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SHORT COMMUNICATION

Dual Anti-cholinesterase Activity of Ajoene by *In silico* and *In vitro* Studies

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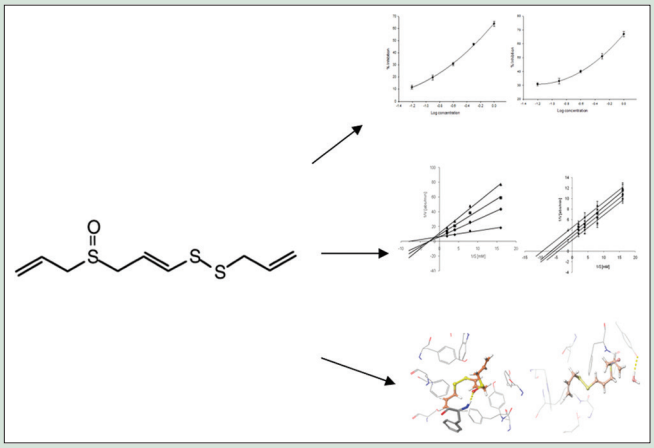
ABSTRACT

The two major forms of cholinesterase enzymes found in the mammalian brain are acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). BuChE usually found mainly in glial cells and neuron in normal physiological condition, whereas AChE found near nerve synapse and axons, both are involved in the breakdown of acetylcholine (ACh) in the brain. The dual inhibition of these enzymes is considered as a promising strategy for the treatment of a neurological disorder such as Alzheimer's disease, senile dementia, ataxia, and myasthenia gravis. The objective is to study the dual anticholinesterase activity of ajoene using *in silico* and *in vitro* methods. The anticholinesterase activity of ajoene was evaluated using Ellman's assay, and molecular docking was performed on Schrödinger suite software. The present study demonstrated ajoene ([*E*, *Z*]-4, 5, 9-trithiadodeca-1, 6, 11-triene-9-oxide) inhibited both AChE and BuChE in a concentration-dependent manner. The IC_{50} value of ajoene was 2.34 mM for AChE and 2.09 mM for BuChE. Kinetic studies showed mixed noncompetitive inhibition of AChE and uncompetitive inhibition of BuChE. Molecular docking studies revealed that ajoene interacts hydrophobically with catalytic residues of AChE while in case of BuChE the interaction is through noncatalytic binding site residues. Ajoene exhibits dual inhibitory activity against both AChE and BuChE enzymes.

Key words: Acetylcholinesterase, ajoene, Alzheimer's diseases, butyrylcholinesterase, molecular docking

SUMMARY

- The present study demonstrated ajoene inhibited both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in concentration-dependent manner
- Mode of inhibition showed mixed noncompetitive and uncompetitive inhibition for AChE and BuChE, respectively, by kinetic study
- Ajoene interacted by catalytic and noncatalytic site residues in case of AChE and BuChE, respectively.



Abbreviations Used: AChE: Acetylcholinesterase; BuChE: Butyrylcholinesterase; AD: Alzheimer's disease; Ach: Acetylcholine; ChAT: Choline acetyltransferase; ATChI: Acetylthiocholine iodide; BuChI: Butyrylcholine iodide; DTNB: 5,5-Dithiobis-2-nitrobenzoic acid; PDB: Protein data bank; RMSD: Root mean square deviation; OPLS: Optimized potentials for liquid simulations; ADME: Absorption, distribution, metabolism, excretion; DADS: Diallyl disulfide; QPlogPo/w: Predicted octanol/water partition coefficient; donorHB: Number of hydrogen bond donors; accptHB: Number of hydrogen bond acceptors; MW: Molecular weight of the compounds.

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INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in the elderly people which are mainly characterized by a disturbance of multiple cortical functions, including memory loss, judgment, orientation, comprehension, learning capacity, and language deficit.[1,2] The major symptoms of all types of dementia are presumed to be related to progressive deterioration of widespread and dense cholinergic innervations of the human cerebral cortex which contributes to the behavioral and cognitive disturbances in the AD. It is also associated with the reduced levels of the neurotransmitter, acetylcholine (ACh), choline acetyltransferase , and acetylcholinesterase (AChE).[3] Apart from AChE, ACh is also hydrolytically destroyed in the brain by another enzyme, butyrylcholinesterase (BuChE).[4] The most important enzyme which regulates the level of ACh in the healthy brain is AChE while BuChE plays a minor role. In the case of AD patients, BuChE activity increases whereas that of AChE activity decreases, the levels changing between BuChE and AChE from 0.6 in the healthy brain to as high as 11 in the cortical areas affected by the disease.[5] These observations lead to dual inhibition strategy of these enzymes has been proposed to increase the efficacy of treatment and broaden the indications.

Ajoene is derived from allicin which is a key phytoconstituent of garlic (*Allium sativum*). Garlic is used as a food, spice, and folk medicine worldwide for centuries. It is used to cure various diseases,

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such as arthritis, cancer, heart disease, worm infestation, diarrhea, and pulmonary complaints.[6,7] Antioxidant is one of the important properties of garlic, which not only reduce the risk of injury to essential molecules but also defend against the oxidative damage and to varying degree may assist in preventing the onset and development of age-related neurodegenerative diseases.[8] This lead to focus our interest on the processed constituent of garlic like ajoene, a derivative of allicin ((*R*, *S*)-diallyldisulfid-*S*-oxide), whose inhibitory activity against AChE and BuChE has already been reported.[9-11] The present study demonstrated dual cholinesterase inhibitory effect of ajoene by *in vitro* and *in silico* studies.

MATERIALS AND METHODS

Chemicals/reagents

AChE (EC 3.1.1.7) and BuChE (EC 3.1.1.8) from bovine erythrocytes, acetythiolcholine iodide (ATChI), butyrylcholine iodide (BuChI), 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and sodium bicarbonate were purchased from Sigma, India. Ajoene was purchase from Triveni chemicals, Chennai, India.

Cholinesterase assays

Cholinesterase inhibition was assessed using the colorimetric method of Ellman[12] as adapted Okello[13] in a flat-bottom 96-well microtiter plates. A typical run consisted of 200 µL of 0.1 M phosphate buffer pH 8; 5 µL of bovine AChE/BuChE solution, at final assay concentrations of 0.03 U/mL; 5 µL of DTNB at a final concentration of 0.3 mM prepared in 0.1 M phosphate buffer pH 7 with 0.12 M of sodium bicarbonate; and finally, 5 µL of the test solution. The reactants were mixed and preincubated at 30°C for 15 min. The reaction was initiated by adding 5 µL of ATChI/BuChI at final concentrations of 0.5 mM. The buffer solution was added instead of inhibitor as the control, which was assayed in triplicates. Two blanks for each run were prepared in triplicates to monitor any nonenzymatic hydrolysis in the reaction. One blank consisting of enzyme instead of buffer and other contains substrate. Change in absorbance at 412 nm as measured on SpectraMax M2, 96-well plate reader for 6 min at 30°C. IC_{50} of the compound that inhibited the substrate ATChI/BuChI by 50% was calculated using linear regression analysis.

Kinetic analysis was also performed, where different concentration of the substrate ATChI/BuChI, i.e., from 0.5 mM to 0.0625 mM, was preincubated with the enzyme AChE/BuChE in the absence (control) and the presence of different concentration of ajoene, i.e., from 0.2 to 4 mM.[14]

Protocol for molecular docking

Protein preparation

X-Ray crystallographic structure of AChE co-crystallized with galantamine was retrieved from Protein Data Bank (PDB ID: 4EY6) with a resolution of 2.4Å.[15] Before molecular docking, the structure of human AChE was prepared using "Protein Preparation Wizard" workflow in Schrödinger Suite.[16] This included assignment of bond orders, addition of hydrogen atoms to the protein, it shows the position of water molecules and the unnecessary water molecules can be deleted. As in this case, seven water molecules were found to be important so they were kept, rest were deleted.[17] Disulfide bonds were created, side chains were added followed by the addition of missing atoms and the assignment of the partial charges. Finally, restrained minimization was done to obtain a root mean square deviation (RMSD) of 30Å using OPLS_2005 (optimized potentials for liquid simulations) force field. A similar protocol was repeated for BuChE, which was also downloaded from protein data bank (PDB ID: 1P0M) with a resolution of 2.38Å.[18] The only difference, in this case was two water molecules were retained, rest others were removed.

Grid generation

The active site of AChE/BuChE was identified by the presence of the ligand bound in the crystal structure. Using the receptor grid generation workflow in Schrödinger Glide, an atom of the ligand was chosen, which created a box (grid) around the ligand, receptor grid was generated keeping all the functional residues.

Ligand preparation

The structure of ajoene was downloaded from PubChem (PubChem CID: 5386591). Preprocessing of the ligand was done using Ligprep in Schrödinger Maestro. It includes generation of tautomers and ionization states at pH 7.0 ± 2.0 using Epik, the addition of hydrogen atoms, structure filtration, neutralization of charged groups, and finally optimizing the geometry of the ligand molecule.

Molecular docking

To predict the affinity and binding orientation of the ligand with the target protein, molecular docking was performed. Using Glide, grid based docking was performed. Extra precision docking was done by keeping the ligand flexible, to generate various conformations. OPLS force fields were used to perform these calculations.[19]

Pharmacokinetic parameters

ADME (absorption, distribution, metabolism, and excretion) studies of ajoene were predicted using QikProp from Schrödinger software. In this various parameters such as predicted octanol/water partition coefficient (QPlogPo/w), the number of hydrogen bond donors (donorHB), acceptors (accptHB), molecular weight of the compounds (MW) and percentage human oral absorption, Violations of Lipinski's rule of five were checked.

RESULTS

Estimation of IC_{50} value of ajoene with acetylcholinestrase and butyrylcholinestrase

The results showed concentration-dependent inhibition of AChE and BuChE with ajoene (0.2 mM to 4 mM) [Figure 1]. The maximum concentration tested showed 64% inhibition for AChE and 67% inhibition for BuChE. The IC_{50} value of ajoene was 2.34 mM for AChE and 2.09 mM for BuChE. Lineweaver-Burk plot was used to study mode of inhibition of ajoene. Furthermore, kinetic studies showed that ajoene demonstrated mixed noncompetitive mode of inhibition for AChE whereas BuChE was inhibited in uncompetitive manner [Figure 2].

Validation of molecular docking protocol

Re-docking of co-crystallized ligand to the protein was done for the validation of the docking protocol. In this analysis, a co-crystallized structure of the target protein was downloaded from Protein Data Bank (PDB ID 4EY6 for AChE and 1P0M for BuChE). Ligand was extracted from the target protein and was then allowed to re-dock into the active site of the protein. Once docking was completed, the co-crystallized ligand was aligned with the best pose of the ligand and RMSD was calculated. This helps in determining the reproducibility and reliability of the docking protocol used.[20] RMSD of the best pose of ligand with that of the co-crystallized ligand was below the range of 1Å in both the cases indicating the validation of the protocol.

Molecular docking study

In silico studies showed that ajoene interacts hydrophobically with residues such as Tyr72, Tyr124, Ala204, Trp236, Trp286, Val294, Phe295, Phe297, Tyr337, Phe338, and Tyr341. Polar interactions were also observed with the catalytic residues such as Ser203 and His447 [Figure 3]. In the case of BuChE, polar interactions were observed with residues such as Ser198, Asn397, and hydrophobic interactions with Ala199, Trp231, Pro285, Leu286, Val288, Ala328, Phe329, Tyr332, and Phe398 [Figure 3]. Ajoene has a dock score of-5.04 and-2.899 in the case of AChE and BuChE, respectively [Table 1].

Physio-chemical properties of ajoene

In silico models has allowed estimating several ADME properties by developing a deeper relationship with molecular structure and ADME parameters.[21] Therefore, various physical and chemical properties of ajoene were also calculated so as to ensure they are within the acceptable range for a drug molecule. According to the Lipinski's rule of five, drugs are orally administered only if molecular weight >500, donorHB ≤5, accptHB ≤10 and QPlogPo/w ≤5.[22] It follows Lipinski's rule of five [Table 2].

Percentage human oral absorption was calculated on the scale on 0–100 to predict oral absorption. Less than 25% is considered to be poor while more than 80% is good orally absorbed. In this case, it is 82.253% which is considered to be good.

Table 1: Binding interactions of ajoene with acetylcholinesterase and butyrylcholinesterase

	Glide score	Glide E_{vdw} (kcal/mol)	Glide E_{coul} (kcal/mol)	Glide energy (kcal/mol)
AChE	−5.037	−27.407	−2.868	−30.275
BuChE	−2.898	−23.699	−1.137	−24.836

AChE: Acetylcholinesterase; BuChE: Butyrylcholinesterase

DISCUSSION

Ajoene ([*E*, *Z*]-4,5,9-trithiadodeca-1,6,11-triene-9-oxide), an organosulfur compound obtained from garlic is identified as one of the strongest anti-platelet compound.[23] Apart from ajoene, garlic contains alliin ([1]-*S*-allyl-L-cysteine sulfoxide) as a major sulfur-containing compound. When the raw garlic is crushed, an enzyme allinase hydrolyzes alliin to allicin, sulfenate, pyruvate, and ammonia. Furthermore, allicin due to its instability rapidly metabolized into diallyl disulfide, diallyl trisulfide, ajoene, *S*-allymecaptocysteine, *S*-allyl cysteine, and vinyl dithiines.[24-28] Ajoene is a derivative of allicin was first isolated from the methanol extract of garlic. *Z*-ajoene has also been demonstrated to have antibiotic and anti-tumor

Table 2: Physiochemical properties of ajoene

Compound name	MW	QPlogPo/w	DonorHB	AccptHB	Percentage human-oral absorption	Violation of Lipinski's rule
Ajoene	234.389	2.531	0	5	82.253	0

MW: Molecular weight of the compounds; QPlogPo/w: Predicted octanol/water partition coefficient; DonorHB: Number of hydrogen bond donors; AccptHB: Number of hydrogen bond acceptors

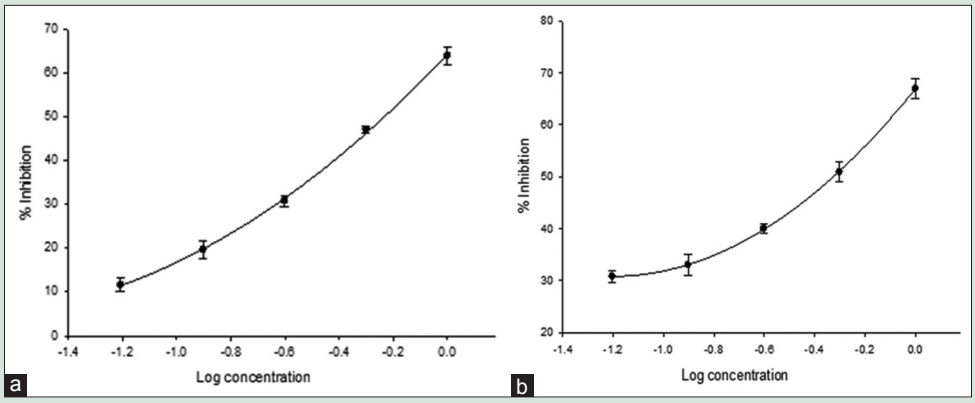

Figure 1: Concentration-dependent inhibition of cholinesterase enzyme. (a) Acetylcholinestrase and (b) butyrylcholinestrase by different concentration of ajoene

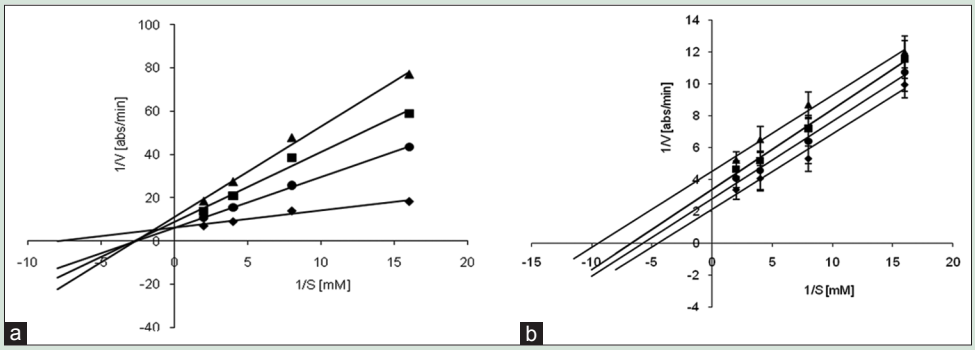

Figure 2: (a) Kinetic studies of AChE with different concentrations of ajoene ●42 µm; ■127 µm; ▲255 µm; ♦control. (b) Kinetics studies of butyrylcholinestrase with ajeone at different concentrations ●266 µm; ■533 µm; ▲1000 µm; ♦control

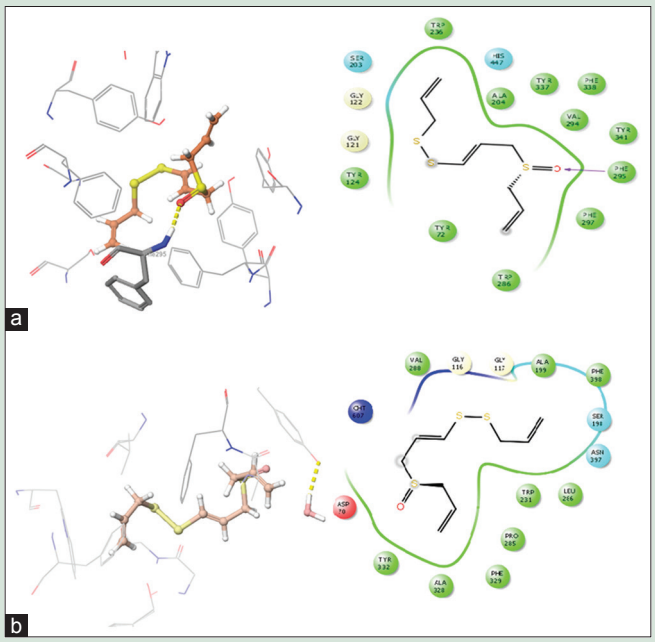

Figure 3: Molecular interaction of ajoene with cholinesterase enzyme (a) Acetylcholinestrase and (b) butyrylcholinestrase

effects. A recent study has shown that ajoene reduced the mortality and cerebral injury in stroke-prone hypertensive rats.[29] *Z*-ajoene has shown to inhibit scopolamine-induced memory impairment *in vivo*.[30]

Few drugs such as tacrine, rivastigmine, physostigmine, pyridostigmine, and neostigmine provide symptomatic relief to AD patients, but there are no drugs clinically available which can cure, prevent, or stop the progression of the AD. Moreover, the drugs available in the market, are many side effects such as gastrointestinal disturbances, short half-life, hepatotoxicity, systemic cholinergic actions, and bioavailability.[31] Hence, there is an immediate need for new anticholinesterase drugs with lesser side effects. This led us to investigate the role of ajoene in dual anti-cholinesterase activity.

The present study demonstrated that ajoene inhibits both AChE and BuChE in a concentration-dependent manner. Kinetic studies using Lineweaver burk plot shows the mixed noncompetitive mode of inhibition for AChE and uncompetitive mode of inhibition for BuChE enzyme. *In silico* studies shows that ajoene interacts with the active site residues of AChE but with BuChE, it interacts through noncatalytic residues. It also follows Lipinski's rule of five and has a good oral absorption, hence showing potential drug quality for the mentioned targets, but further pharmacological and *in vivo* studies are needed to validate and support its significance in animal models. These results may provide an interesting lead with regard to the beneficial effects claimed for ajoene and may be of therapeutic value in an AD in future.

CONCLUSION

Ajoene has a potential to ameliorate the decline of cognitive function and memory loss associated with a decline in levels of neurotransmitters by selectively inhibiting cholinesterase enzymes (AChE and BuChE) in central nervous system.

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Conflicts of interest

There are no conflicts of interest.

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