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Kinetics and computational analysis of cholinesterase inhibition by REVERC3, a bisdemethoxycurcumin-rich *Curcuma longa* extract: Relevance to the treatment of Alzheimer's disease

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Abstract

Objectives: Cholinesterase inhibition is a common strategy to treat Alzheimer's disease. In this study, we have investigated the cholinesterase inhibitory effects of a first-of-its-kind turmeric extract (REVERC3) having enriched content of bisdemethoxycurcumin as major active curcuminoid. **Methods**: The inhibition studies were performed using Ellman's colorimetric assay. The kinetics of acetylcholinesterase and butyrylcholinesterase was determined in the presence of REVERC3 using the Lineweaver–Burk double reciprocal plots. Furthermore, we used AutoDock tools to predict the binding of bisdemethoxycurcumin with the active sites of cholinesterases. **Results**: REVERC3 showed 4.8- and 5.39-fold higher inhibitory potential of acetylcholinesterase and butyrylcholinesterase with IC50 values of 29.08 and 33.59 µg/mL, respectively, compared to the regular turmeric extract. The mode of binding of REVERC3 was competitive in the case of acetylcholinesterase while it was uncompetitive for the inhibition of butyrylcholinesterase. Docking analysis revealed that bisdemethoxycurcumin, the major constituent of REVERC3, has different preferences of binding in the active sites of acetylcholinesterase and butyrylcholinesterase. However, the best binding pose predictions are in line with the experimental binding mode of the cholinesterases. **Conclusion**: These results indicate that bisdemethoxycurcumin-enriched turmeric extract could improve the cholinergic functions via dual inhibition of cholinesterases. However, the predominant role of bisdemethoxycurcumin in REVERC3 must be further validated using preclinical studies and clinical trials.

Keywords

Cognition deficits, cholinesterases, bisdemethoxycurcumin, turmeric

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Introduction

Alzheimer's disease (AD) is the most common form of dementia affecting the geriatric population worldwide.[1] AD is characterized by the progressive loss of cognitive function leading to irreversible neurodegenerative disorder.[2] AD pathology includes the apoptotic death of cholinergic neurons in the neocortical and limbic regions subsequently resulting in the decline of neurotransmission.[3] Presumably, the cholinergic deficits result from tau hyperphosphorylation and amyloid plaque formation.[4–6] Patients suffering from AD often experience the symptoms that typically include repetitive questioning, confusion, and mood swings.[7] The symptoms worsen over time with the patient experiencing delusion and aphasia.[7]

Reduced production of acetylcholine (ACh) leads to cognitive impairments such as learning, memory, and attention.[8] Acetylcholinesterase (AChE), the key enzyme involved in ACh metabolism is a prime target in the treatment of AD.[9] In

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addition, butyrylcholinesterase (BuChE) has also been reported to play a role in the ACh metabolism at the later stages in AD pathology.[10] Hence, it is of great interest to use the interventions that can act as dual inhibitors of AChE as well as BuChE in the therapeutic strategy against AD. Several drugs such as donepezil, galantamine, rivastigmine, and tacrine function as inhibitors of AChE, thereby increasing the ACh levels.[11] Rivastigmine and galantamine are known drugs for the inhibition of both AChE and BuChE.[12,13] Despite being efficacious, these drugs are associated with unwanted side effects which include gastrointestinal irritations.[14,15]

Complementary medicinal research in the development of effective treatment strategy to combat AD and other neurodegenerative conditions is greatly appreciated. Such treatments should ideally improve the quality of life and reduce the burden of financial concerns experienced by the patients. Natural AChE inhibitors possess other pharmacological attributes such as anti-inflammation and anti-oxidant activities that make them useful as multi-target approaches against AD progression.[16,17]

Turmeric is a culinary spice used long since in Indian food and folkloric medicine.[18] The medicinal properties of turmeric such as anti-inflammatory, anti-tumor, and anti-oxidant effects are mostly attributed to the presence of the yellow pigment curcumin.[19–21] Other curcuminoids such as bisdemethoxycurcumin (BDMC) and demethoxycurcumin (DMC) exhibit synergistic effects with curcumin.[22] The physiological benefits of BDMC include anti-inflammatory[22] and anti-carcinogenic activities.[23,24] Previously, Kalaycıoğlu et al.[25] have reported the preliminary findings on the inhibitory potential of BDMC against AChE. Here we have studied the kinetics of AChE and BuChE enzyme inhibition by a BDMC-rich proprietary turmeric extract (REVERC3). Furthermore, in silico docking studies were performed to predict the binding interaction of BDMC with the catalytic sites of cholinesterases. Data from this study provide valuable information on the potentials of BDMC as dual inhibitor of cholinesterases and its possible role in slowing down cognitive decline.

Materials and methods

Materials

AChE (EC 3.1.1.7) from *Electrophorus electricus*, BuChE (EC 3.1.1.8) from equine serum, acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), 5:5-dithiobis-2-nitrobenzoic acid (DTNB) were procured from Sigma Aldrich (St. Louis, MO, USA). All chemicals used were of analytical grade.

Plant extracts

REVERC3™, a standardized turmeric extract enriched with BDMC, and regular turmeric extract were procured from the Department of Phytochemistry, R&D Center for Excellence, Vidya Herbs Pvt. Ltd., Bangalore, India.

High-performance liquid chromatography analysis

Quantification of BDMC in REVERC3 was performed by high-performance liquid chromatography (HPLC) on a Shimadzu LC2030C Prominence-i (Japan) system at UV detection of 420 nm. Separation was carried out in Kinetex C-18 column (100 Å, 150 mm × 4.6 mm, 5 µm pore size) with a mobile phase of water: tetrahydrofuran (60:40) flowed at 1 mL/min. BDMC peak was identified based on retention time (RT) matched with the corresponding reference standard.

Enzyme inhibition by Ellman assay

The inhibition of cholinesterase activities was determined using Ellman's assay[26] with modifications in the concentration of the substrate and enzyme used. Briefly, 200 µL of the reaction mixture in a 96-well plate contained 5 µL of AChE (0.012 U/mL, pH 7.8 sodium phosphate buffer) or BuChE (0.05 U/mL, pH 7.8 sodium phosphate buffer), 100 µL of DTNB (1.5 mM in pH 7.8 sodium phosphate buffer), and 20 µL of different concentrations of REVERC3 or galantamine. The reaction mixture was incubated at 25°C for 10 min and then 5 µL of the respective substrate solutions for AChE (0.75 mM ATC) or BuChE (0.75 mM BTC) assays were added to initiate the reaction. After 15 min incubation, the absorbance was measured at 405 nm using Ascent Multiskan EX plate reader. The assays were performed in triplicates. The percentage of inhibition was calculated as follows

$$\%\ \text{Inhibition} = \frac{(A-a)\ -\ (B-b)}{A-a} \times 100$$

where, A is the enzyme activity without inhibitor; B is the activity with inhibitor; a and b are the negative controls without and with inhibitor, respectively.

IC50 was determined by nonlinear regression analysis performed using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA, USA).

Kinetic analysis of enzyme inhibition

The kinetics of inhibition of AChE and BuChE activities at various substrate and inhibitor concentrations were studied. Two concentrations of REVERC3 and galantamine were evaluated for inhibition of enzyme activity using different ATC (0.1, 0.2, and 0.4) and BTC (0.1, 0.25, 0.5, 0.75, and 1 mM) substrate concentrations. The changes in reaction velocity were determined as a function of maximum velocity (V_{max}) and Michaelis constant (K_m). The pattern of inhibition was determined using Lineweaver–Burk (L-B) double reciprocal plot, where a graph of 1/change in absorbance (ΔAb/min) was plotted against 1/[substrate]. The identification of the type of inhibition was based on point of intersection of lines. The L-B plots and kinetic parameters K_m and V_{max} were obtained using GraphPad Prism.

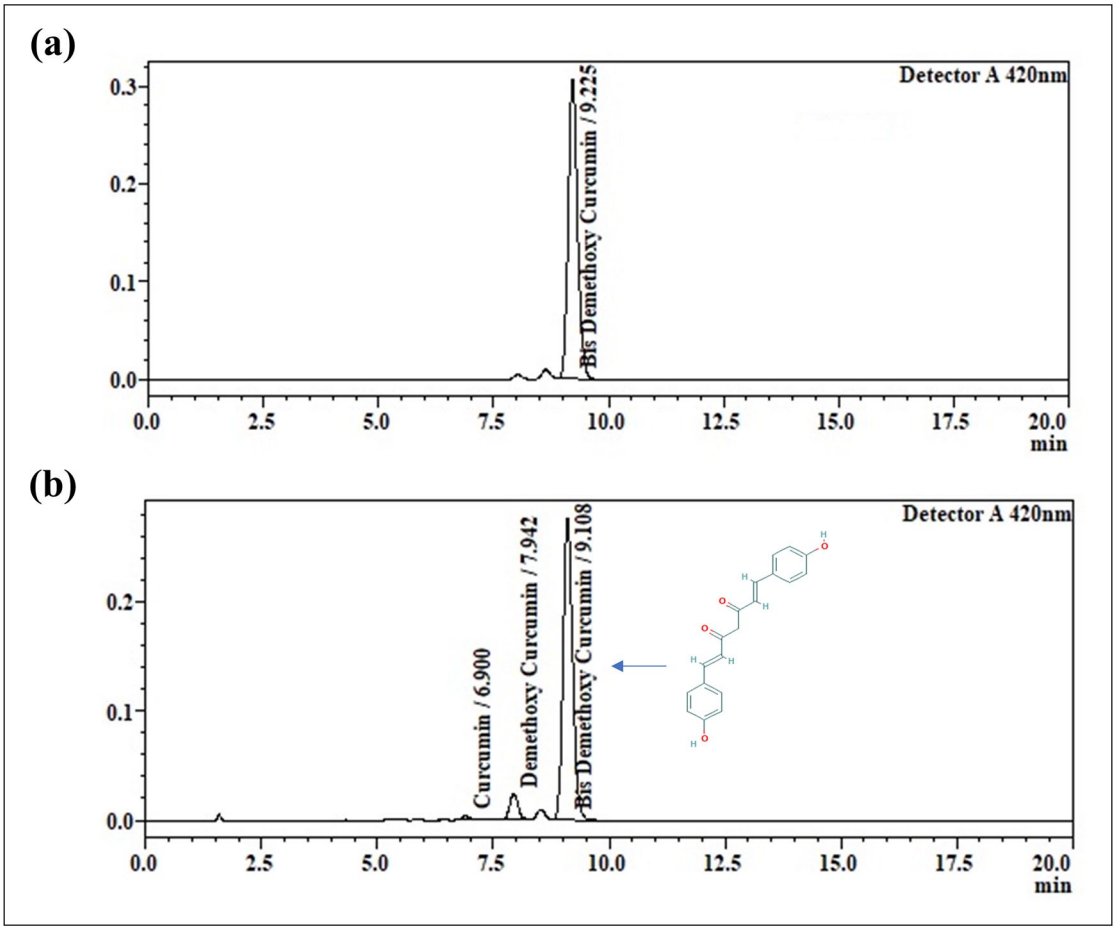

Figure 1. HPLC chromatogram of bisdemethoxycurcumin reference standard (a) and REVERC3 (b).

Molecular docking

The crystal structures of recombinant human AChE bound to donepezil (PDB ID: 4EY7, R = 2.35 Å) and BuChE (PDB ID: 1P0P, R = 2.30 Å) bound to BTC were downloaded from PDB database (http://www.rcsb.org/) in .pdb format. The coordinates of PDB structures were prepared for molecular docking by removing the water ions and ligands using Python molecule viewer. AutoDock tool (ADT 1.5.4) was used to add polar hydrogens and Gasteiger charges. The three-dimensional (3D) structure of BDMC was obtained from Pubchem (https://pubchem.ncbi.nlm.nih.gov). The druggability was determined using SWISSADME prediction (http://www.swissadme.ch/). 3D coordinates were prepared using PRODRG server.

The active site amino acid residues of AChE and BuChE were retrieved from the literature.[27,28] Molecular docking was performed using AutoDock 4.2. Autogrid was utilized to prepare the grid maps using a grid box size of $50 \times 50 \times 50$ xyz points and the active site of AChE (x = 20.823, y = 16.078, and z = 18.939) and BuChE (x = 137.156, y = 113.437, z = 43.769). The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters.

Statistical analysis

IC50 was determined using nonlinear regression analysis and Lineweaver–Burk plots were drawn using linear regression analysis. The analyses were performed, and the graphics generated by GraphPad Prism 5.0.

Results

Quantitative analysis of curcuminoids in REVERC3

The curcuminoids were quantified in REVERC3 by HPLC analysis (Figure 1). The extract has >70% BDMC, >2% curcumin, and >6% DMC.

Determination of cholinesterase inhibition activity

The cholinesterase inhibitory potentials of REVERC3 was determined and compared with regular turmeric extract. Galantamine was used as the standard inhibitor. Table 1 and Figure 2 shows the results of the inhibition assay performed against AChE and BuChE enzymes. As expected, galantamine was far the most potent inhibitor of

Table 1. Comparison of IC50 values of inhibitors against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).

Inhibitor	AChE IC50 value (µg/mL)	Fold difference relative to galantamine	BuChE IC50 value	Fold difference relative to galantamine
Galantamine	0.31	1	9.9	1
REVERC3	29.08	93.8	33.59	3.39
Regular turmeric extract	139.2	449.03	180.9	18.27

AChE: acetylcholinesterase; BChE: butyrylcholinesterase.

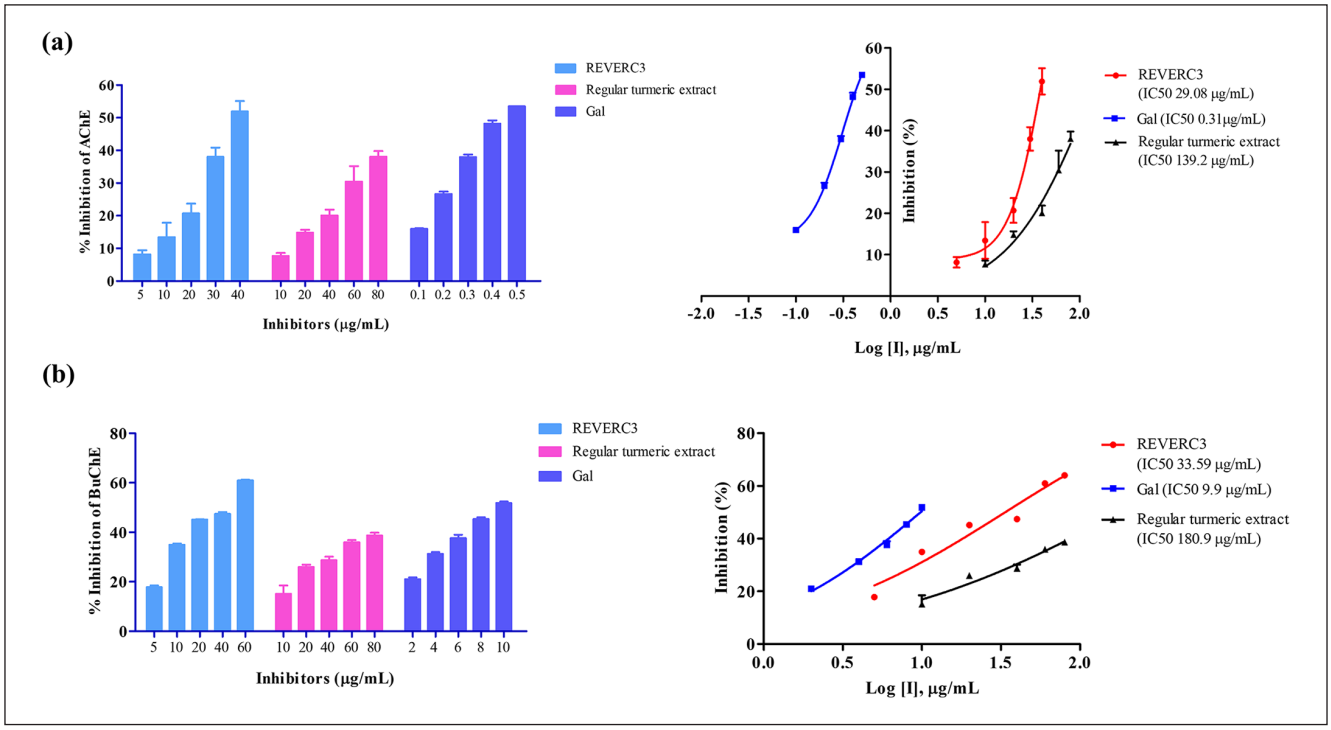

Figure 2. Determination of inhibitory effect of REVERC3 against the cholinesterases. The activities of acetylcholinesterase (AChE) (a) and butyrylcholinesterase (BuChE) (b) were measured in the presence of different concentrations of inhibitors.

cholinesterases with IC50 values of 0.31 and 9.9 µg/mL for AChE and BuChE activities, respectively. REVERC3 exhibited higher AChE inhibitory activity (IC50 29.08 µg/mL) compared to regular turmeric extract (IC50 139.2 µg/mL). REVERC3 and turmeric extract showed 93.8- and 449.03-fold difference relative to galantamine, respectively. A similar trend was observed in BuChE inhibition. REVERC3 demonstrated greater potency with an IC50 value of 33.59 µg/mL compared to regular turmeric extract (180.9 µg/mL).

Inhibition kinetics of cholinesterases

AChE kinetic analysis was performed using different substrate and inhibitor concentrations. Figure 3a shows the Michaelis–Menten graph and the reciprocal L-B plot of AChE activity in the presence and absence of galantamine. From the data, it appears that galantamine exhibits mixed inhibition of AChE. On the contrary, it was observed from the kinetic analysis that REVERC3 demonstrated a competitive mode of inhibition (Figure 3b).

Different concentrations of galantamine and REVERC3 were further tested for the inhibition of BuChE enzyme and the kinetic parameters determined. Galantamine was found to inhibit the enzyme activity competitively (Figure 4a), whereas REVERC3 exhibited uncompetitive inhibition (Figure 4b). REVERC3 had reduced V_{max} (0.04) and K_m (139 µM) values compared to the enzyme activity without inhibitor (V_{max} 0.08, K_m 289.4 µM).

Molecular docking

BDMC is the major active constituent in REVERC3. Here, we have investigated the binding position of BDMC into the active sites of cholinesterases. Initially, SWISSADME was used to predict the druggability of the molecule based on Lipinski's rule of five. We found that BDMC satisfied the druggability criteria (Table 2).

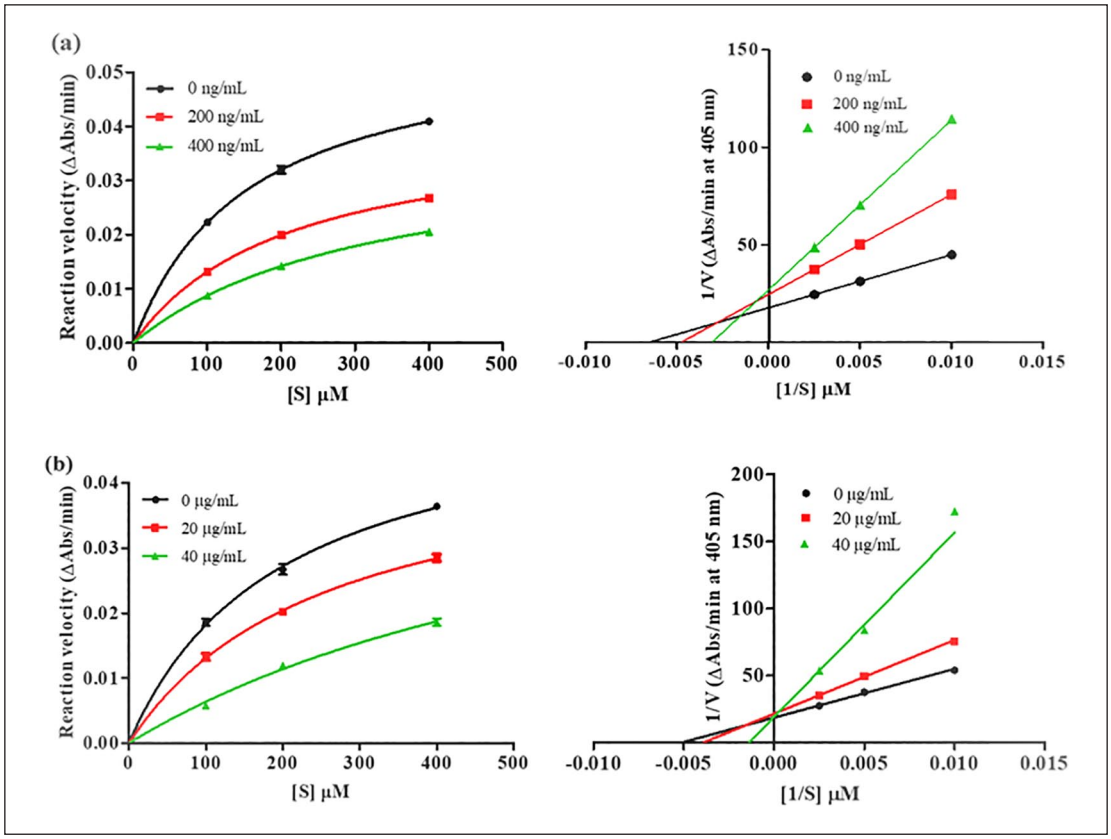

Figure 3. Inhibition kinetics of acetylcholinesterase (AChE) activity in the presence of different concentrations of substrate and the inhibitor. The data are presented as graphics of nonlinear (Michaelis–Menten graph) and linear regressions (Lineweaver–Burk plots) in the presence or absence of two concentrations of galantamine (a) and REVERC3 (b).

AutoDock 4.2 was used to perform the molecular docking analysis. Figure 5 shows the 3D crystal structures of the cholinesterases.

AChE and BuChE enzymes have several domains involved in the substrate binding.[26] In the case of AChE, the catalytic triad is formed by Ser203, Glu334, and His447. The anionic site involved in the binding of choline moiety of ACh contains the aromatic amino acids: Tyr130, Trp86, Tyr337, and Phe338. Another important region in the binding site is the acyl pocket required for the selective binding of ACh (Phe295 and Phe297). Furthermore, the oxyanionic hole formed by Gly121, Gly122, and Ala204 is the site where the structural water molecule stabilizes the enzyme-substrate complex. There exist peripheral anionic site (PAS) in proximity with the catalytic site of the enzyme which allosterically regulates the catalysis. PAS is formed by five residues: Asp74, Tyr72, Tyr124, Trp286, and Tyr341 (Figure 6a). The human BuChE active site contains binding domains like AChE. However, the small structural differences in the active site are evident due to the difference in several amino acid residues determining the binding domains in the active site (Figure 6b).

BDMC was docked into the active sites of the cholinesterases and the top 10 binding poses were analyzed. The best binding conformation of BDMC with AChE active site showed profound interaction of the molecule with the substrate-binding site of the enzyme. BDMC exhibited hydrogen bond interaction with the key residue Phe295 in the acyl pocket of the active site (Figure 7a). The lowest binding energy was –7.3 kcal/mol with K_i value of 4.47 µM (Table 3).

The preference of BDMC for the binding site of BuChE was different as compared to that for AChE. Here, we have used the substrate (BTC) bound enzyme as the receptor. BDMC was found to have H-bond interactions with the key residues of the catalytic triad His438 and Ser198. The affinity of BDMC with the enzyme-substrate complex was appreciable (Ki = 4.73 µM) with a binding energy of –7.27 kcal/mol.

Discussion

Age-related deterioration of health conditions includes loss of cognitive functions due to the decline in cholinergic transmission as a result of disturbances in the ACh metabolism in the brain.[29] In this regard, cholinesterases are the prime targets in the treatment of AD.[30] AChE inhibitors including natural plant alkaloid galantamine have been clinically tested to improve the cholinergic functions.[31] In this study, we have

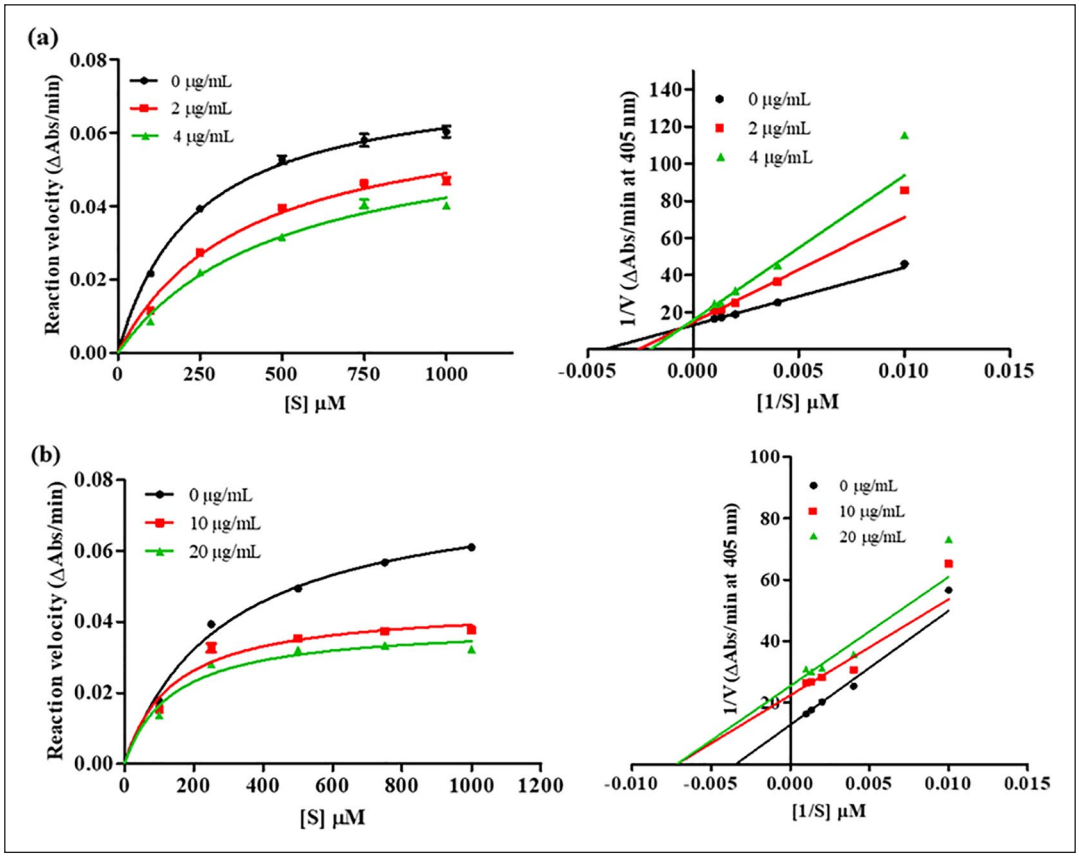

Figure 4. Kinetic analysis of BuChE inhibition. Different concentrations of galantamine (a) and REVERC3 (b) were incubated with various concentrations (100–1000 µM) of butyrylthiocholine iodide (BTC). The data are presented as graphics of nonlinear (Michaelis–Menten graph) and linear regressions (Lineweaver–Burk plots).

evaluated the cholinesterase inhibitory effect of a standardized turmeric extract containing more than 70% BDMC. Conventionally regular turmeric extract contains curcumin as the major curcuminoid and the content of BDMC is only 2%–3%. Here, we have used a BDMC-enriched extract (REVERC3) to study the possible mechanism of cholinesterase inhibition. Initially, the cholinesterase enzyme inhibition assays were performed using different concentrations of REVERC3 and the regular turmeric extract. Interestingly, REVERC3 demonstrated higher inhibitory activity compared to the regular turmeric extract. Furthermore, the BDMC-enriched extract was found to inhibit both AChE and BuChE activities appreciably. The respective IC50 values of REVERC3 for the inhibition of AChE and BuChE were 4.8- and 5.39-fold lower relative to regular curcumin. The drastic increase in the inhibitory ability of REVERC3 could be largely attributed to the enriched content of BDMC in the extract.

Furthermore, we have determined the mode of inhibition of the cholinesterases by galantamine and REVERC3 using LB reciprocal plots. The extract exhibited competitive inhibition of AChE activity. The K_m value was increased while V_{max} remained unaffected in the presence of REVERC3.

Table 2. Drug-like properties of BDMC and galantamine.

Lipinski's rule of five	BDMC	Galantamine
Molecular weight (<500 Da)	308.33	287.35
MLog P (<4.15)	2.13	1.74
H-Bond donor (5)	2	1
H-Bond acceptor (<10)	4	4
Violation	0	0

BDMC: bisdemethoxycurcumin.

Previously, galantamine was found to competitively inhibit AChE activity.[32] However, in this study, galantamine demonstrated mixed inhibition. This could be explained by the difference in the experimental conditions. REVERC3 showed the uncompetitive mode of inhibition of BuChE activity as the V_{max} and K_m were reduced in the presence of the extract. Galantamine appeared to exhibit competitive inhibition.

Based on the inhibition data, we further rationalized the role of BDMC in REVERC3 by predicting its binding into the active sites of cholinesterases. Molecular docking analysis revealed that BDMC could strongly interact with the

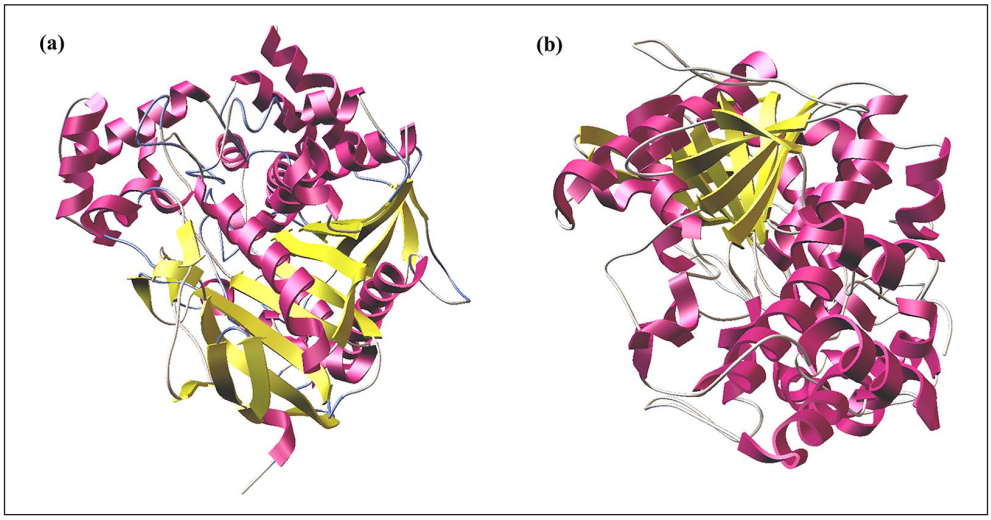

Figure 5. 3D crystal structures of acetylcholinesterase (AChE, PDB ID: 4EY7) (a) and butyrylcholinesterase (BuChE, PDB ID: 1P0P) (b). The protein structures were retrieved from RCSB Protein Data Bank (www.rcsb.org/).

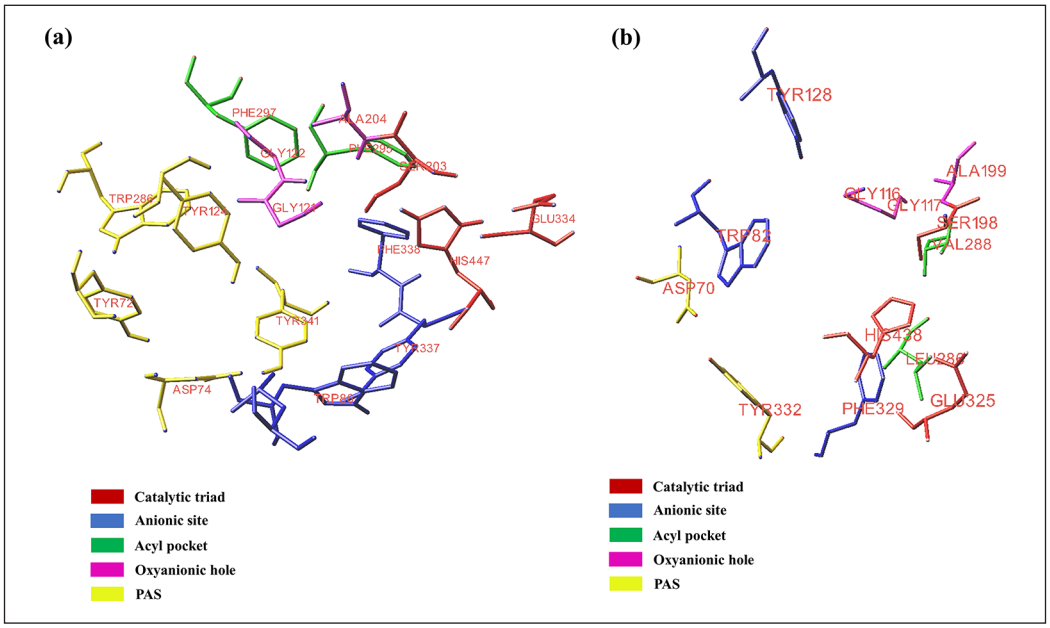

Figure 6. Active sites of recombinant human acetylcholinesterase (PDB ID: 4EY7) (a) and human butyrylcholinesterase (PDB ID: 1P0P) (b).

catalytic site of AChE with low binding energy and K_i value. It was observed that the molecule had H-bond interaction with Phe295 residue in the acyl pocket of the active site. The competitive nature of inhibition of REVERC3 is well supported by the docked binding poses.

Further to investigate the interactions of BDMC with the active site residues of BuChE, we have used the substrate-bound protein as the receptor. The substrate was found to have interactions with the active gorge residues especially the Asp70 in the PAS region. BDMC could strongly interact with Ser198 and His438 residues in the catalytic domain, in presence of the substrate. This could explain the mode of binding of BDMC exerting the uncompetitive inhibition of BuChE.

There are several studies reporting the beneficial role of curcumin in cognitive improvement and Alzheimer's pathological manifestations such as Aβ aggregation.[33,34] However, the poor bioavailability of curcumin cannot be ignored.[35] This study unveils the improved benefits of BDMC over curcumin in inhibiting the key enzymes associated with neurodegeneration. Previously, it has been shown that BDMC is comparatively more stable than curcumin in physiological conditions.[36] Findings from our study rationalize the use of a BDMC-rich turmeric extract as a functional ingredient in

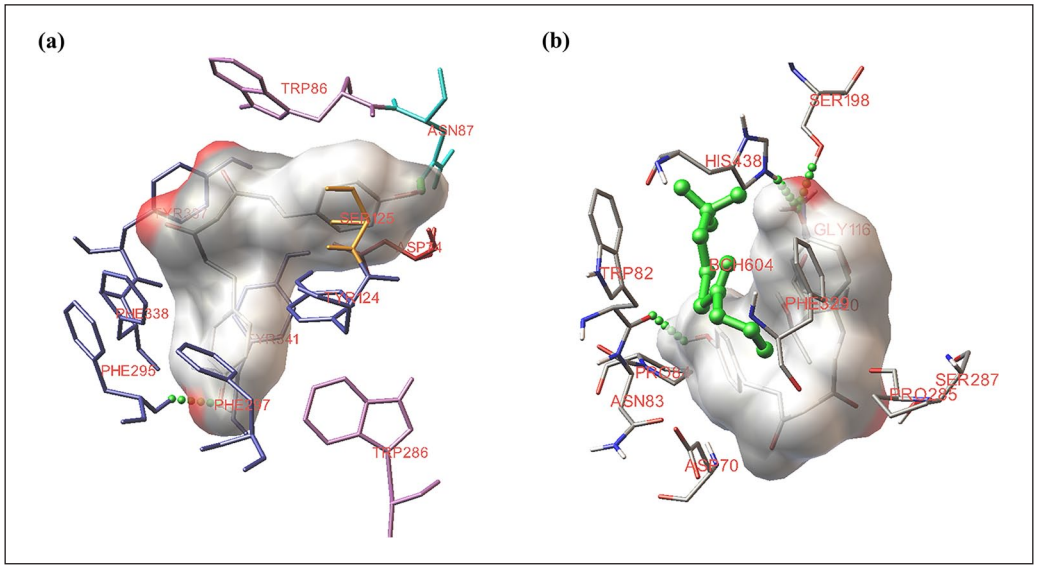

Figure 7. Interaction of BDMC with the active site of cholinesterases. Representative images of BDMC (molecular structure) binding pose conformation with the active site of AChE (a) and BuChE (b). BDMC was docked in the active site of BuChE in the presence of the bound substrate, butyrylthiocholine (Green).

Table 3. Molecular docking analysis of BDMC against cholinesterase active sites.

Enzyme	Binding energy (kcal/mol)	Ligand efficiency	Inhibition constant (μM)	Intermolecular energy	VDW-H bond desolvation energy
AChE	–7.3	–0.32	4.47	–10.28	–10.16
BuChE	–7.27	–0.32	4.73	–10.25	–10.09

AChE: acetylcholinesterase; BChE: butyrylcholinesterase; BDMC: bisdemethoxycurcumin; VDW-H: Van der Walls-H-bond.

food and health supplements for brain health. The present investigation however is limited to the in vitro studies. Further insights into the molecular aspects related to neuronal health such as Aβ aggregation and toxicity, tau hyperphosphorylation and neurotransmission using cellular and preclinical models are required to validate the neuroprotective properties of BDMC.

Conclusion

Data from this study provides preliminary evidence on the possible role of a BDMC-rich turmeric extract in mitigating the cognitive deficits as a function of cholinesterase inhibition. Furthermore, it is important to note that REVERC3 could act as a dual inhibitor of cholinesterases which substantiates the potential neuroprotective effects of BDMC.

Authors' contributions

S.K. contributed to conceptualization. S.H.V., G.K., and A.R. contributed to inhibition and kinetic studies. S.H.V. contributed to computational analysis. C.S. contributed to analytical data analysis. S.K. contributed to supervision. S.H.V. and A.R. contributed to writing—original draft. S.H.V. and S.K. contributed to writing—review and editing.

Declaration of conflicting interests

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