Exploring Natural Products and their Semisynthetic Derivatives for the Management of Alzheimer's Disease

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By

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7.1. Summary

The present study summarized that a couple of pyrroloquinazoline alkaloidal compounds were isolated from DCM fraction of AVME, viz, vasicinone (VAS) and vasicine (VA). These compounds showed stable binding interactions on AChE and BuChE enzymes in *in silico* studies. Both VAS and VA alleviated the AChE, BuChE, and Aβ aggregation inhibition in *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 is 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 have been reported on *Adhatoda vasica* Nees in rats and monkeys for six months and no toxicity symptoms were identified [224]. In previous reports, VA was found to be safe in human volunteers up to 16 mg dose [225]. These studies have proven that alkaloidal compounds (pyrroloquinazolines) from Vasaka are safe medications and can be used for further development.

The levels of AChE and BuChE activities increase drastically in AD conditions, 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 [226]. In 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 Aβ aggregates and presence of hydrophobic amino acid residues in the Aβ structure which is mainly responsible for its aggregation potential [227]. 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 [177]. Therefore, inhibition of Aβ aggregation is the leading option for the treatment of AD. The present study reveals that VAS and VA inhibited the Aβ aggregation in *in vitro* studies suggesting that these compounds

were able to decrease the Aβ 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 Aβ suggesting that VAS and VA can protect the neuronal cells which are exposed to Aβ. VA was previously reported to inhibit the AChE [228,229]. However, VA was not explored for its amyloid beta inhibition activity. In the present work, *in vitro* Aβ studies indicate that VA can also inhibit Aβ aggregation suggesting that VA impedes AD pathogenesis in multiple ways.

Cognition and memory impairment are the key features of AD [226]. 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 [168] 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 [181]. In Aβ 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 [226]. 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 Aβ 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 [230,231]. 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) [232,233]. Vasicinone is an 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 Aβ aggregates as well as neuroprotection activity. The lack of disease-modifying therapeutics for AD, and the add-on use of plant-based pharmacologically effective leads can help effectively manage the disease symptoms.

Further, multifunctional 3-OH pyrrolidine analogs (VA01-VA25) were prepared through a semisynthetic approach using pyrroloquinazoline alkaloid vasicine as a precursor compound. The current study reports VA10 is a potential molecule among all the synthesized

derivatives, which was identified through *in silico*, *in vitro*, and *in vivo* studies. All the compounds exhibited drug-likeness properties, no toxic effects as well as positive ADME profile in *in silico* predictions as per swissADME and preADMET tools. The preliminary data from *in silico* studies, compound VA10 showed most stable interactions and well resided at AChE and BuChE active sites. Further, compound inhibited the eeAChE, hAChE, eqBuChE, and $A\beta_{1-42}$ aggregation, and showed membrane permeability in PAMPA assay, and anti-oxidant activity in *in vitro* studies. In addition, compound specifically binds to AChE PAS site, as confirmed by propidium iodide displacement assay. Moreover, Compound VA10 showed neuroprotection properties on $A\beta_{1-42}$ treated SH-SY5Y cell line. These *in silico* and *in vitro* experimental data suggested that compound VA10 exhibit drug-like properties and acts via multiple targets i.e. exhibiting cholinesterases inhibition potency by binding to PAS site, inhibiting AChE-induced Aβ aggregation by binding to AChE active site, probably exerting antioxidant activity through free radical scavenging potency by transferring hydrogen atoms to their substrates, PAMPA assay permeability, and neuroprotective action on $A\beta_{1-42}$ treated human neuroblastoma cell line (SH-SY5Y).

Before performing *in vivo* studies, an acute oral toxicity test was conducted to assess toxicity of novel compound VA10 and it was found to be safe in rats. Further, *in vivo* screening of lead compound VA10 was performed in AD rat model. Scopolamine induced amnesia model revealed that VA10 (10 mg/kg, p.o.) was able to improve cognition in Y maze test and improving ACh levels by inhibiting AChE activity in rats. It is evident that compound VA10 acts through cholinergic pathway and specifically binds to AChE site. Furthermore, improved learning and memory capacity of VA10 was observed through Morris water maze test in $A\beta_{1-42}$ induced Alzheimer model at a dose of 10 mg/kg (p.o). Ex vivo studies reveal increased ACh levels in brain by inhibiting AChE activity. In addition, histopathological examination suggested that neuronal cell density in the hippocampus region was recovered and

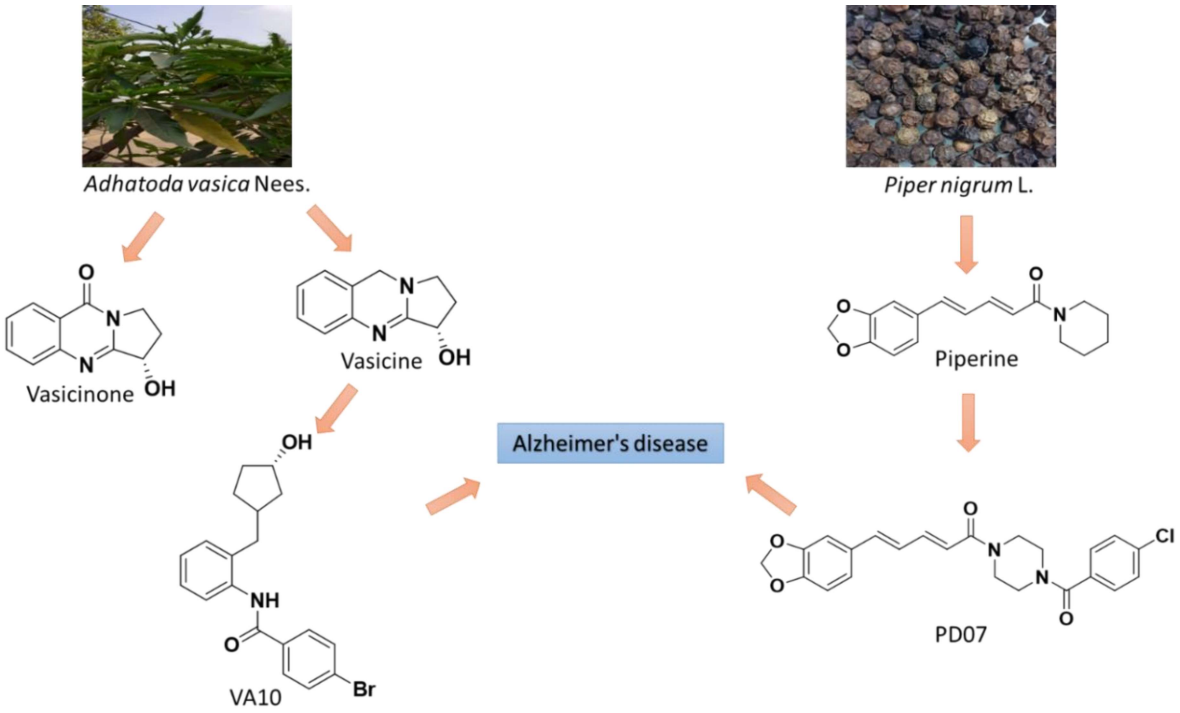
demonstrated neuroprotective potential in $A\beta_{1-42}$ induced Alzheimer's model. Thus, it was concluded compound VA10 improved learning and memory by enhancing ACh levels through AChE inhibition, and also the compound was able to recover the neuronal cell loss. The promising data from the *in silico*, *in vitro,* and *in vivo* experiments allow us to report compound VA10 as a potent semi-synthetic multifunctional lead for the treatment of AD.

Furthermore, A semisynthetic approach was used to synthesize multifunctional piperine derivatives (PC01–PC10 and PD01–PD26) using alkaloid piperine as a precursor molecule. The compound PD07 exhibited most significant results in *in vitro*, *in silico*, and *in vivo* studies among all tested compounds. In *in vitro* experiments, compound PD07 showed lipophilic nature and displayed inhibitory activity on eeAChE, hAChE, eqBuChE, $A\beta_{1-42}$ aggregation, BACE1, and DPPH assay. The compound PD07 effectively displaced the propidium iodide at the AChE PAS site. Additionally, PD07 also showed neuroprotective action on $A\beta_{1-42}$ treated SH-SY5Y cell line. The compound PD07 showed drug-likeness, ideal ADME profile, and no toxic properties in *in silico* predictions. Further, DFT studies were performed to assess the structural properties of the compound PD07. Furthermore, molecular docking and molecular dynamic simulation suggested that PD07 formed most stable interactions and was well-resided at the active sites of the proteins AChE, BuChE, and BACE1. These *in vitro* and *in silico* data suggest compound PD07 have drug-like properties and acts through multiple targets i.e., inhibition of cholinesterases and amyloid beta aggregation by binding to AChE PAS region and binding to BACE1 active site, probably showing antioxidant activity through free radical scavenging activity by transferring hydrogen atoms to their substrates, and neuroprotective action. Moreover, acute oral toxicity and CNS toxicity tests suggests compound PD07 is safe in rats. The scopolamine-induced amnesia experiments indicated that PD07 treatment enhanced rats cognition and memory. Ex vivo experiments revealed that compound PD07 increased ACh levels by inhibiting the AChE activity in the brain. The *in vitro*, *in silico*, and

in vivo suggested that synthesized piperine derivative PD07 is a potent molecule among all synthesized compounds and it is acting through multiple targets in AD.

7.2. Conclusion

- A couple of leads were isolated through bioactivity-guided fractionation, such as vasicinone (VAS) and vasicine (VA) from DCM fraction of AVME. Both compounds showed similar potency on tested targets for the management of AD.
- Further, multifunctional 3-OH pyrrolidine compounds (VA01-VA25) were prepared through a semisynthetic approach using pyrroloquinazoline alkaloid vasicine. The compound VA10 was found to be more potent in AD among 25 novel compounds.
- Furthermore, a semisynthetic approach was used to create the multifunctional piperine derivatives (PC01–PC10 and PD01–PD26) using alkaloid piperine. Among 36 synthesized compounds, PD07 showed potent anti-AD activity.



The work provided new evidence on vasicinone which acts multi-target manner in the treatment of AD. The promising data from the *in silico*, *in vitro,* and *in vivo* experiments allow us to report compounds VA10 and PD07 as potent semi-synthetic multifunctional leads for the treatment of AD. Further, more advanced and rigorous biological and toxicological investigations need to be established for these identified leads to process them further for clinical trials.