



## Original article

# Cholinesterase inhibitory activity of tinosporide and 8-hydroxytinosporide isolated from *Tinospora cordifolia*: *In vitro* and *in silico* studies targeting management of Alzheimer's disease

Mohiminul Adib<sup>a</sup>, Rashedul Islam<sup>a</sup>, Monira Ahsan<sup>a</sup>, Arifur Rahman<sup>b</sup>, Mahmud Hossain<sup>c</sup>, Md Mustafizur Rahman<sup>d</sup>, Sultan M. Alshehri<sup>e,f</sup>, Mohsin Kazi<sup>e</sup>, Md Abdul Mazid<sup>a,\*</sup>

<sup>a</sup> Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka 1000, Bangladesh

<sup>b</sup> Department of Pharmacy, Jagannath University, Dhaka 1100, Bangladesh

<sup>c</sup> Department of Biochemistry and Molecular Biology, University of Dhaka, Dhaka 1000, Bangladesh

<sup>d</sup> Pharmacy Discipline, Khulna University, Khulna 9208, Bangladesh

<sup>e</sup> Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia

<sup>f</sup> Department of Pharmaceutical Sciences, College of Pharmacy, Almaarefa University, Riyadh 11597, Saudi Arabia



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## ABSTRACT

Tinosporide and 8-hydroxytinosporide isolated from *Tinospora cordifolia* were evaluated for acetylcholinesterase (AChE) and butylcholinesterase (BuChE) inhibitory activities. The structure of the compound was confirmed by spectroscopic analysis, whereas cholinesterase inhibition was investigated by Ellman method using donepezil as standard drug and the data were presented as IC<sub>50</sub> (μg/ml ± SEM). Furthermore, donepezil, tinosporide and 8-hydroxytinosporide were executed for docking analysis. The results from the isolated compounds TC-16R confirmed as tinosporide promisingly inhibited AChE with IC<sub>50</sub> value of 13.45 ± 0.144, whereas TC-19R confirmed as 8-hydroxytinosporide moderately inhibited AChE with IC<sub>50</sub> value of 46.71 ± 0.511. In case of BuChE inhibition, the IC<sub>50</sub> values were found to be 408.50 ± 17.197 and 317.26 ± 6.918 for tinosporide and 8-hydroxytinosporide, respectively. The *in silico* studies revealed that the ligand tinosporide fit with the binding sites and inhibited AChE. Overall, the study findings suggested that tinosporide would be a complementary noble molecule of donepezil which is correlated with its pharmacological activity through *in vitro* studies, while 8-hydroxytinosporide modestly inhibited BuChE and the results are very close to the standard donepezil.

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## 1. Introduction

As a multifactorial syndrome having different relatable proteins that are responsible for the etiology, Alzheimer's disease (AD) is a degenerative disorder which progressively causes the loss of basal forebrain neurons, ultimately lower the cortical and hippocampal level of acetylcholine (ACh) (Melnikova, 2007). AD occurs due to the cholinesterase induced declination of neurotransmitters most

commonly to the people in their mid-60s. Enzymes involved in breaking down of neurotransmitters like ACh and butyrylcholine (BuCh) are respectively called acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Yusoff et al., 2014). The therapeutic use of acetylcholinesterase inhibitors (AChEI) has been provided by observing a correlation between cholinergic dysfunction and AD severity (Sabbagh, 2009). The inhibition of AChE has been proven as a successful way to relieve some cognitive and behavioral symptoms of the AD and therefore, several AChE inhibitors, such as tacrine (Davis et al., 1992), galanthamine (Thomsen and Kewitz 1990), huperzine A (Xu et al., 1995) and donepezil (Courtney, Farrell et al. 2004) have been used mainly for the clinical treatment of AD. However, the level of cognitive decline is associated with these drugs and further brain studies of the advanced stage age and AD have consistently experienced damage and anomalies specially in basal forebrain projections.

\* Corresponding author at: Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka 1000, Bangladesh.

E-mail address: [ma.mazid@du.ac.bd](mailto:ma.mazid@du.ac.bd) (M.A. Mazid).

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With the development of “cholinergic hypothesis”, the current pharmacotherapy for AD aims to increase the levels of ACh through the AChE and BuChE inhibition. As BuChE is capable of compensating for missing AChE catalytic functions in the synaptic cleft with the increase in activity by 30–60% during AD (Jhee, Shiovitz et al., 2002; Mesulam, Guillozet et al., 2002; Darvesh, Hopkins et al., 2003; Roger and Roger, 2007; Kamal, Klein et al., 2008), cholinesterases (ChEs) inhibition using a dual inhibitor result in increased levels of ACh in the brain, which provide more successful clinical efficacy of AD (Basiri, Murugaiyah et al., 2013; Khoobi, Alipour et al., 2013). Search for newer molecules from natural products has gained much attention by the researchers’ worldwide despite of having synthetic drugs available for the symptomatic treatment of AD.

Majority of the secondary metabolites with potential AChE and BuChE inhibitory activity are alkaloids followed by terpenes, sterols, flavanoids, and glycosides and compounds having potential anticholinesterase activity are isolated from *Buxaceae*, *Amaryllidaceae*, *Lycopodiaceae*, *Lamiaceae*, *Chenopodiaceae*, *Papaveraceae*, *Apocynaceae*, and *Labiatae* species (Ata, Iverson et al., 2010; Ahmed, Ghalib et al., 2013). Variety of secondary metabolites show AChE activity include vasicinone, vasicine, harmine, deoxyvasicinone, deoxyvasicine, harmaline, harmol, and harmane (Zhao, Ding et al. 2013). For a large number of enzyme-ligand complexes, it is determined by X-ray crystallography that the three-dimensional structure of AChE, reveals two main binding sites: the catalytic active site (CAS), comprising the Ser-His-Glu catalytic triad, and the peripheral anionic site (PAS), connected by a deep, hydrophobic gorge (Sussman, Harel et al., 1991; Bourne, Grassi et al., 1999; Kryger, Harel et al., 2000).

In this study, two secondary metabolites, named tinosporide and 8-hydroxytinosporide were isolated from *Tinospora cordifolia* and identified them by <sup>1</sup>D and <sup>2</sup>D NMR spectroscopy using <sup>1</sup>H, <sup>13</sup>C, HSQC, HMBC, COSY, and NOESY. *In vitro* studies were carried out to develop these two metabolites as potential anticholinesterase agents by determining the inhibitory activities using Ellman’s colorimetric method. Finally, with the help of molecular docking, we have explored the ability of the metabolites as potent inhibitors of AChE and elucidated the possible mechanism of action through *in silico* study.

## 2. Experimental

### 2.1. Instrumentations

The column was initially packed with fine vacuum liquid chromatography (VLC) grade silica (Kiesel gel 60H), then some column and VLC fractions were further fractionated by gel permeation chromatography using Sephadex-LH20. The <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded with Bruker instrument (Rheinstetten, Germany) spectrometer using deuterated chloroform (CDCl<sub>3</sub>) as a solvent. Reaction progress and the product mixtures were routinely checked by thin-layer chromatography (TLC) on Merck silica gel 60F254 (Darmstadt, Germany) aluminum plates.

### 2.2. General procedure for the isolation of tinosporide and 8-hydroxytinosporide

The methanolic extract (1 gm) of the stem of *T. cordifolia* (Willd.) Hook. f. and Thoms. (Family: Menispermaceae) were subjected to vacuum liquid chromatography (VLC) and gravity column chromatography. The column was packed with fine VLC grade silica (Kiesel gel 60H, Merck). The ethyl acetate extract was adsorbed with column grade silica and then added to VLC column. The column with the extract was first washed with 100% petroleum ether

and the polarity of the eluent was increased by using dichloromethane, ethyl acetate and methanol in appropriate amount. Finally the column was washed with 100% methanol.

After initial screening by thin layer chromatography, ethyl acetate soluble VLC fractions were further fractionated by gravity column chromatography. The VLC column fraction further on gravity column eluted with toluene and ethyl acetate in 40:60% yielded white residues. Upon repeated washing with hexane–ethyl acetate afforded compound coded as TC-16R (6 mg) on TLC followed by 1% vanillin-sulfuric acid spray, it showed purple color with the R<sub>f</sub> value of 0.78 [mobile phase- toluene: ethyl acetate (4:1)]. On the other hand VLC fraction on gravity column eluted with 30:70% toluene: ethyl acetate provided compound coded as TC-19R (5.5 mg) which on TLC followed by 1% vanillin-sulfuric acid spray showed purple color and the R<sub>f</sub> value was 0.59 [mobile phase: toluene: ethyl acetate (4:1)]. The isolated pure compounds were then characterized by extensive spectroscopic studies like <sup>1</sup>H NMR, <sup>13</sup>C NMR, HSQC, HMBC, <sup>1</sup>H–<sup>1</sup>H COSY and <sup>1</sup>H–<sup>1</sup>H NOESY experiments. Compound TC-16R was identified as tinosporide and TC-19R was identified as 8-hydroxytinosporide.

### 2.3. Chemicals and reagents

Tris-HCl and bovine serum albumin were from Merck, Germany and, 5-dithio-bis-(2-nitro) benzoic acid (DTNB), acetylcholine iodide, butyrylcholine iodide, Triton X-100, BCA (bicinchoninic acid) kit, and trichloroacetic acid were purchased from Sigma-Aldrich, Germany. As a kind gift from Incepta Pharmaceuticals Ltd., donepezil was collected. Other chemicals required were collected from local sources.

## 3. Methods

### 3.1. *In vitro* AChE inhibition assay

With minor modification using acetylthiocholine iodide as a substrate, inhibition of AChE was assessed following Ellman method (Ellman, Courtney et al. 1961; Rashedul Islam 2019). Rat brains were homogenized in a homogenizer with 5 volumes of a homogenization buffer [10 mM Tris-HCl (pH 7.2), as a source of AChE, that contained 1 M NaCl, 50 mM MgCl<sub>2</sub> and 1% Triton X-100]. The mixture was then centrifuged at 10,000 rpm for 15 min and the supernatant was used as an enzyme source. The process of enzyme preparation was carried out at 4 °C and the protein concentration was measured using the BCA (bicinchoninic acid kit) using BSA as a protein standard. To carry out the reaction, each tested compound or standard (500 μl) was mixed with an enzyme solution (500 μl) and incubated at 37 °C for 15 min, and then Ellman’s reaction mixture [(3.5-ml) 0.5 mM acetylthiocholine, 1 mM 5, 5-dithio-bis (2-nitro benzoic acid) in a 50 mM sodium phosphate buffer (pH 8.0)] were added. Immediately after adding Ellman’s reaction mixture, the rate of hydrolysis by acetylcholinesterase were monitored spectrophotometrically reading absorbance at 405 nm. From the absorbance of control and test sample, AChE inhibition was calculated and expressed in percentage (Rashedul Islam, 2019).

### 3.2. *In vitro* BuChE inhibition assay

BuChE assay was performed by following the AChE inhibition assay method, using butyrylthiocholine iodide as a substrate. Nevertheless, human blood was homogenized in a homogenizer as a source of the enzyme with 5 volumes of a homogenization buffer [10 mM Tris-HCl (pH 7.2), that contained 1 M NaCl, 50 mM MgCl<sub>2</sub> and 1% Triton X-100, and centrifuged at 10000 rpm for 15 min. The

resulting supernatant was used as an enzyme. BuChE inhibition was calculated and expressed in percent as mentioned above for AChE inhibition assay.

#### 4. In silico molecular docking analysis

To explore intermolecular interactions, docking studies were performed for tinosporide and 8-hydroxytinosporide. The interacted molecular properties were then compared with standard donepezil. Docking of the two compounds were subjected against AChE and BuChE. Docking studies were performed using software (AutoDock vina) and docking server (Mcule). Followed by, each docking job was carried out for “Docking score” comparison studies. Receptor-ligand interaction studies were performed through different visualization tools like PyMOL, Discovery Studio Visualizer, and PyRx to catch up the best output.

The crystal structure of AChE (4pqe) and BuChE (6esy), were downloaded from the RCSB protein databank (PDB). All the crystallographic water molecules, as well as all heteroatoms were removed as they may interfere in binding. Proteins were prepared by adding polar hydrogens as well Gasteiger charge using Auto Dock Tools 1.5.6. The crystal structure of PDB ID: 4pqe and 6esy were used as the target for the docking studies. Selected structures were also prepared for docking studies by adding polar hydrogen and Gasteiger charge. Finally, the energy of candidates was minimized for docking purpose.

##### 4.1. ADMET analysis

Tinosporide and 8-hydroxytinosporide were subjected to ADMET studies to find the best pharmacokinetic profile with lower toxicity. The ultimate objective of the *in silico* ADMET studies was to accurately predict the *in vivo* pharmacokinetics of these lead molecules (Gleeson, Anne et al., 2011; Foster D, 2014). In this study, our goal was to focus on the physicochemical properties, lipophilicity, water solubility, pharmacokinetics, drug-likeness (Lipinski's rule of five, Ghose rules etc.) of the tested compounds (Gleeson, Anne et al. 2011). Moreover, AMES toxicity, acute oral toxicity, carcinogenicity, etc. were also evaluated using admetSAR and SwissADME servers.

##### 4.2. Statistical analysis

The data analyzed by one-way ANOVA with  $p < 0.05$  and  $p < 0.01$  were considered statistically significant. The results presented as mean  $\pm$  SEM (standard errors) of the triplicate experiment. The  $IC_{50}$  values of the compounds were evaluated by non-linear regression analysis using GraphPad Prism Data Editor for Windows, Version 6.0 (GraphPad Software Inc., San Diego, CA).

## 5. Results

### 5.1. NMR spectroscopic data and identification of the isolated compounds

The isolated compound TC-16R was obtained as white residue. The  $^1H$  NMR spectrum of compound TC-16R displayed signals at  $\delta$ 6.65 (br s),  $\delta$ 7.62 (br s) and  $\delta$ 6.69 (br s) that was assigned to the protons of the  $\beta$ -substituted furan moiety. Two tertiary methyl groups resonating at  $\delta$ 1.48 and  $\delta$ 1.29 were observed as singlets. The signals at  $\delta$ 5.96 (1H, dd,  $J = 12.2, 4.1$  Hz), was assigned to the C12 proton, which bear the  $\beta$ -substituted furan moiety. C-11 methylene protons attributed signals with aliphatic region at  $\delta$ 2.46 (1H, dd,  $J = 14.7, 4.1$  Hz) and  $\delta$ 2.29 as multiplet. The HOMO-COSY correlation profile displayed two multiplets at  $\delta$ 2.88

and 2.03 were assigned to protons at C-7. The experience of anisotropic effect of the lactone ring had made this downfield signal at 2.88 which was allocated to an axial proton on C-7. The signals at  $\delta$ 5.23 (d, 2.6 Hz),  $\delta$ 3.97 (dd, 2.6, 4.3) and  $\delta$ 4.01 (d, 4.3 Hz) were given to protons on C-1, C-2 and C3 of ring A. Furthermore, the spectrum exhibited two oxymethine protons at  $\delta$  5.23 (d,  $J = 2.6$  Hz,  $\delta$ c 71, H-1) and  $\delta$  5.96 (dd,  $J = 12.2, 4.1$  Hz,  $\delta$ c 70.9, H-12), as well as a number of methylenes and methines appearing in the high field region from  $\delta$ 2.31 to 2.88. The  $^{13}C$  NMR spectrum showed 20 carbons including two carbonyl carbons at  $\delta$ c172.5 and  $\delta$ c173.8. The presence of two oxymethine protons together with two carbonyl protons suggested the presence of two lactone rings which was further substantiated by HMBC experiment. Location of two lactone groups and furan ring was established by HMBC experiment as H-1 and H-8 protons revealed 3 J and 2 J correlations to  $\delta$ c172.5 and 173.8, and H-12 proton showed 2 J and 3 J correlations to C-13 ( $\delta$ c125.9) and C-16 ( $\delta$ c140.3), respectively.  $^1H$ - $^1H$  COSY and  $^1H$ - $^1H$  NOESY experiments showed the expected correlation. On the basis of the spectroscopic data (Table 1), compound TC-16R was identified as tinosporide (Fig. 1). Compound TC-19R also exhibited a similar proton and carbon NMR profile, except the presence of carbon bearing the tertiary hydroxyl group (Table 1, Fig. 1).

### 5.2. In vitro AChE and BuChE inhibitory activity

The inhibitory effects of the tested compounds are shown in Table 2. From the obtained data, it was observed that tinosporide showed promising activity in inhibiting AChE with the  $IC_{50}$  value of  $13.45 \pm 0.144$  ( $\mu$ g/ml  $\pm$  SEM), whereas 8-hydroxytinosporide exhibited moderate activities with the value of  $46.71 \pm 0.511$  ( $\mu$ g/ml  $\pm$  SEM) (Table 2). None of them showed any significant BuChE inhibitory activity.

### 5.3. In silico molecular docking analysis for AChE and BuChE

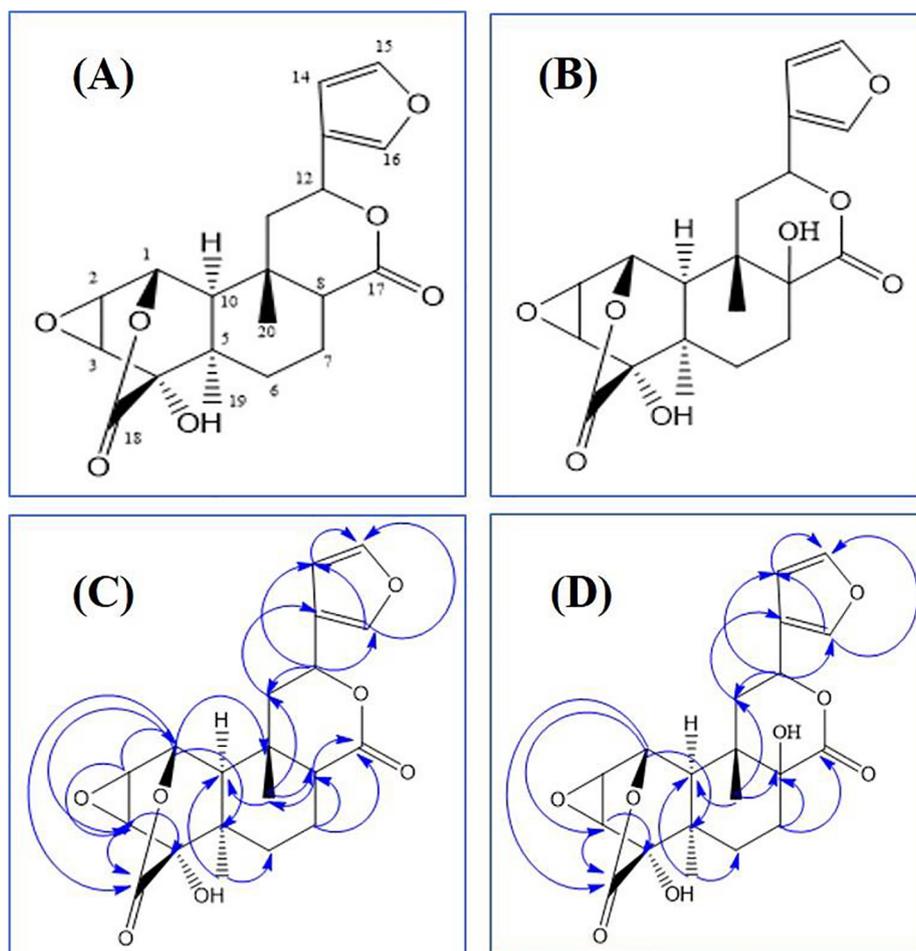
To investigate the interaction of *in vivo* studied ligands, interactions with the respective target macromolecule *in silico* molecular studies were carried out (Fig. 2). The protein AChE crystal structure comprises of chain A and BuChE crystal structure comprises of both chain A and B in its structure were docked using AutoDock Vina and Mcule along with the standard drug donepezil with target protein chain. The subsequent binding energy was found to be  $-8.3$  kcal/mol and  $8.2$  kcal/mol for AChE, and  $9.9$  kcal/mol and  $-9.3$  kcal/mol for BuChE target, respectively thus having  $< 200$  RMSD value (Fig. 2, Table 3). Whereas in between bioactive molecule 8-hydroxytinosporide and AChEdocked conformation, the ligand generously embedded in the binding pocket of AChE attaining binding energy of  $-9.0$  kcal/mol (AutoDock vina) and  $-8.7$  kcal/mol (Mcule) and for BuChE, the binding energy was of  $-9.5$  kcal/mol (AutoDock vina) and  $-9.9$  kcal/mol (Mcule) correspondingly having  $< 2.00$  RMSD value. Elsewhere, the ligand tinosporide appeared to be embedded in the deeper core of the protein binding pocket seems like in the heart of the pocket having a binding energy of  $-8.7$  kcal/mol (AutoDock vina) and  $9.0$  kcal/mol (Mcule), whereas with BuChE, the values were  $-8.8$  kcal/mol (AutoDock vina) and  $-9.4$  kcal/mol (Mcule) with the same RMSD limit (Table 3). Post-docked binding mode of the selected compound exposed to be smoothly embedded in the target protein binding pocket (Fig. 3, and Fig. 4).

## 6. Discussion

According to the NMR spectroscopic data isolated compound TC-16R was confirmed as tinosporide. On the other hand, isolated

**Table 1**<sup>1</sup>H NMR, <sup>13</sup>C NMR and HMBC data for Compounds TC-16R identified as tinosporide and TC-19R identified as 8-hydroxy tinosporide.

SI. TC-16R			TC-19R		
$\delta_C$	$\delta_H$	HMBC	$\delta_C$	$\delta_H$	HMBC
1	71.0 5.23 d (2.6)	34.9 (C-9), 41.6 (C-5), 52.1 (3), 50.0 (2), 172.5 (C-18)	70.9 5.37br s		41.6 (C-5), 52.3 (3), 172.6 (C-18)
2	50.0 3.97 dd (2.6, 4.3)	52.1 (3), 71.0 (1)	50.2 4.10 m		–
3	52.1 4.01 d (4.3)	50.1 (2), 81.3 (C-4), 172.5 (C-18), 71.0 (1)	52.3 4.03br s		50.2 (2), 81.6 (C-4), 172.6 (C-18)
4	81.3 –	–	81.6 –		–
5	41.6 –	–	41.6 –		–
6	26.5 1.99 m, 1.75 m	17.0 (C-7), 23.3 (C-19), 41.6 (C-5), 81.3 (4)	27.7 2.04 m		23.7 (C-19), 27.2 (C-7), 41.6 (C-5), 47.4 (C-10), 72.8 (C-8)
7	17.0 2.88 m, 2.03 m	26.5 (C-6), 34.9 (C-9), 44.0 (C-8), 173.8 (C-17),	27.2 3.43 m, 2.03 m		27.7 (C-6), 40.0 (C-9), 72.8 (C-8), 174.3 (C-17),
8	44.0 2.55 dd (11.1, 1.5)	17.0 (C-7), 26.5 (C-6), 27.9 (C-20), 34.9 (C-9), 46.3 (C-10), 173.8 (C-17)	72.8 –		–
9	34.9 –	–	40.0 –		–
10	46.3 2.31 s	23.3 (C-19), 27.9 (C-20), 34.9 (C-9), 41.6 (C-5), 50.0 (C-2), 81.3 (C-4)	47.4 2.41 s		21.0 (C-20), 23.7 (C-19), 27.7 (C-6), 36.3 (C-11), 40.0 (C-9), 41.6 (C-5), 50.2 (C-2), 70.9 (C-1), 81.6 (C-4)
11	41.2 2.46 dd (14.7, 4.1) 2.01 m	27.9 (C-20), 34.9 (C-9), 44.0 (C-8), 46.3 (C-10), 70.9 (C-12), 125.9 (C-13)	36.3 2.80dd (14.5, 11.6) 2.32 dd (14.5, 5.5)		21.0 (C-20), 40.0 (C-9), 47.4 (C-10), 71.4 (C-12), 72.8 (C-8), 126.5 (C-13)
12	70.9 5.96 dd (12.2, 4.1)	41.2 (C-11), 109.1 (C-14), 125.9 (C-13), 140.3 (C-16)	71.4 6.00 dd (11.6, 5.5)		36.3 (C-11), 109.3 (C-14), 126.5 (C-13), 140.3 (C-16)
13	125.9 –	–	126.5 –		–
14	109.1 6.65 br s	125.9 (C-13), 140.3 (C-16), 144.1 (C-15)	109.3 6.69br s		126.5 (C-13), 140.3 (C-16), 144.1 (C-15)
15	144.1 7.62 br s	109.1 (C-14), 125.9 (C-13), 140.3 (C-16)	144.1 7.59br s		109.3 (C-14), 126.5 (C-13), 140.3 (C-16)
16	140.3 7.69 br s	109.1 (C-14), 125.9 (C-13), 144.1 (C-15)	140.3 7.71br s		109.3 (C-14), 126.5 (C-13), 144.1 (C-15)
17	173.8 –	–	174.3 –		–
18	172.5 –	–	172.6 –		–
19	23.3 1.48, 3H s	26.5 (C-6), 41.6 (C-5), 46.3 (C-10), 81.3 (C-4)	23.7 1.64, 3H s		27.7 (C-6), 41.6 (C-5), 47.4 (C-10), 81.6 (C-4)
20	27.9 1.29, 3H s	34.9 (C-9), 41.2 (C-11), 44.0 (C-8), 46.3 (C-10),	21.0 1.53, 3H s		36.3 (C-11), 40.0 (C-9), 47.4 (C-10), 72.8 (C-8)

**Fig. 1.** Structure and key HMBC correlations of tinosporide (A, C) and 8-hydroxytinosporide (B, D).

**Table 2**

AChE and BuChE inhibitory activity of secondary metabolites tinosporide and 8-hydroxytinosporide obtained from *T. cordifolia* where donepezil was used as standard drug.

Tested compounds	AChE inhibition (IC <sub>50</sub> (μg/ml) ± SEM)	BuChE inhibition (IC <sub>50</sub> (μg/ml) ± SEM)
Donepezil	6.31 ± 0.089	11.93 ± 0.129
Tinosporide	13.45 ± 0.144	408.50 ± 17.197
8-Hydroxytinosporide	46.71 ± 0.511	317.26 ± 6.918

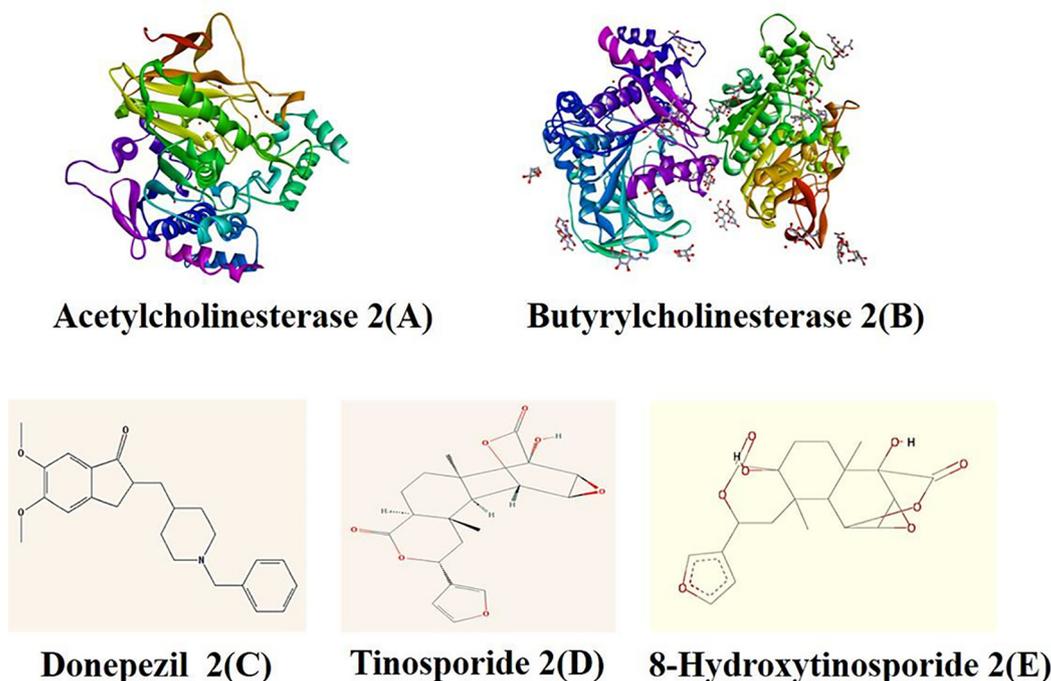
compound TC-19R exhibited a similar proton and carbon NMR profiles. On careful comparison of the NMR data of TC-16R and TC-19R, it is clear that TC-19R was similar in all respects to TC-16R, except the presence of carbon bearing the tertiary hydroxyl group. From the literature reports we could find that 6-OH and 8-OH substituted tinosporide have been isolated from *T. cordifolia* (Sivasubramanian, Gadepalli Narasimha et al. 2013). Based on the spectroscopic data and by comparison with published literature, the compound TC-19R was confirmed as 8-hydroxytinosporide (Table I, Fig. 1).

Choline esters, such as ACh and BuCh, play an important role in the transmission of nerve impulse, are lysed by cholinesterase enzyme. For this reason inhibition of cholinesterases has become an important target for the treatment of neurodegenerative disorders, including AD (Reisberg, Doody et al. 2003). In this study, these

two isolated secondary metabolites were assessed *in vitro* for AChE and BuChE inhibitory activities as described under experimental section and none of them were reported with any significant BuChE inhibitory effects.

The molecular interaction profile of donepezil with acetylcholinesterase includes a single H-bond with TYR A: 72 having 2.84 Å bonding distance, pi-sigma bond with TYR A: 341 along with many other Van der Waals, pi-pi T-shaped, pi-pi stacked interactions with ASN A: 283, PHE A: 295, PHE A: 338, VAL A: 294, TYR A: 124, TYR A: 337, TYR A: 72, TRP A: 286. However, molecular interaction profile of donepezil with BuChE includes three C-H bonds with TRP A: 82, PRO A: 285, SER A:287 having 3.74 Å, 3.37 Å, 3.75 Å bonding distance individually, pi-sigma bond with TRP A: 82 along with many other Van der Waals, pi-pi T-shaped, pi-pi stacked and pi-alkyl interactions with GLY A: 121, THR A: 122, LEU A: 125, THR A: 120, SER A: 198, PHE A: 398, TRP A: 231, GLY A:117, HIS A: 438, GLY A: 116, GLY A: 439, GLU A: 197, TYR A: 128, GLY A: 115, PHE A: 329, TRP A: 82, LEU A: 286 (Fig. 4, Table 4). Ligand-protein interaction analysis was performed based on electrostatic interactions and hydrogen bonds. Observed interactions most possibly are responsible for contributing to the binding affinity of donepezil with the target as well prevail its pharmacologic effect against Alzheimer's disease.

Besides, compare to that of donepezil, the molecular interaction of 8-hydroxytinosporide with AChE claimed two H-bond with catalytic site residue ASN A: 533 and GLN A: 403 having 2.06 Å, 2.23 Å



**Fig. 2.** Structure of target molecule (A) AChE, (B) BuChE, (C) donepezil, (D) tinosporide and (E) 8-hydroxytinosporide.

**Table 3**

Consensus docking affinity score of ligands against two target proteins.

Target	Ligand	Binding affinity (kcal/mol)		
		AutoDock Vina	Mcule	Consensus Docking
AChE	Donepezil	-8.3	-8.2	-8.2
	Tinosporide	-8.7	-9.0	-8.8
	8-hydroxytinosporide	-9.0	-8.7	-8.8
BuChE	Donepezil	-9.9	-9.3	-9.6
	Tinosporide	-8.8	-9.4	-9.1
	8-hydroxytinosporide	-9.5	-9.9	-9.7

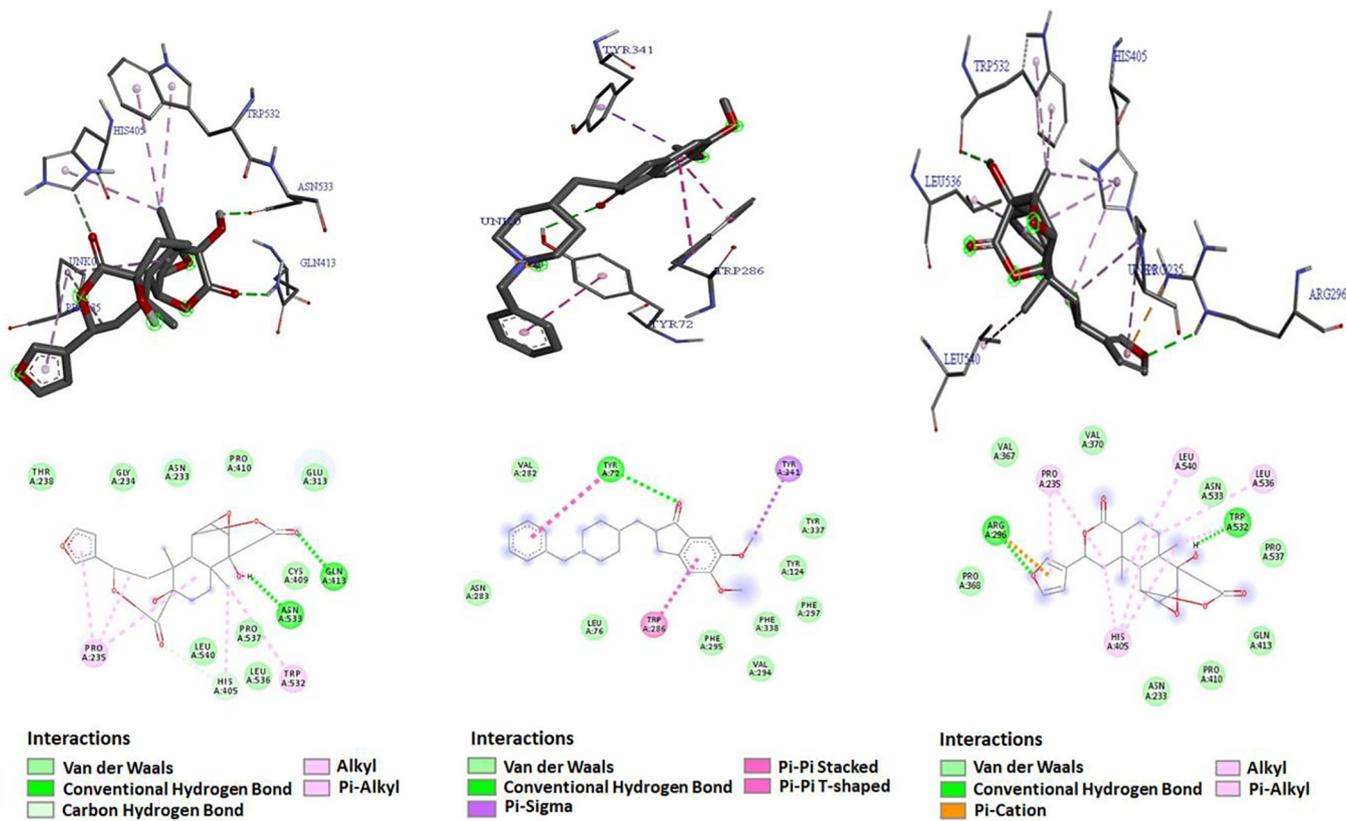


Fig. 3. 3D and 2D interaction of donepezil, tinosporide, 8-hydroxytinosporide with AChE.

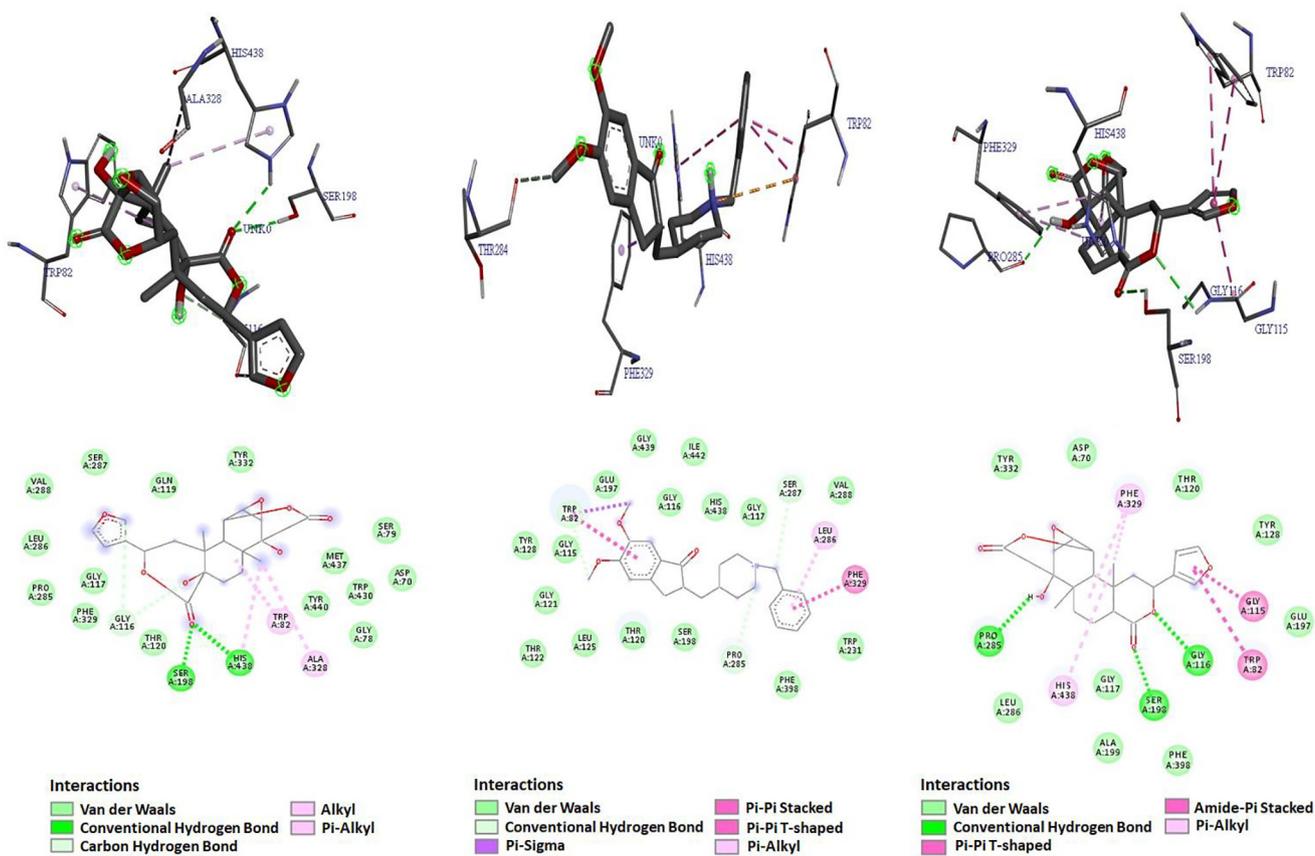


Fig. 4. 3D and 2D interaction of donepezil, tinosporide, 8-hydroxytinosporide with BuChE.

**Table 5**  
ADMET analysis data.

Molecule → Parameters ↓	Donepezil	Tinosporide	8-hydroxytinosporide
MR	115.31	198.19	166.61
TPSA	38.77	32.23	35.5
MLOGP	3.06	3.32	2.97
GI absorption	High	High	High
BBB permeant	Yes	Yes	Yes
Lipinski #violations	0	0	0
Ghose #violations	0	1	0
Veber #violations	0	0	1
Bioavailability Score	0.55	0.30	0.27
PAINS #alerts	0	0	0
Leadlikeness #violations	2	3	1
Synthetic Accessibility	3.62	2.55	3.38
Ames Mutagenesis	Negative	Negative	Negative
Acute oral toxicity	III	III	III
Eye Irritation	–	–	–
hERG	+	–	–

bonding distance as well a single C-H bond with HIS A: 405 along with many other Van der Waals, pi-alkyl, alkyl interactions with binding pocket catalytic residue THR A: 238, ASN A: 233, PRO A: 410, GLU A: 313, LEU A: 540, PRO A: 235, TRP A: 532. During the comparison with donepezil, the molecular interaction of 8-hydroxytinosporide with BuChE claimed two H-bond with catalytic site residue SER A: 198 and HIS A: 438 having 2.06 Å, 2.23 Å bonding distance as well a single C-H bond with GLY A: 116 along with many other Van der Waals, pi-alkyl, alkyl interactions with binding pocket catalytic residues SER A: 287, TYR A: 332, SER A: 79, ASP A: 70, TRP A: 430, GLY A: 70, TYR A: 440, THR A: 120, GLY A: 117, PRO A: 285, TRP A: 82, HIS A: 438, ALA A: 328 (Fig. 4, Table 4).

While another ligand tinosporide molecular interaction with AChE showed two H-bond with ARG A: 296, TRP A: 532 having 2.73 Å, 2.22 Å bonding distance along with many other Van der Waals, pi-alkyl, pi-cation, alkyl interactions with binding pocket catalytic residues ASN A: 233, GLN A: 413, PRO A: 637, VAL A: 370, PRO A: 368, LEU A: 536, HIS A: 405, PRO A: 235, ARG 296, HIS A: 405, TRP A: 532, LEU A: 540. Additionally with BuChE, tinosporide molecular interaction showed three H-bond with PRO A: 285, SER A: 198, GLY A: 116 having 2.44 Å, 2.14 Å, and 3.05 Å bonding distance GLY A: 117, ALA A: 199, GLU A: 197, TYR A: 128, THR A: 120, ASP A: 70, TYR A: 332, GLY A: 115, TRP A: 82, PHE A: 329, HIS A: 438. (Fig. 3, Table 4).

AChEs also inhibit BuChE because AChE and BuChE both share 65% amino acid sequence similarity even though being encoded by different genes on human chromosomes (Inestrosa and Alarcón 1998). Functionally, both enzymes hydrolyze acetylcholine efficiently but at a different rate even at the same temperature and pH. But AChE has higher hydrolytic acetylcholine activity than BuChE (Houghton, Ren et al. 2006). In this study, we observed optimum affinity of tinosporide and 8-hydroxytinosporide towards AChE and BuChE, respectively in a manner similar to that of standard drug donepezil. In the analysis of ligand interaction, the ligand tinosporide comparatively showed a maximum number of interactions with amino acid residues including five (05) types of non-covalent interactions than the other two. On the other hand, 8-hydroxytinosporide appeared to be in ligand interaction mostly the same phenomena of donepezil having five (05) types of interactions along with H-bonds. From the literature review, non-covalent interactions of a drug must place with the protein to reduce the ability of a drug to dissociate from its binding site through enhancing binding affinity and binding constant (Zhou, Huang et al., 2012; Tang, Roberts et al., 2017). Therefore, such interactions greatly impact in drug design, prominently in the syn-

thesis of many organic molecules (Schneider and Böhm, 2002; Cockroft and Hunter, 2007; Mitra and Dash, 2018). A satisfactory number of Van der Waals interactions in both ligands predicts their moderate solubility in lipid. Besides, 1.72–2.85 Å range of H-bond distances predicts good docking simulation results (Schneider and Böhm 2002) in which the requirements were desirably achieved by both of the experimental ligands than donepezil.

Compounds under investigation satisfy all the characteristics of CNS acting drugs like good brain penetration and other potential toxicity profiles (Table 5). These compounds have the possibility to be the best drug-like characteristics. Further on, both test ligands fulfill the criteria of Lipinski's Rule of Five, confer that both are perfect for the orally active drug (Table 5). As a result, overall study findings suggest that the ligand 8-hydroxytinosporide would be the best choice for the treatment of cognitive impairment ruling as a potent inhibitor of AChE as a complementary noble molecule of donepezil which is correlated with its pharmacological activity through *in vitro* studies, while 8-hydroxytinosporide modestly inhibited BuChE and results are very close to the standard donepezil.

## 7. Conclusion

The study involved the isolation of metabolites from *T. cordifolia* and their evaluation of potential role in AD. AChE and BuChE inhibitory activity of tinosporide and 8-hydroxytinosporide obtained from methanol extract of *T. cordifolia* has been investigated. Tinosporide exhibited promising anti-AChE activity. The docking study findings suggest that the ligand tinosporide would be a lead for the development of drugs to be used in the treatment of cognitive impairment ruling as a potent inhibitor of AChE enzyme as a complementary noble molecule of donepezil. Furthermore, the results of 8-hydroxytinosporide with standard donepezil suggest that it would be the choice as lead molecule for development of BuChE inhibitors to be used in the BuCh related disorders which is correlated with its activity through *in vitro* studies. These preliminary data suggest that these two natural metabolites might be used as lead to develop new drugs for AD and also warrant us for further *in vivo* experiments using animal model.

## Author contributions

Abdul Mazid and Monira Ahsan designed and coordinated the research, Mohiminul Adib isolated the metabolites and Rashedul Islam carried out *in vitro* experiments. Arifur Rahman did *in silico* studies, Mahmud Hossain helped in data analysis, Mustafizur Rahman, Sultan M. Alshehri and Mohsin Kazi helped in drafting, review and editing the manuscript. All authors have gone through the final manuscript and approved it.

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## Declaration of Competing Interest

Authors declare no conflicts of interest.

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