In-silico and In-vitro Evaluation of Acetylcholinesterase Activity of Methanolic Extract of *Vitex negundo*

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ABSTRACT

Introduction: *Vitex negundo* has a myriad of medicinal uses, such as antioxidant, analgesic and anthelminthic properties, and it is used for dysmenorrhoea. The plant contains various phytochemicals, such as flavonoids, vitamins and casticin. All components of the plant are utilised medicinally due to the minimal occurrence of adverse drug reactions, making it a potential drug target for various chronic diseases. However, the role of this plant in neurodegenerative disorders, such as Alzheimer's Disease (AD), has not yet been extensively studied.

Aim: To evaluate the in-silico and in-vitro activity of the anti-Alzheimer properties of the Methanolic Extract of *Vitex negundo* (MEVN) leaves.

Materials and Methods: The present in-silico docking study and in-vitro study were conducted in the Department of Pharmacology at Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai, Tamil Nadu, India, over a period of two months. The in-silico analysis was performed to determine the intermolecular interactions between the ligand and protein of the top five scored molecules using PyMol software. To assess the Acetylcholinesterase (AChE) inhibitory property, the findings were presented as docking scores. Cell viability and cytotoxicity were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, with results expressed as percentages for various concentrations: 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL, and 100 µg/mL of MEVN.

Results: The MEVN exhibited competitive inhibition of the AChE enzyme. There were active interactions between Agnuside, Isochlorogenic Acid B, Isochlorogenic Acid C, Kaempferol-3-O-rutinoside, and Quercetin found in the MEVN with AChE. The percentage of cell viability for the concentrations of 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL, and 100 µg/mL, as determined by the MTT assay, was 98.62%, 98.86%, 97.19%, 96.66%, 91.86%, and 78.82%, respectively. The results indicated that MEVN does not exhibit any toxicity.

Conclusion: The findings demonstrated that MEVN possesses AChE inhibitory properties and maintains cell viability, both of which are indicative of anti-Alzheimer activity. Therefore, MEVN can be further evaluated for its potential in preventing cell cytotoxicity and treating AD.

Keywords: Alzheimer's disease, Cell cytotoxicity, Cell viability, Docking study, Vero cell line

INTRODUCTION

The term "Ethnobotany" defines the relationship between plants and people. Most traditional medicines used in healthcare are derived from plant sources, which have further evolved in modern times [1]. Semisynthetic and synthetic derivatives of plant sources are used as first-line drugs for many existing chronic diseases worldwide, as they are safer and have a minimal occurrence of adverse drug events. However, these drugs must be selected with adequate scientific evidence of their remedial potential and must be standardised with proper research and validation techniques [2]. A study conducted by Purohit S et al., states that there are more than 700,000 species of plants that have been used as herbal medicines so far [3].

Vitex negundo Linn (Verbenaceae), commonly known as the Chinese chaste tree, is a woody, aromatic shrub that can grow into a small tree. It is found widely in regions of Afghanistan, India, Pakistan, Sri Lanka, Thailand, Malaysia, Madagascar, and Africa. In traditional medicine, various parts of this plant have been used by people as an anthelminthic and have been applied as a poultice to cure headaches and sinusitis [4]. It has significant use in treating dysmenorrhoea in Ayurveda [5]. Anti-inflammatory medications like Ibuprofen and Phenylbutazone; analgesics like Meperidine, Aspirin, Morphine, and Pethidine; sedative-hypnotic medications like Pentobarbital, Diazepam, and Chlorpromazine; and anti-convulsive medications like Diphenylhydantoin and Valproic acid have all been enhanced by the administration of this plant's extract [6]. Some of the phytochemicals found in *Vitex negundo* leaves include several flavonoids, oleanolic acid, casticin, and vitamin C. The seeds, roots, flowers, and fruits are rich in sitosterol, hexadecenoic acid, and several flavonoids [7].

Various studies analysing the phytochemical constituents of this plant have revealed its anti-inflammatory, analgesic, antioxidant, and enzyme inhibitory properties. The phytochemical components of Vitex negundo have been found to act individually, synergistically, or additively in the improvement of several medical ailments [8,9]. This paves the way for further research regarding its role in degenerative neurological disorders such as AD, which have not been studied extensively so far [10,11].

There may be abnormal cholinergic innervation in the early stages of AD. This degeneration particularly affects cholinergic neurons. Acetylcholinesterase (AChE) inhibitors or agonists of nicotinic and muscarinic receptors can enhance cholinergic function [12]. Since it has been identified that the peripheral anionic site is involved in AChE as an allosteric regulator, the inhibition of AChE has become increasingly important. Patients with AD are treated using these approaches [13]. Enzyme inhibitor therapy can prolong a patient's life, but it is purely symptomatic; that is, it delays the onset of symptoms and is not a complete cure [14].

In-vitro tests are useful for guiding the fractionation of plant extracts through chromatographic separation techniques. Hence, they can be effectively used to assess the various properties of plant extracts and the properties of their phytochemicals [15]. The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) can be used to check the metabolic activity of a cell, estimate the count of viable cells, and research cell cytotoxicity [16]. This method can be effectively applied in research on neurodegenerative disorders

such as Alzheimer's disease. The present study was conducted to evaluate the role of MEVN in Alzheimer's disease.

MATERIALS AND METHODS

The present in-silico and in-vitro study was carried out in the Department of Pharmacology at Sri Ramachandra Institute of Higher Education and Research in Porur, Chennai, Tamil Nadu, India, from March 2024 to April 2024. The study was approved by the Institutional Ethical Committee (CSP-MED/24/FEB/98/46).

Procurement and authentication of the plant: The plant was collected and authenticated by the Xavier Research Foundation at St. Xavier's College, Palayamkottai-627002, Tamil Nadu, India. The registration number of the certificate is XCH-40596.

Study Procedure

Molecular Docking Study: A study conducted by Anusha D et al., revealed a comprehensive search of all possible studies on *Vitex negundo* [17]. Based on this, a total of five ligands with AChE inhibitory attributes were selected, and the molecular structures of the reported compounds were extracted from the PubChem database. The three-dimensional crystal structure of human AChE (ID: 4EY6) was retrieved from the Protein Data Bank [18]. After the compounds were selected, Molinspiration was utilised to identify the basic physical characteristics of the selected compounds, such as the number of hydrogen bond acceptors and donors, log P, and molecular weight. The shortlisted compounds were then chosen for further study and analysis. Following the preparation of the ligands and proteins, docking was carried out using the AutoDock Vina software programme, which is based on a novel hybrid search algorithm called guided differential evolution [19]. The guided differential evolution algorithm combines the cavity prediction method with the differential evolution optimisation approach. The predicted cavities used in the search process facilitate rapid and accurate binding identification. The PyMOL software was used to analyse the intermolecular interactions between the ligands and proteins of the top five scored molecules [20].

Determination of AChE Inhibition in the Samples:

Materials: Acetylthiocholine iodide (ATCI), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and electric eel AChE were purchased from Sigma Aldrich Chemicals Co., USA.

Preparation of MEVN: The *Vitex negundo* extract was prepared using a Soxhlet apparatus, using 20 g of dried *Vitex negundo* leaf powder in 250 mL of methanol for 12 hours. The prepared extract was then dried and used for enzyme inhibition assays.

AChE Enzyme Activity and Inhibition Analysis: The AChE esterase activity assay mixture comprised 5 µg/mL enzyme preparations, 1 mM ATCI, 2 mM DTNB, and 100 mM Potassium Phosphate (KPO_4) buffer (pH 7.0) in a total volume of 1000 µL with distilled water. The reaction mixture was incubated at 37°C for 10 minutes, and the yellow colour that developed was detected at 412 nm using UV-visible spectroscopy [21].

The enzyme solution in the buffer was incubated at room temperature for 30 minutes with varying concentrations (50 µg/mL to 250 µg/mL) of MEVN for the enzyme inhibition assay. Following incubation, the activity of the AChE enzyme was determined.

Kinetic Analysis of AChE Inhibition: This study aimed to ascertain the kinetics of AChE enzyme inhibition at various concentrations of ATCI (0.5 mM to 1.00 mM), both in the absence and presence of MEVN. The inhibitory constant (Ki) was determined using the Dixon graph, and the type of inhibition was determined using the Lineweaver-Burk (LB) plot [22,23].

Determination of Cytotoxicity Using the MTT Assay: The MTT assay is a colorimetric method used to measure cytotoxicity and cell proliferation. It works by reducing the yellow-coloured, water-soluble tetrazolium dye MTT to formazan crystals. The insoluble formazan crystals are produced by mitochondrial lactate dehydrogenase, which is generated by living cells and gives rise to a purple colour when dissolved in Dimethylsulfoxide (DMSO) solvent at room temperature. This colour change is proportional to the number of viable cells and can be measured spectrophotometrically at 570 nm. Using Vero cell lines, this assay is performed for both the test sample and the standard, in accordance with the procedures used by Alley MC et al., and Mosmann T [24,25]. The Vero cell line (African Green Monkey Kidney) is subcultured every two days in Dulbecco's Modified Eagle Medium (DMEM) high glucose media supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic-antimycotic solution. The cells are maintained in an atmosphere of 5% CO_2 and 18-20% O_2 at a temperature of 37°C in a CO_2 incubator [26].

The materials required for the assay are:

- Cell lines: Vero- African green monkey kidney cell lines
- Cell culture medium: DMEM- High Glucose
- A pipettor and adjustable multichannel pipettes
- Fetal Bovine Serum
- MTT Reagent (5 milligrams/ml)
- DMSO solvent
- Dulbecco's Phosphate-buffered Saline (D-PBS)
- Doxorubicin
- A 96-well plate for cell culture
- T25 flask for cell culture
- 50 mL centrifuge tubes
- 1.5 mL centrifuge tubes
- 10 mL serological pipettes
- 10 to 1000 µL tips

The equipment required for the assay procedure includes the following:

- Centrifuge
- 100-1000 µL, 10-100 µL, and 2-10 µL pipettes.
- Inverted binocular biological microscope
- Biosafety hood
- An incubator set at 37°C with a 5% CO2 humidified atmosphere.
- 96 well plate reader

The assay controls that were used include the following:

- Medium control (medium devoid of cells)
- Negative control (medium with cells but devoid of the experimental drug/compound)
- Positive control (medium with cells treated with Triton-X-100 act as positive control for cell cytotoxicity)

Cell viability and cytotoxicity were estimated using the in-vitro MTT assay and expressed as percentages for various concentrations of MEVN, including 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL, and 100 µg/mL.

RESULTS

In-silico study-molecular docking analysis: In this study, 27 constituents previously identified in the leaf extract of *Vitex negundo* [27] were selected for molecular docking analysis to evaluate their binding affinity to the enzymatic protein AChE. The binding energies for the selected constituents were determined, as shown in [Table/Fig-1]. Of all the constituents tested, five compounds-namely, Agnuside, Isochlorogenic Acid B, Isochlorogenic Acid C, Kaempferol-3-O-Rutinoside, and Quercetin-exhibited highly negative binding energies,

with Agnuside exhibiting the most negative binding energy corresponding to active interaction with AChE. The intermolecular interactions contributing to the affinity between these five compounds and the amino acid residues of AChE were ascertained as shown in [Table/Fig-2a-f,3].

Ligand	Binding energy (kcal/mol)
Agnuside	-11.0
Apigenin-7-glucoside	-9.8
Apigenin	-9.5
Aucubin	-8.5
Caffeic-acid	-7.1
Casticin	-9.0
Chlorogenic acid	-9.1
Cryptochlorogenin acid	-9.3
Flavosativaside	-7.4
Hyperoside	-7.7
Isochlorogenic acid A	-9.5
Isochlorogenic acid B	-10.4
Isochlorogenic acid C	-9.9
Isoschaftoside	-8.3
Isovitexin	-9.5
Kaempferol	-8.1
Kaempferol 3 Orutinoside	-10.3
Luteolin	-7.4
Luteoloside	-9.6
Neochlorogenic acid	-9.5
p-Hydroxybenzoic acid	-6.3
Protocatechualdehyde	-6.1
Protocatechuic acid	-6.4
Quercetin	-9.9
Schaftoside	-8.3
Vitexin 2" rhamnoside	-8.4
Vitexin	-7.4

[Table/Fig-1]: List of ligands with binding energy for the protein AChE.

In-vitro study:

AChE Enzyme Activity and Inhibition Analysis: In the AChE inhibition analysis, MEVN showed a linear dose-dependent increase in inhibitory activity against AChE, as shown in [Table/Fig-4], with the highest dose (250 µg/mL) demonstrating a maximal inhibitory percentage of 88%. The half-maximal Inhibitory Concentration (IC50) value of MEVN against AChE was determined to be 143.33 µg/mL.

Kinetic analysis of AChE inhibition:

Lineweaver-Burk (LB) plot: The LB plot showed a constant $1/V_{max}$ (Y-axis intercept) and decreasing $1/K_m$ (X-axis intercept) values for increasing concentrations of MEVN, ranging from 0 to 200 µg/mL. This indicates that V_{max} remained unchanged while K_m increased with MEVN, thus denoting a competitive type of inhibition of AChE by MEVN, as shown in [Table/Fig-5].

Dixon plot: The Dixon plot indicated the inhibition constant value (Ki) of MEVN against AChE to be 52.26 µg/mL, as shown in [Table/Fig-6].

Effect on cell cytotoxicity using MTT assay: In the MTT assay, no cytotoxicity was observed in the Vero cell line when treated with various concentrations of MEVN, as reflected by high percentages of cell viability. The concentration of 75 µg/mL demonstrated 91.86% cell viability, as shown in [Table/Fig-7]. Hence, the findings of the MTT assay suggest that MEVN is non toxic.

DISCUSSION

The plant *Vitex negundo* possesses a broad spectrum of therapeutic characteristics and has been utilised for a variety of medical disorders. It can be further studied for various chronic diseases, according to an analysis of the plant's diverse phytochemical components [28].
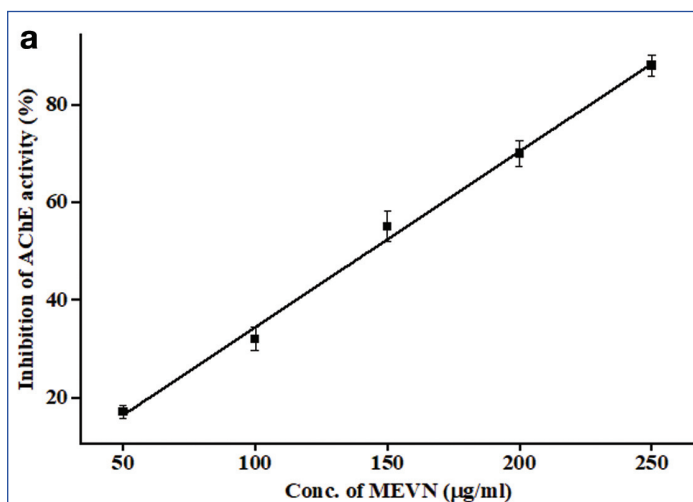
The leaves of this plant are found to have antibacterial, antioxidant, astringent, antipyretic, sedative, tonic, and vermifuge properties. According to a study by Tiwari OP and Tripathi YB, the juice from the leaves can be used to treat sinusitis and scrofulous sores [29], while the oil derived from the leaf extract can be used to eliminate worms and foetid discharges from both acute and chronic ulcers. A study by Chattopadhyay P et al., suggests that leaf decoction may enhance vision [30]. Additionally, a crushed leaf poultice is used to treat sinusitis, tubercular neck swellings,
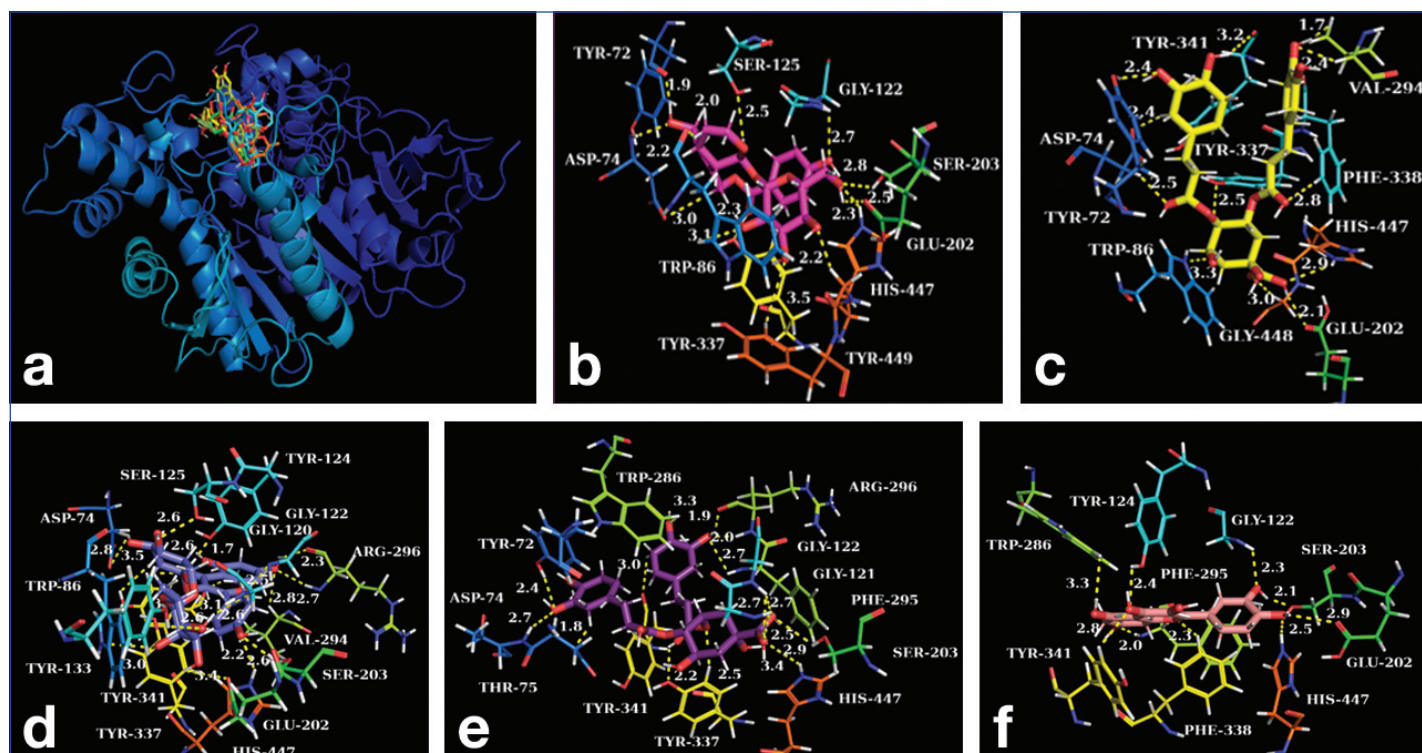
[Table/Fig-2]: (a) Superimposed ribbon structure of AChE showing the orientation of the ligands in the active site. Intermolecular interactions between agnuside (b), isochlorogenic acid-B (c), kaempferol-3-O-rutinoside (d), isochlorogenic acid-C (e) and quercetin (f) and AChE in the docking.

Protein	Interacting amino acid residues (distance Å)	
	Hydrogen bond	Hydrophobic interaction
Agnuside	Tyr72 (1.9), Asp74 (2.2), Trp86 (3.0), Gly122 (2.7), Ser125 (2.5), Glu202 (2.5), Ser203 (2.5), His447 (2.3), Tyr449 (2.2)	Trp86 (3.7)
Isochlorogenic acid B	Try72 (2.4), Asp74 (2.4), Trp86 (3.3), Glu202 (2.1), Val294 (1.7), Tyr337 (2.5), Phe338 (2.8), His447 (2.9), Gly448 (3.0)	Trp86 (5.1), Val294 (5.0), Tyr337 (5.2) Tyr341 (4.6),
kaempferol-3-O-rutinoside	Asp74 (2.8), Trp86 (3.0), Gly120 (2.6), Tyr124 (1.7), Gly122 (2.5), Tyr133 (2.6), Tyr136 (2.6), Glu202 (3.4), Ser203 (2.6), Val294 (2.8), Arg296 (2.3), Tyr337 (3.1), Tyr341 (3.5), His447 (2.2)	Trp86 (4.2), Tyr124 (5.7), Tyr133 (5.2)
Isochlorogenic acid C	Tyr72 (2.4), Asp74 (2.7), Thr75 (1.8), Gly121 (2.4), Gly122 (2.7), Ser203 (2.5), Trp286 (3.3), Phe295 (2.7), Arg296 (1.9), Tyr337 (2.2), Tyr341 (3.0), His447 (3.4)	Tyr72 (4.9), Tyr124 (5.4), Trp286 (4.3), Tyr341 (5.1)
Quercetin	Gly122 (2.3), Tyr124 (2.4), Glu202 (2.9), Ser203 (2.1), Trp286 (3.3), Phe295 (2.0), Phe338 (2.3), His447 (2.5)	Tyr124 (5.5), Trp286 (5.2), Tyr337 (5.5), Phe338 (5.4), Tyr341 (5.0)

[Table/Fig-3]: Details of interaction between agnuside, isochlorogenic-acid-B, isochlorogenic-acid-C, kaempferol-3-O-rutinoside and quercetin with AChE.
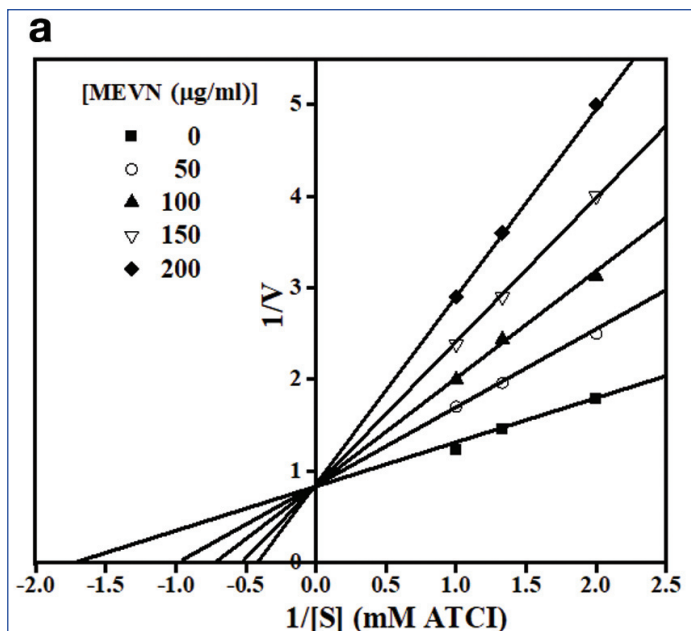

1/S	0	50	100	150	200
	1/V	1/V	1/V	1/V	1/V
2	1.79	2.5	3.125	4	5
1.33	1.45	1.96	2.44	2.9	3.6
1	1.23	1.7	2	2.38	2.9

[Table/Fig-5]: a) and b) Lineweaver Burk plot showing type of inhibition.

[Table/Fig-4]: a) and b) Inhibition of AChE by MEVN.

MEVN:	
Concentration (µg/mL)	Inhibition (%)
50	17
100	32
150	55
200	70
250	88

Sample	IC50 (µg/mL)	Type of inhibition	Inhibitory constant (Ki) (µg/mL)
Vitex negundo {Methanolic extract of Vitex negundo (MEVN)}	143.33	Competitive	52.26

[Table/Fig-6]: a) and b) Dixon plot of AChE inhibition by MEVN Showing Ki value.

headaches, and neck gland sores, as reported by Lemire J et al., [31]. The anti-inflammatory activities of *Vitex negundo* leaf extracts have been demonstrated by Venkateswarlu K and Jana et al., in both acute and subacute inflammation, which is thought to be due to the inhibition of prostaglandin synthesis [32]. Ullah Z et al., evaluated the antioxidant and therapeutic potential of *Vitex negundo* flavonoids in modulating solenoid-induced cataracts and found it to be effective [33]. In another study, it was found that *Vitex negundo* potentiated the activity of the anticonvulsant drug valproic acid, although its stand-alone activity was not found to be equivalent to that of standard anticonvulsant drugs [34]. Furthermore, *Vitex negundo* leaf extracts are associated with anti-hyperglycaemic activity [35].
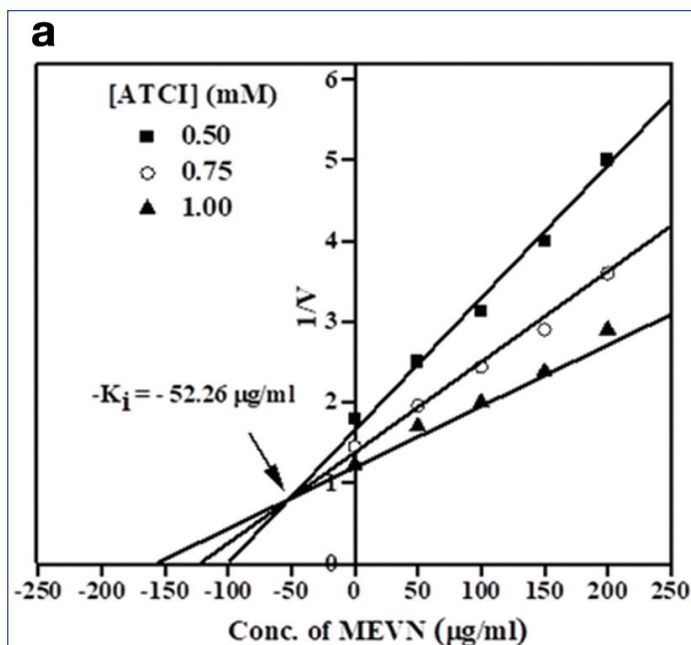
All the properties of this plant and its phytochemicals pave the way for further research on its medicinal uses regarding neurodegenerative disorders such as AD, which has not yet been extensively explored.

The present study evaluates the enzyme kinetics and enzyme inhibitory activity of MEVN on AChE using in-silico studies. It is further evaluated for its cytotoxic potential through in-vitro studies using the MTT assay.

The in-silico interaction of AChE with the target ligands of MEVN inhibition is required for the anti-Alzheimer potential demonstrated by molecular docking in the present study. This study evaluated the speed and accuracy of docking, utilising efficient optimisation and multithreading of complexes with pharmacologically

[Table/Fig-7]: a) Effect of MEVN on Vero cell viability; b) Representative bright field microscopy image showing the morphology of Vero cells from control and various concentrations of MEVN treated groups; c) The cell viability using the MTT assay.
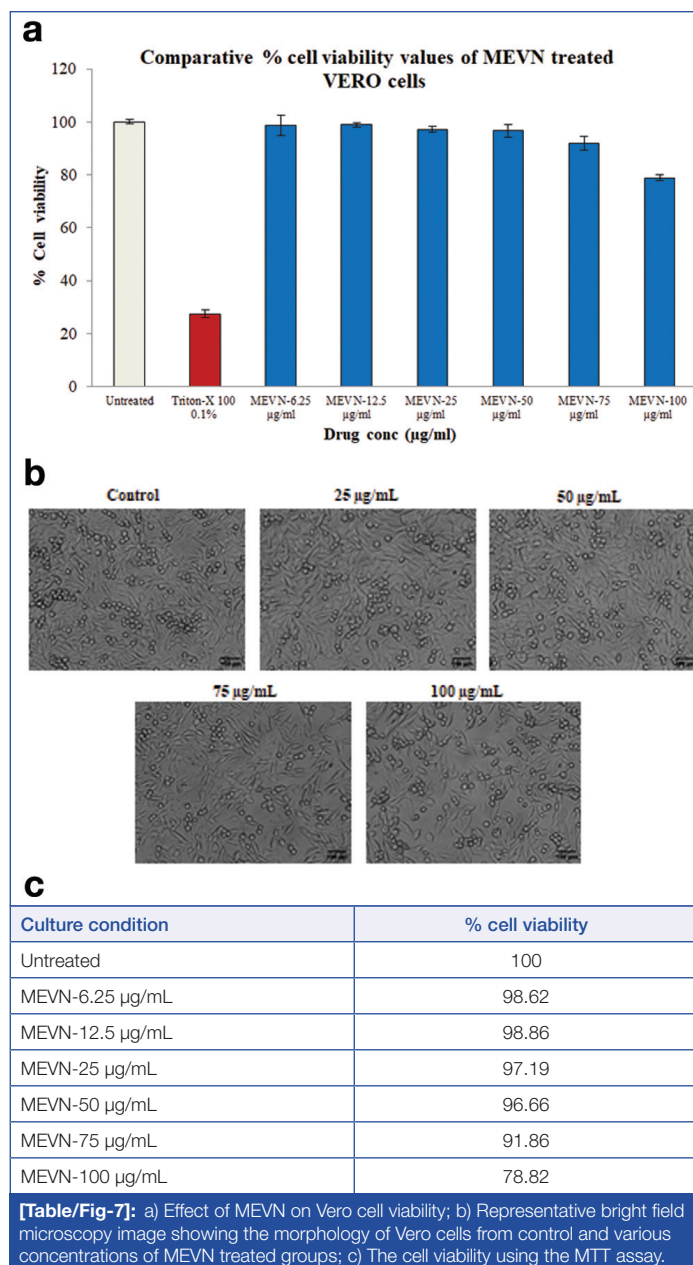
important ligands. Positive results were found for the interaction between Agnuside, Isochlorogenic acid B, Isochlorogenic acid C, Kaempferol-3-O-rutinoside, and Quercetin from MEVN with AChE [36]. It was found that targeting multiple proteins, rather than a single protein, has enhanced AChE inhibitory activity. In line with this, the interaction of AChE with multiple proteins targeted to inhibit its activity would be advantageous [37]. The phytochemicals of the *Vitex negundo* plant have been found to possess anti-inflammatory, antioxidant, sedative, and hypoglycaemic properties against various conditions in both in-vitro and in-silico analyses [38].

In addition, the AChE inhibitory activity demonstrated in this study was elicited using the AChE inhibition assay, which showed concentration-dependent inhibition of AChE, with maximum inhibition at a concentration of 200 μg/mL. The Lineweaver-Burk plot is indicative of effective enzyme kinetics, and the Dixon plot was used to determine the inhibitory constant. This provides conclusive evidence of effective AChE inhibition using the in-vitro assay. Further understanding of the effect of AChE inhibition through additional in-vitro studies of MEVN is warranted to fully understand its therapeutic effect against neurodegenerative disorders such as AD.

The in-vitro analysis of MEVN further revealed that it is non toxic, with cell viability increasing in a concentration-dependent

manner in Vero cell lines treated with increasing concentrations of MEVN. Cell viability even at lower concentrations of 6.25 μg/mL was 98.62%, demonstrating its ability to inhibit cell cytotoxicity. The prolongation and improvement of cell viability can be used to assess the inhibition of degeneration and its neuroprotective properties in neurodegenerative disorders such as AD. Previous in-silico and in-vitro studies, such as those conducted with Beta vulgaris, have shown potential anti-Alzheimer properties through AChE inhibition by constituents including Kaempferol and Apigenin [39].

Based on the findings of this present study, it can be well established that MEVN leaves possess anti-Alzheimer properties. However, further confirmation of these findings is needed through various other assays. The results imply that the methanolic extract of the leaves of *Vitex negundo* can be used as a therapeutic drug target for AD.

Limitation(s)

The ligands selected for the docking in-silico study can be further expanded by incorporating Absorption–distribution–metabolism–excretion–toxicity (ADMET) predictions and including many more ligands for evaluation in AChE interaction studies. Additional assays are required to confirm the AChE inhibition by MEVN. Furthermore, the increase in cell viability needs confirmation by other different assays.

CONCLUSION(S)

In the present study, the compounds present in MEVN showed good binding affinity to AChE in the in-silico molecular docking analysis and exhibited competitive inhibition of AChE in a linear dose-dependent manner. It was also found to be non-cytotoxic in the MTT assay conducted in the Vero cell line. These findings confirm the inhibitory activity of MEVN against AChE. The prevalence of AD has been steadily increasing in recent times, and AChE inhibition has been identified as one of the vital drug targets for decreasing the progression of the disease. Thus, MEVN has potential therapeutic use in neurodegenerative disorders such as AD. Further research involving in-vivo animal experiments, clinical trials, and studies targeting other proteins besides AChE is required to confirm the anti-Alzheimer properties of *Vitex negundo* leaves and to establish *Vitex negundo* as one of the mainstay treatment options for AD.

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