding interactions of VAS, VA, and DPZ with BuChE active site. Fig. 4A and 4B, depicts the interaction of VAS with BuChE, Fig. 4C, and 4D depicts the interaction of VA with BuChE, and Fig. 4E and 4F depicts the interaction of tacrine with BuChE.

3.4. VAS and VA exhibited low binding energy to BuChE

The compound VAS (Fig. 4A and B) and VA (Fig. 4C and D) showed interactions with BuChE at active site and showed low binding energies (Table S2). These ligands showed similar interaction profiles with reference compound tacrine (Fig. 4E and F). We observed that VAS and VA interacted with many key amino acid residues present in the active site of BuChE by forming various chemical bonds like hydrogen bonds, π - σ , and π -alkyl.

3.5. VAS produced strong molecular interactions with AChE

The protein-ligand interactions were analyzed using following parameters, such as hydrogen bond interactions, hydrophobic interactions, ionic interactions, and water bridges formed between ligand and protein. The hydrogen bond interactions (Fig. 5A) formed between compound VAS and AChE amino acid residues are Trp286, Glu292, Ser293, Phe295, Arg296, and Phe338. The hydrogen bond can influence drug specificity, adsorption, and metabolism. The observed hydrophobic interactions (Fig. 5A) are Tyr72, Leu76, Tyr124, Trp286, Leu289, Phe297, Tyr337, Val340, and Tyr341. There no notable ionic interaction was found between AChE and VAS. The observed water bridges (Fig. 5A) are Tyr72, His287, Gln297, Glu292, Ser293, Arg296, Ser298, Phe338, and Tyr341. The ligand RMSF (Fig. 5B), protein RMSF (Fig. 5C), protein-ligand contacts (Fig. 5D), protein RMSD (Fig. 5E), ligand RMSD (Fig. 5E), and properties of ligand VAS (Fig. 5F) results were strongly suggesting that ligand VAS is most stable at AChE site.

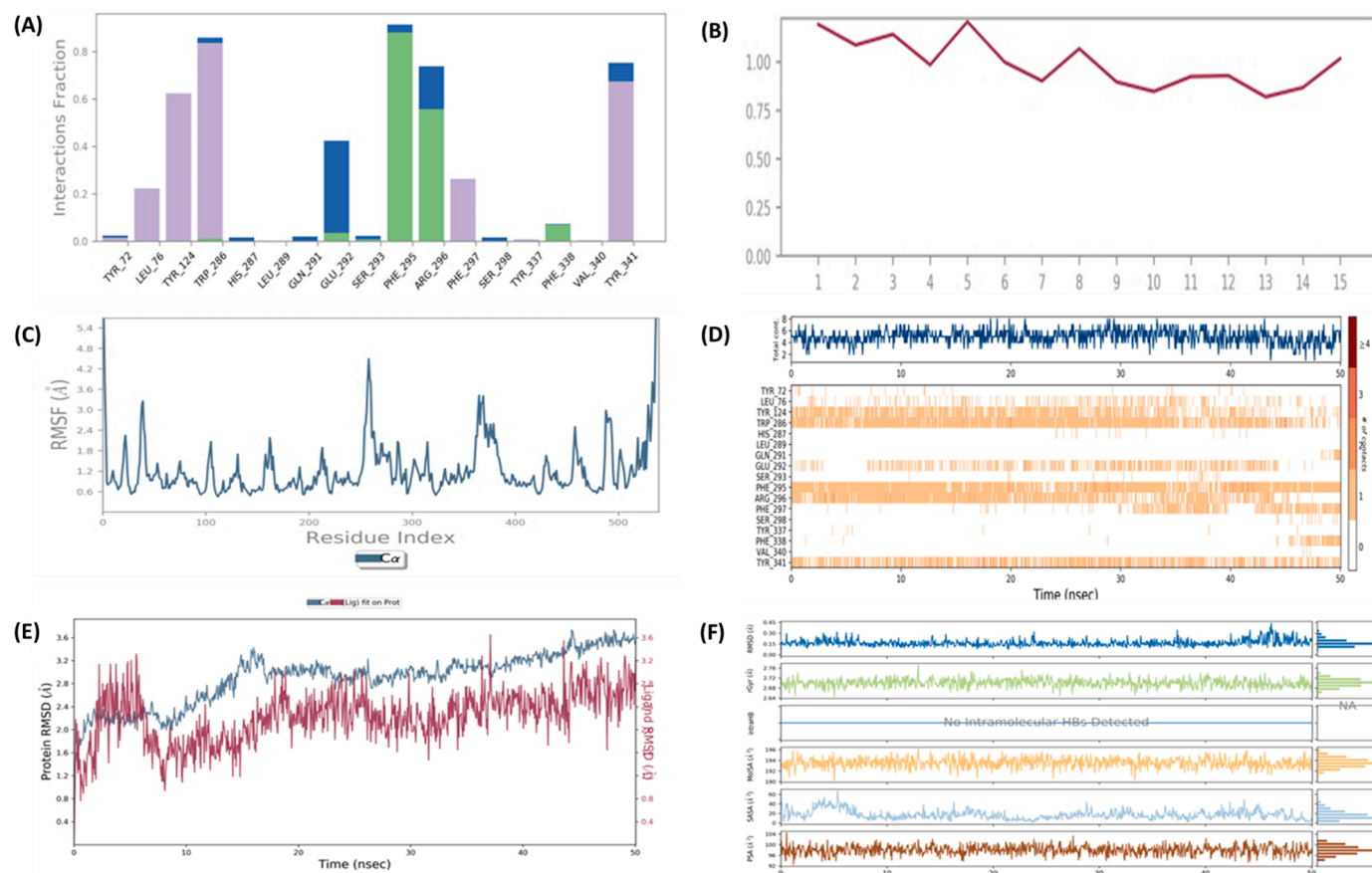


Fig. 5. VAS modulates the AChE interactions on molecular dynamic simulations, Fig. 5A showing bar charts of ligand-protein interactions throughout the simulation (Green- H-bonding; Gray - hydrophobic; Blue - water bridges), Fig. 5B depicts the ligand VAS Root mean square fluctuation (RMSF) and stability at active site, Fig. 5C showing the RMSF plot of protein AChE during simulation, Fig. 5D depicts the AChE-VAS contacts during simulation, Fig. 5E depicts the protein root mean square deviation (RMSD) (left Y-axis) and ligand RMSD (right Y-axis) indicated the stability of ligand VAS with respect to protein AChE, and Fig. 5F showing Ligand VAS properties during simulation.

3.6. VA produced strong molecular interactions with AChE

The compound VA showed stable hydrophobic interactions (Fig. 6A) with AChE active site amino acids. They are Tyr72, Leu76, Tyr124, Trp286, His287, Leu289, Glu292, Phe297, Phe338, and Tyr341 which may play important roles in substrate binding. The ligand VA formed hydrogen bond interaction (Fig. 6A) with Phe295 (100 % simulation time) and Arg296 (10 % simulation time). Water bridges (Fig. 6A) formed between ligand and Phe338 residue. There is no ionic interactions were formed between protein and ligand VA. The ligand RMSF (Fig. 6B), protein RMSF (Fig. 6C), protein-ligand contacts (Fig. 6D), protein RMSD (Fig. 6E), ligand RMSD (Fig. 6E), and properties of ligand VA (Fig. 6F) results suggested that VA have stable interaction profile in the AChE active site.

3.7. IC₅₀ concentrations of fractions, VAS, and VA

The IC₅₀ values of AV methanolic extract, fractions, VAS, and VA against AChE and BuChE, and antioxidant activities were depicted in Tables 1 and 2. The DCM fraction (AChE IC₅₀: 18.37 ± 0.53 µg; BuChE IC₅₀: 30.22 ± 0.82 µg, DPPH IC₅₀: 65.71 ± 1.29 µg) has been identified as highest cholinesterase inhibitory potency (Table 1). The IC₅₀ values of isolated compound VAS (AChE IC₅₀: 3.26 ± 0.070 µM; BuChE IC₅₀: 5.62 ± 0.083 µM, DPPH IC₅₀: 30.44 ± 0.47 µM) and VA (AChE IC₅₀: 2.97 ± 0.061 µM; BuChE IC₅₀: 5.03 ± 0.073 µM, DPPH IC₅₀: 34.12 ± 0.43 µM) showed promising results on AChE and BuChE enzymes (Table 2). These results indicate that DCM fraction containing VAS and VA exhibited significant inhibition on AChE ($P < 0.05$) and BuChE ($P < 0.05$), and

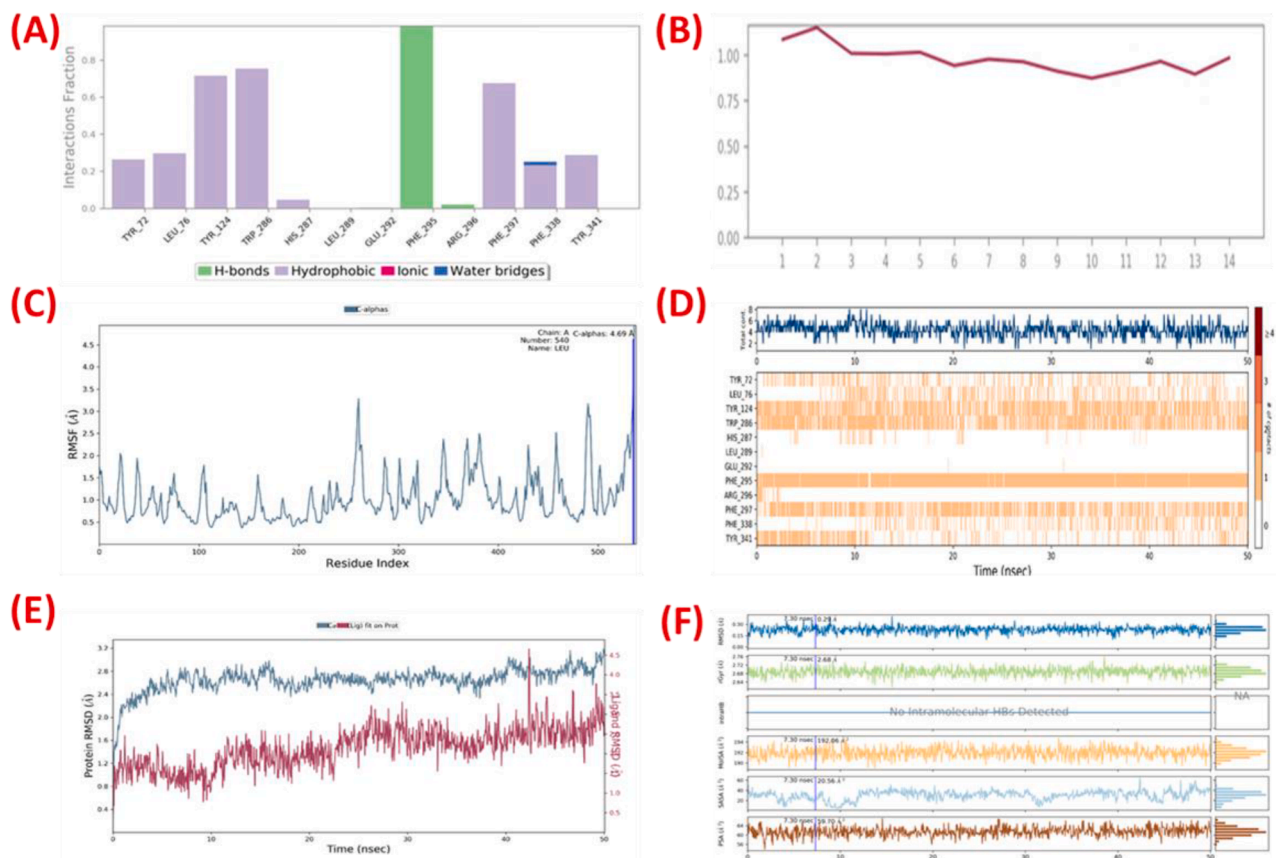


Fig. 6. VA modulates the AChE interactions on molecular dynamic simulations, Fig. 6A showing bar charts of ligand-protein interactions throughout the simulation (Green- H-bonding; Gray- hydrophobic; Blue- water bridges), Fig. 6B depicts the ligand VA Root mean square fluctuation (RMSF) and stability at active site, Fig. 6C showing the RMSF plot of protein AChE during simulation, Fig. 6D depicts the AChE-VA contacts during simulation, Fig. 6E depicts the protein root mean square deviation (RMSD) (left Y-axis) and ligand RMSD (right Y-axis) indicated the stability of ligand VA with respect to protein AChE, and Fig. 6F Showing Ligand VA properties during simulation.

Table 1

Effect of AV extract and its fractions on AChE, BuChE, and DPPH inhibitory activity.

S. No.	Extract/ Fraction	eeAChE IC ₅₀ (μg) ^a	eqBuChE IC ₅₀ (μg) ^a	DPPH IC ₅₀ (μg) ^a
1	Methanolic extract	48.35 ± 1.37	70.41 ± 1.63	100.27 ± 2.08
2	Hexane fraction	78.67 ± 1.49	91.91 ± 2.35	165.16 ± 2.64
3	Toluene fraction	54.14 ± 1.00	70.28 ± 2.06	144.31 ± 2.45
4	Ethyl acetate fraction	39.30 ± 0.86	52.29 ± 1.28	121.43 ± 2.18
5	DCM fraction	18.37 ± 0.53	30.22 ± 0.82	65.71 ± 1.29
6	Butanol fraction	36.46 ± 1.01	49.16 ± 1.34	101.58 ± 1.49

^a Data expressed in three independent experiments (mean ± SD, n = 3)

Table 2

Effect of VAS and VA on AChE, BuChE, and DPPH inhibitory activity.

S. No.	Compound	eeAChE IC ₅₀ (μM) ^a	eqBuChE IC ₅₀ (μM) ^a	DPPH IC ₅₀ (μM) ^a
1	VAS	3.26 ± 0.070	5.62 ± 0.083	30.44 ± 0.47
2	VA	2.97 ± 0.061	5.03 ± 0.073	34.12 ± 0.43
3	DPZ	0.048 ± 0.00056	0.13 ± 0.0032	nd
4	AA	nd	Nd	26.16 ± 0.38

^a Data expressed in three independent experiments (mean ± SD, n = 3). nd = not determined, DPZ = donepezil, AA = ascorbic acid.

DPPH ($P < 0.005$) assays.

3.8. VAS and VA can cross the BBB

The BBB permeation of tested compounds was predicted (Di et al., 2003) by following indications $P_e > 4.0 \times 10^{-6} \text{ cm s}^{-1}$ could cross BBB (CNS+), $P_e < 2.0 \times 10^{-6} \text{ cm s}^{-1}$ not cross the BBB (CNS-), and compounds with $2.0 \times 10^{-6} \text{ cm s}^{-1} < P_e < 4.0 \times 10^{-6} \text{ cm s}^{-1}$ uncertain BBB permeation (CNS±). The permeability of VAS and VA was found to be $5.61 \pm 0.12 \times 10^{-6} \text{ cm s}^{-1}$ and $5.21 \pm 0.11 \times 10^{-6} \text{ cm s}^{-1}$ respectively. These results suggested that compounds (VAS and VA) can cross BBB.

3.9. VAS and VA inhibited the Aβ aggregation

The inhibitory effects of VAS and VA on Aβ aggregation were depicted in Fig. 7. Compounds VAS and VA, exhibited moderate anti-aggregatory properties at a concentration of 10 and 20 μM in Aβ aggregation assay (Fig. 7). At, 30 μM concentration, compounds showed promising results and there is no significant difference with positive control DPZ (20 μM) ($P < 0.0001$). Aβ (control) samples showed large-size aggregates and treated samples showed small-size aggregates confirmed by confocal imaging studies. The confocal images were taken at VAS/VA of 30 μM concentrations, which has shown significant Aβ inhibition.

3.10. VAS and VA displaced the propidium iodide

The test compounds VAS and VA affinity towards the peripheral

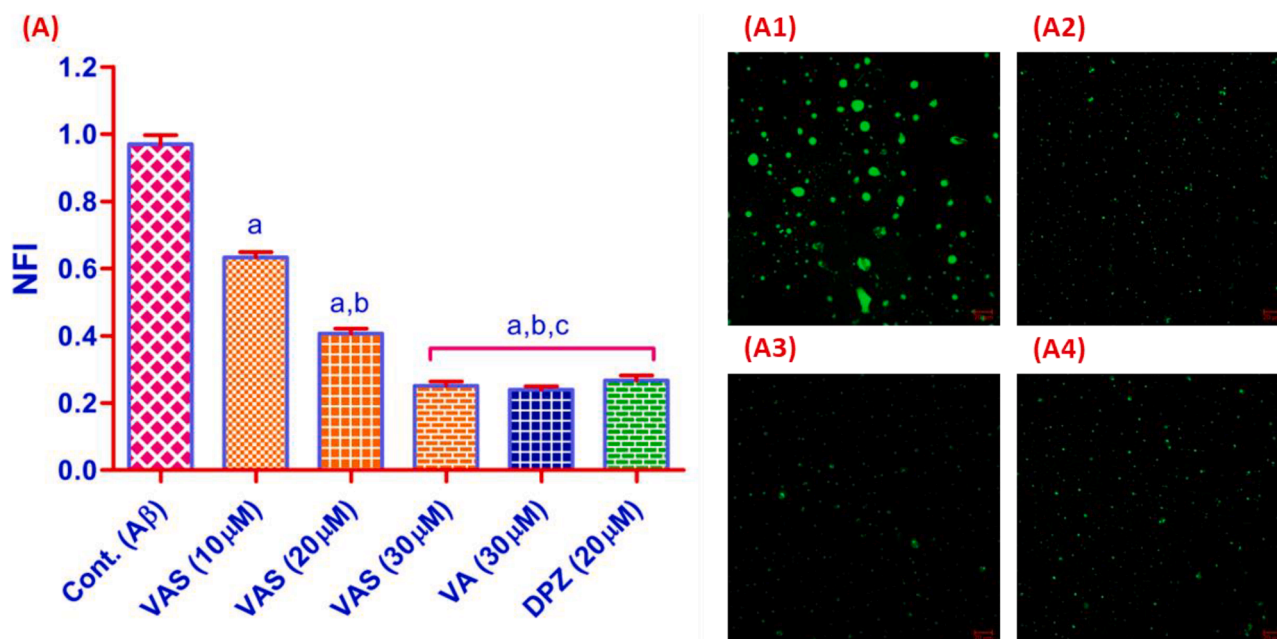


Fig. 7. Effect of VAS and VA on Aβ aggregation. Fig. 7A showing inhibitory effect of isolated compounds on Aβ aggregation, Fig. 7A1 showing effect of Aβ aggregates without isolated compounds, Fig. 7A2 showing effect of VAS (30 μM) on Aβ aggregates, Fig. 7A3 showing effect of VA (30 μM) on Aβ aggregates, and Fig. 7A4 showing effect of DPZ (20 μM) on Aβ aggregates. ^a $P < 0.05$ compared to control, ^b $P < 0.05$ compared VAS (10 μM), ^c $P < 0.05$ compared VAS (20 μM). (Mean $\pm$ SD, $n = 3$. One-way ANOVA followed by Tukey's post hoc test). NFI: nominal fluorescence intensity.

anionic site (PAS) of AChE results were presented in Fig. 8A. The propidium iodide fluorescence was decreased in the presence of VAS/VA, which suggested that compounds can be interrupted by propidium displacement from the PAS site. The obtained results of VAS and VA were significant to reference drug DPZ ($P < 0.0001$).

3.11. VAS and VA showed neuroprotection

The neuroprotective nature of VAS and VA against SH-SY5Y cells were depicted in Fig. 8B. Cell viability was increased in a dose-dependent manner by treatment with VAS and VA (Fig. 8B). The findings demonstrated that there is no significant difference between test compounds VAS/VA (30 μM) with DPZ (20 μM) in cytotoxicity profile ($P < 0.0001$).

3.12. Effect of VAS and VA on acute oral toxicity

The acute oral toxicity studies of VAS and VA were illustrated in Fig. 9. There were no notable toxicity symptoms observed i.e., death, behavior, mortality, body weight, and changes in food and water consumption. It was found that there was no significant histological damage in brain, liver, heart, and kidney up to 300 mg/kg. The organ sections were reported in 10X resolution using bright field microscope.

3.13. VAS and VA improved memory function

The changes in scopolamine-induced cognition and memory function with the treatment of VAS and VA are depicted in Fig. 10. One-way ANOVA revealed significant differences among the groups (Gutti

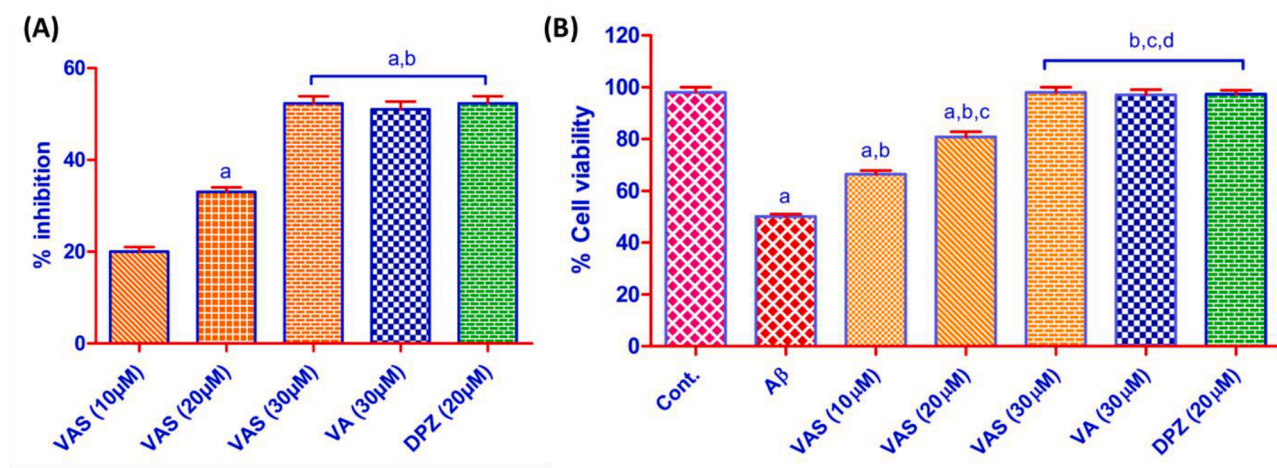


Fig. 8. Effect of VAS and VA on propidium iodide displacement assay and neuroprotection activity. Fig. 8A depicts the effect of VAS and VA on propidium iodide displacement. ^a $P < 0.05$ compared to VAS (10 μM), ^b $P < 0.05$ compared to VAS (20 μM). Fig. 8B depicts the effect of VAS and VA on SH-SY5Y cells. ^a $P < 0.05$ compared to control, ^b $P < 0.05$ compared to Aβ, ^c $P < 0.05$ compared to Aβ+VAS (10 μM), ^d $P < 0.05$ compared to Aβ+VAS (20 μM). One-way ANOVA followed by Tukey's post hoc test ($n = 3$).

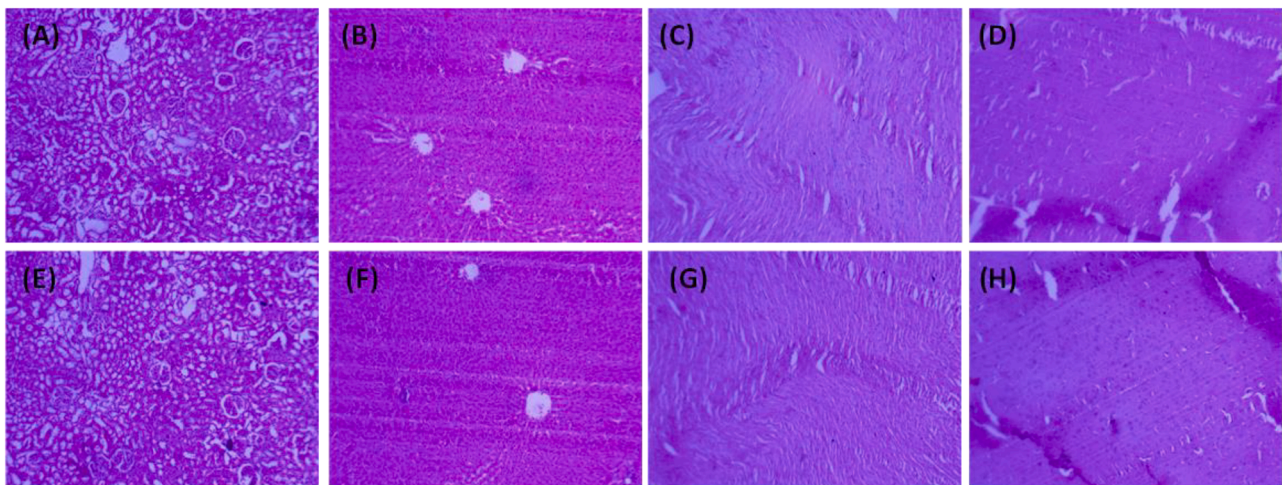


Fig. 9. Effect of VAS and VA treatment on organ toxicity. Fig. 9A–D indicates the effect of VAS treatment on histological abnormalities in kidney, liver, heart, and brain, respectively. Fig. 9E–H indicates the effect of VA treatment on histological abnormalities in kidney, liver, heart, and brain, respectively.

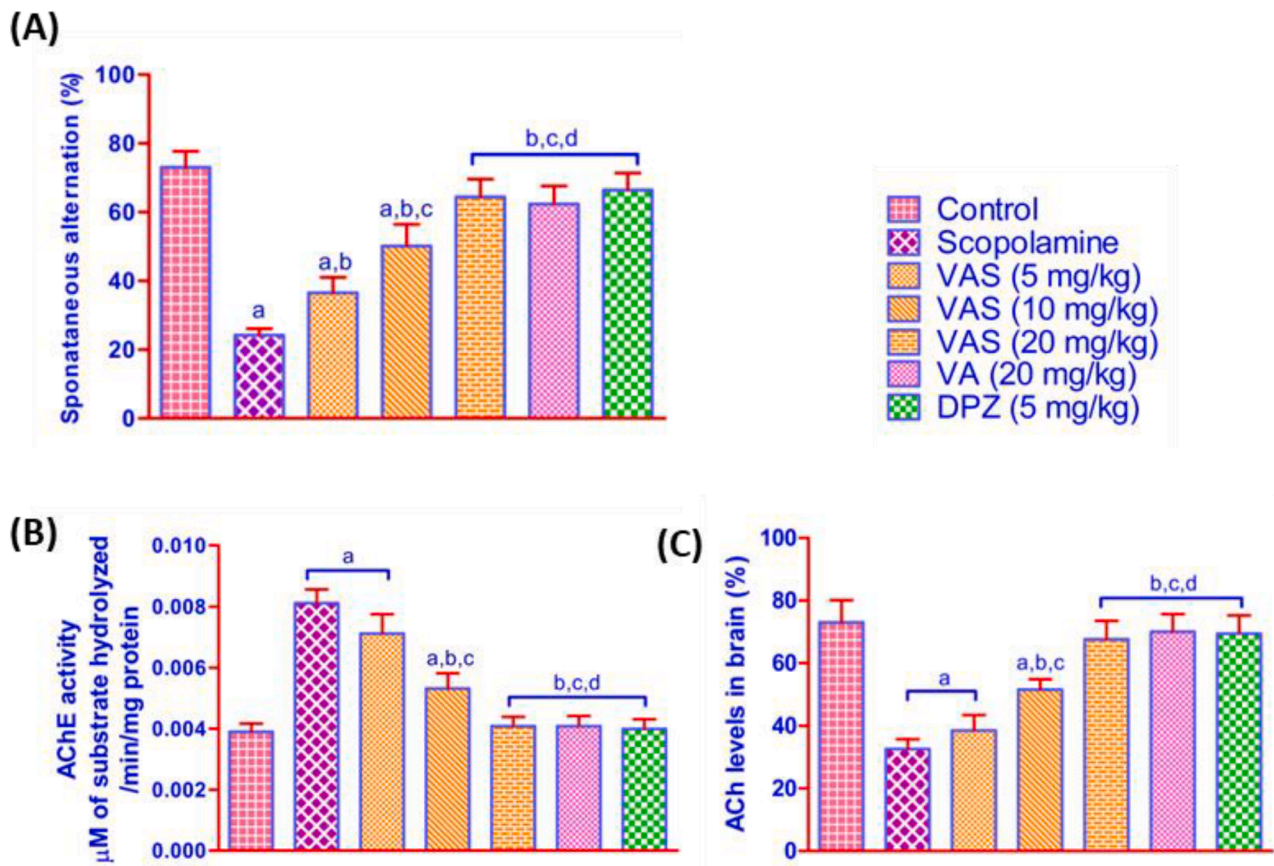


Fig. 10. Effect of VAS on scopolamine-induced cognition and memory impairment. Fig. 10A showing effect of VAS on spontaneous alternations (%), Fig. 10B showing effect of VAS on AChE activity in the brain, and Fig. 10C showing effect of VAS on ACh levels in the brain. ^a $P < 0.05$ vs. control; ^b $P < 0.05$ vs. scopolamine; ^c $P < 0.05$ vs. VAS (5 mg/kg); ^d $P < 0.05$ vs. VAS (10 mg/kg). One-way ANOVA followed by Tukey's post hoc test, $n=6$.

et al., 2019). The percentage of spontaneous alternations and ACh levels were decreased ($P < 0.0001$) and increased AChE activity ($P < 0.0001$) in the scopolamine-treated rats than control rats ($P < 0.0001$). VAS and VA treatment significantly increased the spontaneous alternations (%) ($P < 0.0001$) and ACh levels ($P < 0.0001$); and decreased AChE activities ($P < 0.0001$) than scopolamine treated groups. Further, there were no significant differences in spontaneous alternations (%) ($P < 0.0001$), AChE ($P < 0.0001$) activity, and ACh levels ($P < 0.0001$) between VAS,

VA, and DNZ treated groups. These findings indicate that VAS and VA significantly improved memory function in scopolamine-treated rats.

3.14. VAS and VA ameliorated the memory deficits by alleviating AChE activity

The changes in $A\beta$ induced memory deficits, AChE activity, and ACh levels after treatment with VAS and VA are depicted in Fig. 11. $A\beta$

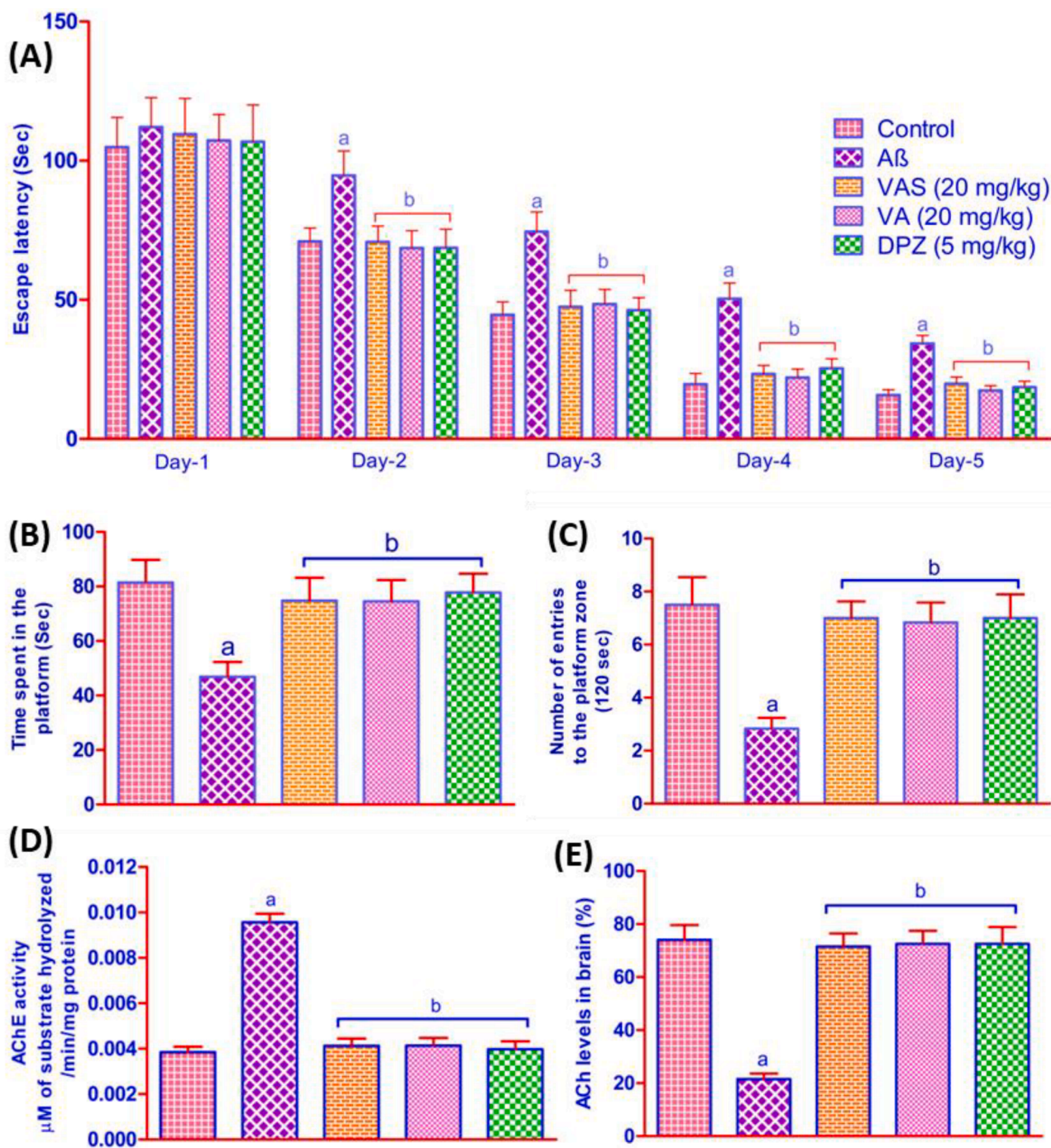


Fig. 11. Effect of VAS and VA on Aβ induced memory deficits. Fig. 11A effect of VAS on escape latency during the training trials, Fig. 11B showing animals time spent on the platform during probe trial, Fig. 11C depicts the number of entries to the platform zone, Fig. 11D showing the effect of VAS on AChE in the brain, and Fig. 11E showing the effect of VAS on ACh in the brain. ^a $P < 0.001$ compared to normal control, ^b $P < 0.05$ compared to Aβ control. One-way ANOVA followed by Tukey's post hoc test ($n = 6$).

treated rats significantly increased the escape latency ($P < 0.0001$) and AChE activity ($P < 0.0001$), and decreased time spent in the platform zone ($P < 0.0001$), number of entries into the platform zone ($P < 0.0001$), and ACh levels than control group ($P < 0.0001$). VAS and VA treated groups significantly decreased the escape latency and AChE activity ($P < 0.0001$), and increased time spent in the platform zone ($P < 0.0001$), number of entries into the platform zone ($P < 0.0001$), and ACh levels in Aβ group ($P < 0.0001$). The dose of VAS at 20 mg/kg (p.o.) showed significant effect when compared to VA (20 mg/kg, p.o.) and DPZ (5 mg/kg, p.o.) groups.

3.15. VAS and VA restored the neuronal cells

The changes in the neuronal cell density by treatment with VAS and VA against Aβ induced memory deficits in rats were illustrated in Fig. 12. Aβ group showed lower number of neuronal cells and neuronal cell density (%) in DG, CA1, and CA3 regions of hippocampus than control groups ($P < 0.0001$). VAS/VA/DPZ treated groups increased the number of neuronal cells and neuronal cell density (%) in DG, CA1, and CA3 regions of hippocampus than Aβ group ($P < 0.0001$). However, these parameters did not show any significant differences between VAS/

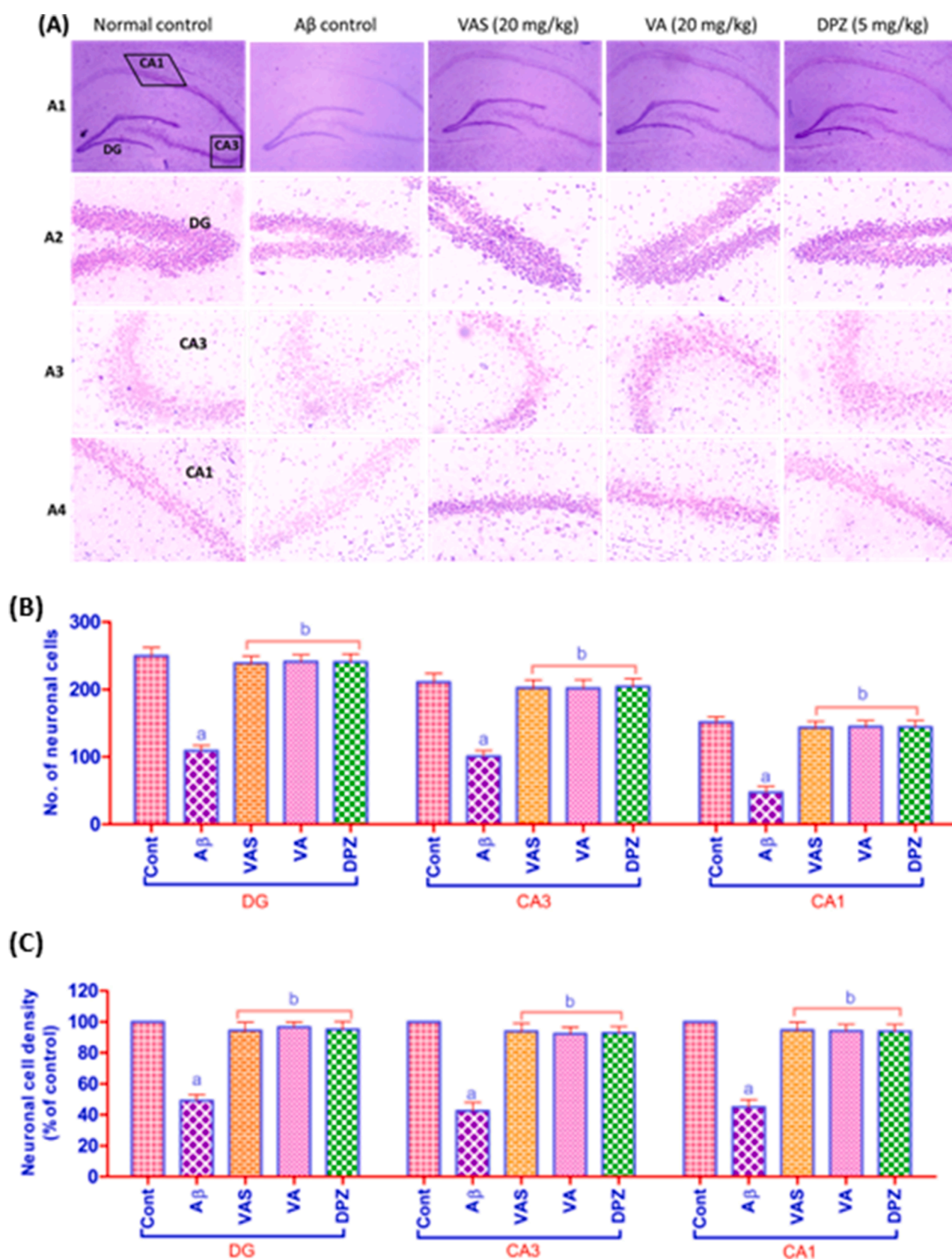


Fig. 12. Effect of VAS and VA on A β induced neurotoxicity in rats. Fig. 12A1 indicates the neuronal density at hippocampus regions. Fig. 12A2 to 12A4 indicates neuronal cell density at dentate gyrus (DG), cornu ammonis 3 (CA3), and cornu ammonis 1 (CA1). Fig. 12B showing the number of neuronal cells and Fig. 12C showing neuronal cell density (% of control). ^a $P < 0.05$ vs. normal control, ^b $P < 0.05$ vs. A β control. One-way ANOVA followed by Tukey's posthoc test ($n = 6$).

VA/DPZ groups. These findings indicate that treatment with VAS and VA significantly increased ($P < 0.0001$) the neuronal density in A β group rats.

4. Discussion

The present study summarized that a couple of pyrroloquinazoline alkaloidal compounds were isolated from DCM fraction, viz, vasicinone (VAS) and vasicine (VA). These compounds showed stable binding interactions on AChE and BuChE enzymes *in-silico* studies. Both VAS and VA alleviated the AChE, BuChE, and A β aggregation inhibition *in-vitro* studies. Further, VAS and VA improved cognition and memory by enhancing ACh levels, inhibiting AChE, and hippocampal neuronal cell density in amyloid-beta-induced cognition and memory-impaired rats. Cumulatively, the present study reports VAS as a new anti-AD agent from *Adhatoda vasica* Nees.

Different fractions (hexane, toluene, ethyl acetate, dichloromethane (DCM), and butanol) of methanolic extract of *Adhatoda vasica* Nees were prepared. These organic solvents were selected on basis of polarity to isolate all types of compounds present in the methanolic extract. Among these, DCM fraction exhibited better inhibitory activity on cholinesterase (ChEs) and DPPH antioxidant assay. Further, it was identified that DCM fraction has rich in alkaloids and might be responsible for the pharmacological actions of DCM fraction. Thereafter, active constituents VAS and VA from DCM fraction were isolated using column chromatography and characterized by using different analytical techniques such as ATR, NMR, and HRMS. Furthermore, the purity of compounds were assessed by HPLC (98 %).

The compounds VAS and VA did not showed toxicity symptoms in acute toxicity studies in rats. In earlier studies, chronic toxicity studies has been reported on *Adhatoda vasica* Nees in rats and monkeys for six months and there was no toxicity symptoms were identified (Pahwa et al., 1987). The VA was found to be safe in humans, as report of previous investigations on 24 human volunteers up to 16 mg dose was evaluated (Wakhloo et al., 1980). These studies has proven that alkaloidal compounds (pyrroloquinazolines) from vasaka are safe medications and can be used for further development.

The levels of AChE and BuChE activities increases drastically in AD condition, which causes cholinergic deficit. The cholinergic inhibitors work to reverse the overactivation of these enzyme activities thereby increasing synaptic levels of ACh. The currently approved ChE inhibitors are not effective for long-term disease modification (Long and Holtzman, 2019). Therefore, present study, the binding efficiency of VAS and VA against AChE and BuChE using *in silico* and molecular dynamics studies were investigated. The docking conformations on ChEs (AChE and BuChE) reveals that these ligands formed interaction at active sites residues and showed similar binding profile with reference compounds DPZ/Tacrine (H-bond, π - π stacked, π - σ , and π -alkyl). Further, molecular dynamics simulations were performed to assess the stability of compounds, and compounds were found to be most stable at AChE binding site. Based on *in-silico* and molecular dynamics studies it can be concluded that VAS and VA formed stable interactions with AChE and BuChE active sites. In support of these *in-silico* findings and *in-vitro* studies found that these two compounds displaced the propidium iodide at AChE PAS site with significant to DPZ, indicating that they are highly efficacious to bind at AChE PAS site. In the view of above findings, *in-vitro* antioxidant, and ChE inhibitions were evaluated. Further, *in-vitro* studies revealed that VAS and VA produced significant antioxidant activity and inhibited AChE and BuChE activities. It was also observed that a significant elevation of ACh levels indicates that compounds may be involved in amelioration of memory deficits.

A pathological hallmark of AD is the presence of A β aggregates and presence of hydrophobic amino acid residues in the A β structure which is mainly responsible for its aggregation potential (Cukalevski et al., 2012). The accumulation of aggregates stimulates neurotoxic pathways such as inflammation, calcium dysregulation, and apoptosis, which

cause the impairment of synapses and neurons. The aggregates can damage cell organelles (Singh et al., 2020). Therefore, inhibition of A β aggregation is the leading option for the treatment of AD. The present study reveals that VAS and VA inhibited the A β aggregation *in-vitro* studies suggesting that these compounds were able to decrease the A β aggregation. In support of these findings, it was also identified that VAS and VA treatment increased the cell viability (%) of SH-SY5Y cells treated with A β suggesting that VAS and VA can protect the neuronal cells which are exposed to A β . VA was previously reported to inhibit the AChE (Ali et al., 2016; Zhu et al., 2018). However, VA was not explored for its amyloid beta inhibition activity. In the present work, *in-vitro* A β studies indicate that VA can also inhibit A β aggregation suggesting that VA impedes AD pathogenesis in multiple ways.

Cognition and memory impairment are the key features of AD (Long and Holtzman, 2019). The prerequisite of anti-AD drugs is that they must be able to ameliorate cognition and memory impairments. The present existing drugs for treating AD are providing symptomatic treatment. Y-maze test is most commonly used to assess the spatial memory function in animals. In the present study, memory impairment in rats was induced by administering scopolamine (Gutti et al., 2019) through i.p. route. The percentage of alternations is considered as memory retrieval. It was found that VAS and VA significantly improved the percentage of alternations in scopolamine-treated rats, indicating that these compounds possess memory-enhancing activity. To support these findings, AChE and ACh levels were measured in scopolamine-treated rats. It was found that VAS and VA increased the levels of ACh by inhibiting AChE activity. These results suggest that VAS and VA are specifically acting on cholinesterase enzymes thereby improving the memory function by inhibiting the AChE activity. Morris water maze (MWM) is routinely used test to evaluate cognition and memory impairment in rodents (Ramakrishna et al., 2021). In A β induced Alzheimer's model, treatment with test compounds VAS, and VA significantly decreased the escape latency, indicates improvement in learning behavior. Further, VAS and VA treatment increased time spent in the platform zone and the number of entries into the platform zone in MWM test indicating the alleviation of spatial memory. During or after AD pathogenesis, hippocampal neuronal cell density is reduced (Long and Holtzman, 2019). Therefore, it is important to prevent progressive hippocampal neuronal loss. Nissl staining data reveals that VAS and VA treatment significantly increased the neuronal cell density of DG, CA3, and CA1 regions of hippocampus. Comprehensively, *in-vivo* studies found that VAS and VA from *Adhatoda vasica* Nees ameliorated the A β induced AD in rats by enhancing ACh enzyme levels and improving hippocampal neuronal cell density.

Adhatoda vasica Nees consists of several phytochemicals, among them vasicine is widely explored for its pharmacological activities. Vasicine was reported to improve cognition and memory function in scopolamine-treated rats and exhibited anti-AD effects by inhibiting the AChE and BuChE (Ali et al., 2016; Liu et al., 2015). Moreover, Ambroxol and bromhexine are developed from vasicine which is active constituent of vasaka, generally used in allergic inflammation, cough, and Parkinson's disease (PD) (Grange and Snell, 1996; Mishra et al., 2018). Vasicinone is a oxidized form of vasicine. However, vasicinone effects on central nervous system are not known. In the present study, we reports vasicinone from *Adhatoda vasica* Nees also eliciting anti-AD effects. This anti-AD effect is almost equal to vasicine. Therefore, *Adhatoda vasica* Nees plant elicits anti-AD effects could be due to phytochemicals such as vasicine and vasicinone.

These promising results from the above studies, compounds VAS and VA can act as multi-target drugs and can be used for further preclinical investigations as anti-AD agents. The currently available treatments for AD are primarily based on the cholinergic hypothesis, providing only symptomatic relief. However, multitargeted agents are likely to emerge as disease-modifying therapeutics for AD. The identified plant-based leads VAS and VA effectively inhibited cholinesterases and A β aggregates as well as neuroprotection activity. The lack of disease-modifying

therapeutics for AD, the add-on use of plant-based pharmacologically effective leads can help effectively manage the disease symptoms.

5. Conclusion

In summary, the present study identified two potent leads vasicinone (VAS) and vasicine (VA)] from DCM fraction of AVME for the management of AD, compounds were screened through *in-silico*, *in-vitro*, and *in-vivo* studies. *In-silico* studies showed effective and stable binding interactions at AChE and BuChE active sites. VAS and VA effectively inhibited the AChE, BuChE, and A β aggregation in *in-vitro* studies. Further, Binding ability at AChE active site was confirmed by propidium iodide assay. The compounds were also exhibited antioxidant potency *in-vitro* DPPH assay. Furthermore, compounds neuroprotection potency was estimated through MTT assay. Moreover, *in-vivo* studies revealed that VAS and VA enhanced working and spatial learning memory by enhancing the ACh levels and preventing hippocampal neuronal loss. The promising data from the *in-silico*, *in-vitro*, and *in-vivo* experiments led us to report that both VAS and VA are potent natural drugs for the development of disease-modifying agents in AD. The work provided new evidence on VAS which acts multi-target manner in the treatment of AD.

CRediT authorship contribution statement

Kancharla Bhanukiran: Methodology, Validation, Conceptualization, Writing – original draft. **Ravi Singh:** Conceptualization. **Gajendra T A:** Investigation, Formal analysis. **Kakarla Ramakrishna:** Writing – review & editing. **Sushil Kumar Singh:** Conceptualization. **Sairam Krishnamurthy:** Formal analysis, Validation. **Ashok Kumar:** Conceptualization. **Siva Hemalatha:** Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.phyplu.2023.100439](https://doi.org/10.1016/j.phyplu.2023.100439).

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