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Unraveling the Neuroprotective Activity of Siddha Herbal Formulation Valuluvai nei Using *In Vitro* Acetylcholinesterase Enzyme Inhibition Assay

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Abstract: The magnitude of patients with Neurodegenerative disorders (NDD) like Alzheimer's disease, Parkinsonism, Huntington's disease, and Lewy body disease is becoming very high worldwide. Ethno-pharmacological studies have shown that the use of traditional medicines for treating NDD shows good prognosis around the world. Valuluvai nei (VN) is a lipid (Ghee) based polyherbal Siddha formulation which is commonly used for treating several neurological disorders. The study was designed to evaluate the Neuroprotective activity of Valuluvai nei through *in vitro* acetylcholinesterase enzyme inhibition assay. The test drug was analyzed through the AChE inhibition assay method in different concentrations such as VN25 µg/ml, VN 50 µg/ml, VN 100 µg/ml, VN 250 µg/ml, VN 500 µg/ml. Physostigmine is used as a standard drug. The percentage of inhibition and IC₅₀ value were calculated and compared with standards. The result obtained from the present study indicates that the test drug VN was effective in inhibiting the AChE enzyme at a stipulated concentration dose-dependently. Maximum percentage inhibition of about 34.19 ± 1.011 % was observed at 500 µg/ml with the IC₅₀ value of 724.4 ± 13.99 µg/ml when compared to that of the Physostigmine, a known AChE inhibitor with a maximum inhibition of 93.81 ± 5.104 % at the concentration of 40 µg/ml with the IC₅₀ value of 14.44 ± 1.285 µg/ml. It was concluded from the current investigation that the Valuluvai nei has shown promising neuroprotective activity through acetylcholinesterase enzyme Inhibition assay and may tend to halt the progression of neurodegeneration in near future.

Keywords: Acetylcholinesterase inhibitor, Alzheimer's disease, Neurodegeneration, Valuluvai nei, Siddha medicine

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Introduction

Neurodegenerative disorders (NDD) affect many of the body's activities such as talking, balance, movement, breathing, memory, and cognitive functions. Many NDDs are genetic. Sometimes the

cause is idiopathic (Lamprey *et al.*, 2022). NDD includes Alzheimer's disease, Huntington's disease, Lewy body disease, Parkinson's disease, and Spinal muscular atrophy, etc. Alzheimer's

disease (AD) is one of the most common progressive neurodegenerative disease which begins slowly after the age of 60. Dementia was developed when the brain is affected by AD. In the current situation, more than 24 million people have dementia worldwide (Ballard *et al.*, 2011). Every year 10 million new cases were reported. People with Alzheimer's disease have trouble with memory and cognitive function. Acetylcholine esterase and Butyrylcholinesterase are the two most common forms of cholinesterase in the human brain. In AD patient's, brain has the most biochemical remarkable changes that may be due to decreased Ach levels in the cortex and hippocampus of the brain. Commercially available Synthetic acetylcholinesterase inhibitors (AChEIs) including rivastigmine, donepezil (David melzer,1998), Galantamine, and tacrine may influence the Ach by the inhibition of AChE and causes side effects like sleep disturbance, gastrointestinal problems, drowsiness etc. Due to the side effects, short half-life, and high cost of Synthetic acetylcholinesterase inhibitors (AChEIs), there is a need for a low-cost, highly effective alternative with fewer side effects (Volker Schulz, 2003; Wollen, 2010).

Siddha system of medicine is one of the ancient renowned pioneer system of medicine. Nowadays, Siddha drugs are becoming widely popular because of their effectiveness with minimal side effects. In Siddha, there are lots of herbal/formulations mentioned for treating neurological disorders by improving brain function and performance. As per the Siddha concept, Thiridhosam (vaatham, Pittham, and kabham) and mukkunam (satthuva, raso, and thamo) are the vital energy to coordinate the physiological and psychological functions of the human body (Venugopal, 1968). In the Siddha system of medicine, diseases are classified based on the derangement of three humours(vaatham, Pittham, and kabham). Nerve impulses are considered as vatham and considered to be the motivating factor for the other two humours (Pittham and Kabham). Increased functions of vatha humour affect the cognitive and memory-

enhancing functions of the brain and it may lead to pathological conditions like Alzheimer's disease. The line of treatment for neurodegenerative disorders is by decreasing the excessive action of vatha humour and regulating the function of the brain. Nei (Medicated Ghee) is one of the most potent dosage among 32 types of internal medicines in Siddha. Ghee-based polyherbal formulations are widely indicated in various neurological conditions. The blood-brain barrier is highly permeable to lipids. The lipid base formulations might have the potential to cross the blood-brain barrier and show beneficial effects on brain tissue (Pandey, 2016). Valuluvai nei (VN) is a multi-component Classical Siddha formulation that is mentioned in Siddha literature Nagamuni thalainoi Maruthuvam. The drug has been indicated for neurological symptoms like putthi miga valarum (memory enhancer), oomai naakkadhuvum purandu mozhi pesum (improves speech), kalai gnanangal adaivikkum (improves cognitive function) (Thiyaga Rajan, 1976). Moreover, most of the ingredients in this formulation have already proven memory-enhancing, neuroprotective, and Ant-Alzheimer activities in various scientific studies. So, the present study was aimed to evaluate the Neuroprotective activity of Valuluvai nei (VN) using an *in vitro* acetylcholinesterase enzyme inhibition assay.

Materials and Methods

Method of preparation:

After purification, the herbal drugs like Valuluvai arisi, Seenthil thandu, Vasambu, Amukkara, Vilamiccha ver, Adathodai ver were made into decoction (Table 1). The decoction was mixed with Birammi ilaicchaaru, ghee and milk. Sivathai, Pangam palai, Naayuruvi ver and Karunjeeragam were grounded separately to a fine powder (*Chooranam*). The herbal powder was added to the above-prepared mixture and kept on medium flame. When it reaches its consistency, it was kept off the flame, then Valuluvai nei was filtered and stored in an airtight glass container.

Table 1: Ingredients of Valuluvai nei

S. No.	Name of the drug	Botanical name	Common name
1	Valuluvai arisi (Celastraceae)	<i>Celastrus paniculatus</i> Wild.	Black oil plant
2	Seenthil thandu (Menispermaceae)	<i>Tinospora cordifolia</i> (Wild) miers ex Hook.f. and Thomas	Guduchi
3	Vasambu (Acoraceae)	<i>Acorus calamus</i> Linn	Sweet flag
4	Amukkara (Solanaceae)	<i>Withania somnifera</i> (Linn) Dunal	Ashwagandha
5	Vilamiccha ver (Labiatae)	<i>Coleus vettiveroides</i> K.C.Jacob	Kuruver
6	Adathodai ver (Acanthaceae)	<i>Justica beddomei</i> (Clarke) Bennet.	Malabar nut
7	Birammi ilaicchaaru (Plantaginaceae)	<i>Baccopa monnieri</i> (Linn) Pennell	Indian pennywort
8	Sivathai (Convolvulaceae)	<i>Operculina turpethum</i> (Linn) Silva Manso.	Turpeth
9	Pangam palai (Aristolochiaceae)	<i>Aristolochia bracteolata</i> Linn	Worm killer
10	Naayuruvi ver (Amaranthaceae)	<i>Achyranthes aspera</i> Linn.	Chaff-flower
11	Karunjeeragam (Ranunculaceae)	<i>Nigella sativa</i> Linn	Black cumin

Procurement of test drug for analysis:

The test drug Valuluvai nei prepared in the above manner was procured from the GMP-certified pharmacy and given for analysis.

Drug dosage : Karandiyalavu (10-15 ml)

Adjuvant: Milk

Duration of treatment : 1 mandalam (48 days)

Indication: Putthi miga valarum (memory enhancer) , oomai naakkadhuvum purandu mozhi pesum (improves speech), kalai gnanangal adaivikkum(improves cognitive function).

In vitro AChE enzyme Inhibition Assay – Methodology:

AChE activity of Siddha herbal formulation

Valuluvai nei (VN) was measured using a modified 96-well microplate assay based on Ellman's method, enzyme hydrolyses the substrate acetylthiocholine resulting in the product thiocholine which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which can be detected at 412 nm. 50 mM Tris-HCl pH 8.0 was used as a buffer throughout the experiment. AChE enzyme stock solution (518 U/ml) was kept at -80°C and the enzyme-dilution was done in 0.1% BSA in buffer. DTNB was dissolved in the buffer containing 0.1 M NaCl and 0.02 M MgCl₂. ATCI was dissolved in deionized water. In the 96 well plates, 100 µl of 3 mM DTNB, 20 µl of 0.26 U/ml of AChE, and 40 µl of buffer (50 mM tris pH 8.0), to which 20 µl of test drug in

Table 2: Percentage Inhibition of AChE Enzyme by test drug-VN

Concentration of VN in µg/ml	Percentage Inhibition of AChE Enzyme by Test Drug
VN 25	4.024 ± 1.578
VN 50	11.38 ± 2.777
VN 100	21.58 ± 1.714
VN 250	28.57 ± 1.715
VN 500	34.19 ± 1.011

Each value represents the mean ± SD. N=3

various concentrations (25, 50, 100, 250 and 500 µg/ml in chloroform) dissolved in buffer containing not more than 10% methanol were added to the wells. After mixing, the plate was incubated for 15 min (25°C). The enzymatic reaction was initiated by the addition of 20 µl of 15 mM acetylthiocholine iodide and the hydrolysis of acetylthiocholine was monitored by reading the absorbance every 5 min for 20 min at 412 nm. Physostigmine (5, 10, 20 and 40 µg/ml) was used as positive control. All the reactions were performed in triplicate (Ellman *et al.*, 1961).

Statistical analysis:

The statistical analysis was done by one-way ANOVA (Graph pad Prism 5 computer program).

Numerical data were expressed as mean ± standard deviation (SD).

Results

Effect of Valuluvai nei in AChE Enzyme inhibition activity:

The result obtained from the study indicates that the Valuluvai nei (VN) was effective in inhibiting the AChE enzyme at the stipulated concentration dose- dependently. Maximum percentage inhibition of about 34.19 ± 1.011 % was observed at 500 µg/ml (Table 2; Fig. 1) with the IC₅₀ value of 724.4 ± 13.99 µg/ml.

Effect of Physostigmine on AChE Enzyme inhibition activity:

Data obtained from the study has been compared with the Standard drug Physostigmine, a known AChE inhibitor, and the results reveal maximum inhibition of 93.81 ± 5.104 % at the concentration of 40 µg/ml (Table 3; Fig. 2) with the IC₅₀ value of 14.44 ± 1.285 µg/ml.

Discussion

Alzheimer's disease (AD) is a devastating, irreversible neurodegenerative condition that is characterized by memory loss, disorientation, increased confusion, and other psychological and physical manifestations. The main histopathological features of AD are the appearance of extracellular amyloid-beta deposits (Aβ) in senile plaques and the development of intracellular neurofibrillary tangles, reactive microgliosis, and astrogliosis (Abdul Manap *et al.*, 2019). Cholinesterase inhibitors are commonly prescribed for the treatment of AD. Physostigmine is a tertiary amine carbamate acetylcholinesterase inhibitor as it crosses the blood-brain barrier and stimulates central cholinergic neurotransmission. The drug is extracted from the seeds of *Physostigma venenosum* and was first synthesized in 1935. Physostigmine inhibits acetylcholinesterase, the enzyme responsible for the breakdown of used acetylcholine. By interfering with the metabolism of acetylcholine, physostigmine indirectly stimulates both nicotinic and muscarinic receptors due to the consequential increase in available acetylcholine at the synapse.

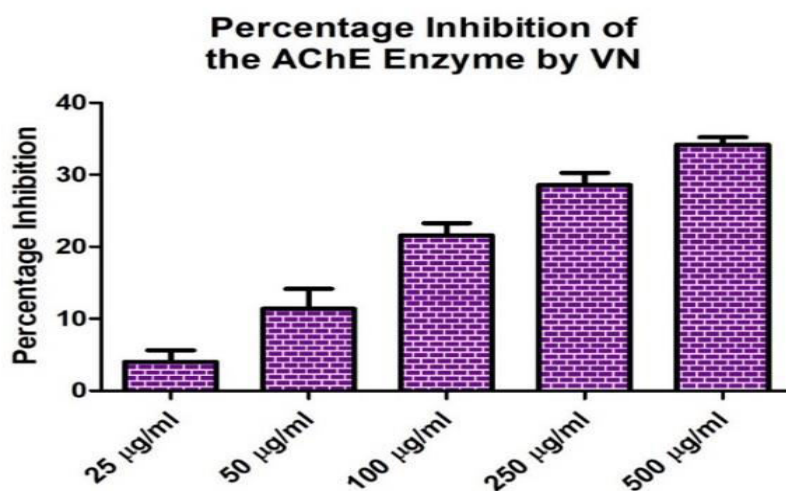


Fig. 1: Percentage Inhibition of AChE Enzyme by the Siddha formulation VN.

Table 3: Percentage Inhibition of AChE Enzyme by Standard Drug

The concentration of physostigmine in µg/ml	percentage Inhibition of AChE Enzyme by Std Drug
5	18.26 ± 3.605
10	45.45 ± 3.687
20	77.07 ± 0.9114
40	93.81 ± 5.104

Each value represents the mean ± SD. N=3

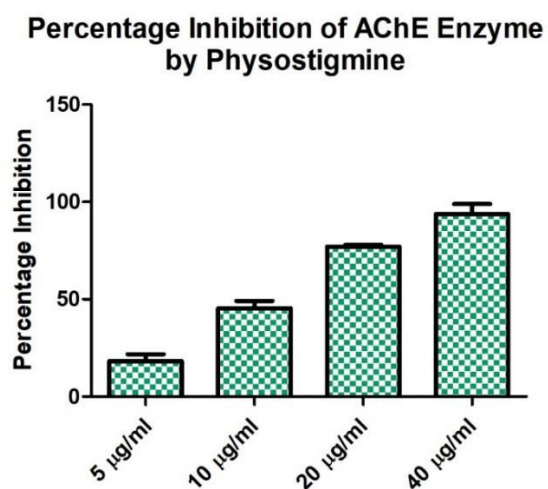


Fig. 2: Percentage Inhibition of AChE Enzyme by Physostigmine.

But due to the narrow therapeutic index and short half-life, this drug causes various adverse effects. The common adverse effects of physostigmine are increased sweating, loss of bladder control, muscle weakness, nausea, vomiting, shortness of breath, tightness in the chest, or wheezing, slow or irregular heartbeat, unusual tiredness or weakness, watering of mouth, blurred vision.

Herbal drugs have been used for centuries in many countries due to their safety, efficacy, and comparably lesser side effects than chemical drugs. The study revealed that the Valuluvai nei (VN) possess convincing AChE enzyme inhibition property in a dose-dependent manner. Among the ingredients *Celastrus paniculatus*, *Bacopa monnieri*, *Withania somnifera*, *Tinospora cordifolia*, *Acorus calamus*, and *Achyranthes aspera* are known for their neuroprotective, Nootropic, and Anti Alzheimer's activities. *Celastrus paniculatus* Wild. (CP) is one of the well-known Siddha nervine tonic, used extensively as a neuro-protective and memory enhancer, and in different central nervous system disorders. Preclinical and clinical studies have shown that CP is beneficial in neurodegeneration and improves intellect and memory. CP enhanced the Intelligence Quotient (IQ) in Mentally retarded children. Previous studies revealed that the aqueous extract of *Celastrus paniculatus* seed has dose-dependent cholinergic activity, thereby improving memory performance. The mechanism of action of CP may be due to increased acetylcholine levels in the brain (Shah *et al.*, 2018). The anti-Alzheimer (AD) activity of the crude methanolic extract of *Celastrus paniculatus* exhibited strong DPPH free radical scavenging as well as inhibition of authentic peroxynitrite (ONOO-) activity and total reactive oxygen species (ROS) generation (Badrul A and Ekramul, 2011). CP has a protective action against 3-NP-induced HD-like symptoms due to its strong antioxidant effect (Malik *et al.*, 2017). This herb is a rich source of several secondary metabolites, such as β -Dihydroagarofuranoids sesquiterpenes, alkaloids (Celastrine, Celapanin, Celapagin, and paniculatin), flavonoids, terpenoid (β -amyrin, Lupeol, Pristimerin), sterols (β -

sitosterol, campesterol, stigmasterol, α -tocopherol, γ -Tocopherol), fatty acid (palmitic, stearic, oleic, linoleic, linolenic acids) and non-fatty acids (Benzoic acid, Cinnamic acid). These active constituents possess potent nootropic activity, anti-Alzheimer, anticonvulsant, antidepressant, and several other properties (Bhagya *et al.*, 2016).

Tinospora cordifolia has protective effects against Alzheimer's disease and other neurodegenerative diseases. Tinosporide and 8-hydroxytinosporide isolated from *Tinospora cordifolia* were evaluated for acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activities (Adib *et al.*, 2021). Extract of this plant ameliorates cognitive functions and drug-induced amnesia as well as it has memory-enhancing properties in normal and in memory deficient animals. *T. cordifolia* ethanol extract (TCEE) possesses significant neuroprotection in 6-OHDA-induced PD by protecting dopaminergic neurons and reducing the iron accumulation due to the presence of phytochemical constituents such as alkaloids, glycosides, diterpenoid lactones, berberine, flavonoids, saponins and trace element contents (Avinash *et al.*, 2004; Kosaraju *et al.*, 2014). This plant helps to enhance the cognition in normal and cognition deficits animals in the Hebb William maze, behavioural test, and the passive avoidance task (Yalla Reddy *et al.*, 2010).

Bacopa monnieri has been used as a therapeutic agent for various disorders especially neurodegenerative disorders. Brahmi Nei is an important formulation that is common in Siddha and Ayurveda clinical practices. This drug helps to rejuvenate the mind and enhance memory and concentration. The memory-enhancing property of bacopa is due to its lipid peroxidation property and it is suggested for low-dose long-term therapy rather than single high-dose therapy (Tripathi *et al.*, 1996). The bioactive components present in the herb like Bacoside A, Bacoside B, Bacosaponins, and Betulinic have a significant role in neuroprotection like reduction of ROS, neuroinflammation, aggregation inhibition of

Amyloid- β and improvement of cognitive and learning behaviour. A pilot, exploratory study with Brahmi gritha in ADHD children showed a significant reduction (almost 66%) in total ADHD score when compared to baseline (Bhalerao, 2013). Neuroprotective and neurorescue properties of *Bacopa monnieri* (L.) Wettst was evaluated in MPTP induced mice model of PD. The extract of this plant may slow down the disease progression and delay the onset of neurodegeneration in PD (Singh *et al.*, 2020).

Studies have shown that *Withania somnifera* extracts exhibit numerous therapeutic effects including oxidative stress reduction, inflammation memory, and cognitive function improvement. In AD, the neuroprotective effect of this plant is mediated by the reduction of beta-amyloid plaque aggregation, tau protein accumulation, regulation of heat shock proteins, and inhibition of oxidative and inflammatory constituents (Das *et al.*, 2021). A total alkaloid extract of this root exhibited a calming effect on the central nervous system (CNS) in several mammalian species (Dhuley, 1998). Withanolide A combats neurodegenerative processes (Sehgal, 2012) and it may help to induce the regeneration of axons, dendrites, and pre-and post-synapses in the neurons (Kuboyama *et al.*, 2005). Withanamides have been shown to scavenge free radicals generated during the initiation and progression of AD, Neuronal cell death triggered by amyloid plaques was also blocked by Withanamides (Jayaprakasam B, 2010). Aqueous extracts of this plant have been found to increase cholinergic activity and increase acetylcholine, and choline acetyltransferase activity in rats (Parihar and Hemnani, 2003). A study conducted on experimental rats demonstrated that vitanon-isolated from the root of *W. somnifera* showed significant improvements in cognitive function as a result of the inhibition of amyloid β -42, and a reduction in pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, and nitric oxide (Pandey *et al.*, 2018).

Esfandiari *et al.* (2018) investigated that the extracts of the *Acorus calamus* prevent memory

loss, anxiety, and oxidative stress among rats exposed to lipopolysaccharide-induced neuroinflammation). The secondary metabolites of the rhizome part of *A. calamus* mainly α - and β -asarone exhibits neuroprotective effects by crossing the blood-brain barrier (Balakrishnan *et al.*, 2022). β -Asarone isolated from *A. calamus* is a powerful neuroprotector as it retained the memory of the animals by protecting the hippocampus after the kainic acid lesion (Venkatramaniah *et al.*, 2016). β -Asarone protects cognitive function by inhibiting ACh esterase and suppressing TNF- α and IL-1 β among Alzheimer's disease-induced rats (Deng *et al.*, 2016).

Achyranthes aspera is widely used as a nootropic agent in traditional medicine and exhibits a protective effect on hippocampus-dependent spatial learning and memory (Girach and Khan, 1992). The nootropic activity of this plant has been reported and confirmed in animal studies using radial arm maze, passive shock avoidance, and novel object recognition tests in mice (Dinesh *et al.*, 2015).

From the literature, it is evident that the ingredients of Valuluvai nei (VN) have proven the Neuroprotective, Nootropic, Anti Alzheimer's activities in various scientific studies. The phytochemicals present in the formulation might be responsible for these pharmacological activities which support the therapeutic claim mentioned in the literature. This research work revealed that the Valuluvai nei has a promising neuroprotective effect and the evidence provides that the VN can be used to treat or delay Neurodegenerative disorders.

Conclusion

This study revealed that the Valuluvai nei has Neuroprotective activity with highly beneficiary value to treat Neurodegenerative disorders. More specific experiments on animal models are required to establish the exact molecular mechanisms of action. Furthermore, wide-based clinical trials must be carried out to prove the neuroprotective effect of the drug in humans also.

In future, the goal is to identify the suitable compound which is responsible for its Neuroprotective activity by different scientific methods.

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