Arabian Journal of Chemistry (2023) **16**, 104408

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

New acetylcholinesterase inhibitors isolated from *Delphinium uncinatum*

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Received 25 August 2022; accepted 7 November 2022
Available online 11 November 2022

KEYWORDS

Norditerpenoids;
Uncinatine B-D;
Alzheimer's disease (AD);
Acetylcholinesterase inhibitory potential;
Molecular docking

Abstract The inhibition of acetylcholinesterase (AChE), the key enzyme in the breakdown of acetylcholine, is presently the most common pharmacological approach available for Alzheimer's disease (AD). Despite research on the molecular bases of AD, potent therapeutic agent against its expansion is still needed. In searching for natural cholinesterase inhibitors, the present study was focused on the isolation of three new norditerpenoid alkaloids, uncinatine **B-D** together with known virescenine from *Delphinium uncinatum*. Chemical structures for all the isolated norditerpenoids (**1**–**4**) were established using latest spectroscopic techniques. The isolated undescribed compounds along with known virescenine were testified for their acetylcholinesterase inhibitory activity supported by docking analyses. Molecular docking simulation showed that the isolated compounds (**1**–**4**) were observed to adhered in the active site of AChE with docking scores − 13.5322 (**1**),

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.arabjc.2022.104408

−11.8173 (**2**), −12.4240 (**3**) and − 8.9352 (**4**) respectively. Overall results demonstrated that these natural norditerpenoids compounds were found as selective inhibitors of AChE. This is the first report regarding the use of bioactive ingredients of *Delphinium uncinatum* in testing against Alzheimer's disease.

1. Introduction

Delphinium uncinatum, a renowned species of genus *Delphinium*, belonging to the family of Ranunculaceae. This species is extensively disseminated in different regions of Pakistan like Dir, Swat, Chitral and Azad (Biosci et al., 2012; Khan et al., 2010, 2015). Genus *Delphinium* is recognized since long, as a natural source of potent diterpenoid alkaloids, that can be applied as therapeutic remedy for different disorders in human worldwide (Ahmad et al., 2017a, 2016; Liu et al., 2019; Shaheen et al., 2015). Natural products especially diterpenoids and norditerpenoids alkaloids, acting as acetylcholinesterase (AChE) inhibitors were obtained from plant species as reported previously in literature (Ahmad et al., 2017c; Hostettmann et al., 2006; Houghton et al., 2006; Mukherjee et al., 2007a; Ranjan* et al., 2017). Four norditerpenoid alkaloids condelphine, 14-acetylvirescenine, delbrusine and 14-acetylperegrine have been previously investigated and isolated from *D. uncinatum* (Ulubelen et al., 1998).

AD is an age-related and progressive neurodegenerative disorder of multifactorial nature, a renowned type of dementia that affects a large number of old age (over 65 years) individuals (Konrath et al., 2013; Mughal et al., 2017; Reflexa et al., 2013). The rate of AD immensely increases in the developing countries with rapid increase in ageing of human population. According to WHO (World Health Organization) report, dementia can currently be found among 36 million of the world's population with a progressive increase in occurrence over the past decades and 71 % dementia cases are expected to occur by 2040 in the inhabitants of developing nations (El-Metwally et al., 2019; Ferri et al., 2005; Kalaria et al., 2008). The basic pathological signs of AD are marked by the growth of senile or amyloid plaques with extracellular deposit of β-amyloid peptide (Aβ) (Kril, 2009; Mughal et al., 2018; Portelius et al., 2006). The collapse of memory with conservation of long-term memory, can lead to cognitive dysfunction is the initial and main behavioral symptom of AD (Schliebs and Arendt, 2006).

The important remedial effect of cholinesterase inhibitors [ChEI] in AD treatment is to fortify cognitive function for at least 1-year period in around 50 % patients. The studies at clinical level illustrates that in a specific ratio of AD patients (about 20 %) cognitive function was stabilized for a period of up to 24 months. Further, those patients of AD who did not show any fruitful response to therapy with one ChEI can be changed into another one with a 50 % success rate. Previous research findings have shown that both AChE and BuChE were found to be associated with the hydrolysis of acetylcholine in brain and the inhibition caused by dual cholinesterase inhibitors might increase the efficiency of treatment (Giacobini, 2004; Mughal et al., 2019).

During extreme condition of AD, the management of ACh depends on BChE. As per clinical examination report, patients using dual cholinesterase inhibitors as medicine revealed minor cortical atrophic changes unlike patients who take selective AChE inhibitor (Ahmad et al., 2017a; Mughal et al., 2021). Cholinesterases, consisting of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) belongs to enzyme family that causes the catalytic hydrolysis of acetylcholine (ACh (Jamila et al., 2015; Obaid et al., 2022). Acetylcholinesterase (AChE) enzyme is the most convenient and attractive target for mechanism-based inhibitors in the rational drug discovery and is useful for neuronic disorder like AD (Choudhary et al., 2006). The acetylcholinesterase (AChE) plays a vital role in AD and hence catalyzes the hydrolysis of ester bond in acetylcholine (ACh) to end the nerve impulse transmitted action of ACh by means of cholinergic synapses (Sultzer et al., 2022). The inhibition of AChE is considered to be the basic therapeutic approach for AD, myasthenia gravis and senile (Atta-ur-Rahman and Choudhary, 2001). Synthetic compounds used for the therapy of memory impairment and cognitive dysfunction had adverse effects and troubles related to bioavailability (Oh et al., 2004; Schulz, 2003) whereas inhibitors extracted from plants were quite safe, having high potency as compared to synthetic inhibitors (Mukherjee et al., 2007a; Owokotomo et al., 2015). Now it is the need of the present era to work for the discovery of excellent AChE inhibitors.

To further discover natural cholinesterase inhibitors, *D. uncinatum* was investigated as a part of our continuous work, which resulted in the isolation of three unreported norditepenoids **1**–**3** along with known compound virescenine (**4**) for the first time.

2. Materials and methods

2.1. General procedures

IR spectrum was acquired on spectrophotometer (Jasco-320-A; Perkinelmer: JASCO, Tokyo, Japan) in KBr and was expressed in cm^{-1}. HR-EIMS [Jeol JMS HX 110] was obtained by mass spectrometer. The 1D and 2D NMR spectrum was obtained on Bruker 500 and 600 MHz for ^{1}H; 125 and 150 MHz for ^{13}C [Bruker; AVANCE III; Karlsruhe; Germany] spectrophotometer with TMS as the internal standard. Chemical shift [δ] was shown in ppm while coupling constant [J] in Hz; deuterated solvents received from Sigma Aldrich was used for NMR analysis. Column chromatography was performed with silica gel (70 230 mesh ASTM, Scharlab S.L, Gato Perez Sentmenat–Spain) whereas TLC (Thin layer chromatography) was run on pre-coated aluminum [silica gel F_{254}] sheets. The developed TLC was then visualized via ultra-violet lamp [254 & 356 nm] or Dragendorff's reagent or by means of Iodine.

Solvents obtained from commercial sources were used for extraction and isolation of pure compounds after proper distillation.

2.2. Plant material

Aerial parts of *Delphinium uncinatum* were collected at Altitude: 3934 feet, Latitude = 34. 8,778,807 and Longitude = 72.2600655; from Kabal valley district Swat, Khyber Pakhtunkhwa, Pakistan, in May 2014. Taxonomical and Botanical identification was made by Dr. Zahidullah Assistant Professor, Center for Plant Science and Biodiversity University of Swat and voucher specimen (no: H.UOM.BG-162) was deposited in the herbarium of University of Malakand for future reference.

2.3. Isolation

Reported procedure was followed for isolation and purification (Ahmad et al., 2017b). 4 kg shade dried, and powder materials of *D. uncinatum* were extracted with 80 % methanol four time at room temperature to get crude methanol extract solution. Solvent was evaporated from extracted solution at reduced pressure to obtain crude methanol extract (200 g). The concentrated methanol extract was suspended in water at pH 1–2 (0.5 N H_2SO_4) and was partitioned with chloroform to get acidic crude extract. Afterwards the acidic aqueous layer basified to pH 8–10 (10 % KOH) and was extracted with chloroform to get crude alkaloid fraction. The crude alkaloid fraction (18 g) loaded onto silica-gel column and was eluted by using *n*-hexane/ chloroform upto 100 % chloroform and the chloroform/methanol upto 20 % methanol to obtain ten sub-fractions (A-J). According to TLC analysis, sub-fractions (A-J) were condensed to four sub-fractions (N1-N4). Compound **1** (46 mg) and **2** (65 mg) were purified from the sub-fraction N3 by using *n*-hexane/ acetone/ di-ethyl amine (Et_2NH) (85:15 + 3 mL Et_2NH per 1 Liter) whereas compound **3** (30 mg) was acquired from sub-fraction N4 by using (80:20 + 3 mL Et_2NH per 1 Liter) of *n*-hexane/ acetone/ Et_2NH.

Uncinatine-B (**1**): White amorphous powder, IR; 3462 (OH); 3355 (NH); 1695 (C═O); 1620, 1595 (C═C) and 1080 cm^{-1} (simple ether bonds); ^1H NMR ($CDCl_3$, 600 MHz) Table 1; ^{13}C NMR ($CDCl_3$, 150 MHz): Table 2; HR-EIMS (*m*/*z*): 628.7580, $C_{34}H_{48}N_2O_9$ calcd. 628.7630).

Uncinatine-C (**2**): White amorphous solid. IR; 3492 (OH); 1083 (Ether bonds); 2900 $cm^{-1}$for C—H stretching. ^1H NMR

Table 1 ^1H NMR data of compounds 1–3 in $CDCl_3$.

Position	1 (600 MHz)	2 (500 MHz)	3 (500 MHz)
1	3.26 (m, 1H)	3.76 (br s, 1H)	3.98 (br s, 1H)
2	2.21, 2.33, (2H, m)	1.66, 1.91 (2H, m)	2.06 (2H, m)
3	1.84, (t, 2H, 2.3)	1.64, 2.21(2H, m)	1.32 (2H, m)
4	–	–	–
5	2.45, (br s, 1H)	1.88, (m, 1H)	2.44 (m, 1H)
6	3.34, (br s, 1H)	1.69, 1.87 (m, 2H)	1.62, (m, 2H)
7	2.13, dd, (4.7)	2.30, (d, 1H)	1.89, m
8	–	–	–
9	2.19, (br s, 1H)	2.09 (m, 1H)	2.36 (m, 1H)
10	1.71 (m, 1H)	1.94, (m, 1H)	2.48 (m, 1H)
11	–	–	–
12	2.06, 2.43 (br s, 2H)	1.73 (m, 2H)	1.35 (m, 2H)
13		2.66 (m, 1H)	1.91(m, 1H)
14	3.47 (d, 1H,4.3)	4.88 (t, 1H,4.8)	4.25 (d 1H, 5.3)
15	2.42 (d, 2H, 3.5)	2.34 (m, 2H)	2.31, t,2H, 3.3)
16	3.22 (t,1H, 6.9)	3.32, (m, 1H)	3.61 (br s,1H)
17	3.51 (br s,1H)	2.76 (br s, 1H)	5.28 (d, 1H, 4.5)
18	4.24 (m, 2H)	3.18,3.04 (dd,2H,8.8)	3.20,3.15 (d, 2H,8.8)
19	2.58, br s	2.11, 2.37, d(7 & 6.6)	2.26, br s
CH_3 CH_2— N-CH_2, N-CH_2	2.53, 2.60(m, 2H) 1.15(t, 3H, 7.1)	2.48 and 2.56(m, 2H) 1.14(t, 3H, 7.1)	4.17(m, 2H) 4.30 (m, 3H)
OCH_3	3.43 (s, 3H)	3.29(s, 3H)	
OCH_3	3.34 (s, 3H)	3.34(s, 3H)	–
OCH_3	3.32 (s, 3H)	–	3.33(s, 3H)
OCH_3	3.03 (s, 3H)	–	3.39(s, 3H)
1‘	–	–	–
2‘	–	–	7.73 (d,1H, 7.6)
3‘	8.69 (d,1H, 7.9)	–	7.55 (t, 1H, 7.4)
4‘	7.55 (m, 1H)	–	7.15 (m, 1H)
5‘	7.06(m, 1H)	–	7.55, (t, 1H, 7.4)
6‘	7.95 (d, 1H, 6.3)	–	7.73 (d, 1H,7.6)
NH	11.08 (s 1H)	–	–
CH_3	2.72 (s, 1H)	–	–

Table 2 ^{13}C NMR data of compounds 1–4 in $CDCl_3$.

Position	1 (150 MHz)	2 (125 MHz)	3 (125 MHz)
1	84.2	72.0	73.2
2	26.8	26.6	27.8
3	29.6	29.7	31.9
4	31.8	36.5	29.2
5	48.5	41.3	34.0
6	82.9	25.0	24.8
7	49.8	44.8	44.7
8	75.6	74.7	72.4
9	47.6	45.5	45.9
10	38.7	43.3	38.7
11	51.0	48.9	46.3
12	44.8	29.1	29.7
13	78.5	37.2	42.2
14	90.1	76.7	75.2
15	36.3	42.6	34.2
16	84.6	82.0	80.7
17	61.5	63.6	68.9
18	68.1	78.9	78.6
19	56.1	56.0	55.2
N-CH_2 / CH_3	48.9 / 13.5	48.5 / 13.0	62.1 / 60.2
N-CH_2-CH_2			
OCH_3	59.5	59.4	–
OCH_3	57.9	56.5	–
OCH_3	56.5	–	59.5
OCH_3	55.5	–	56.5
1′	115.8	–	130.0
2′	141.6	–	129.6
3′	120.2	–	128.8
4′	134.3	–	130.0
5′	122.3	–	128.8
6′	131.0	–	129.6
C═O	167.4	–	–
NH	–	–	–
C═O	169.0	170.4	173.2
CH_3	25.5	–	–

($CDCl_3$, 500 MHz): Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) Table 2; HR-EIMS (*m*/*z*): 449.2777 observed, $C_{26}H_{43}NO_6$, 449.2783 calculated.

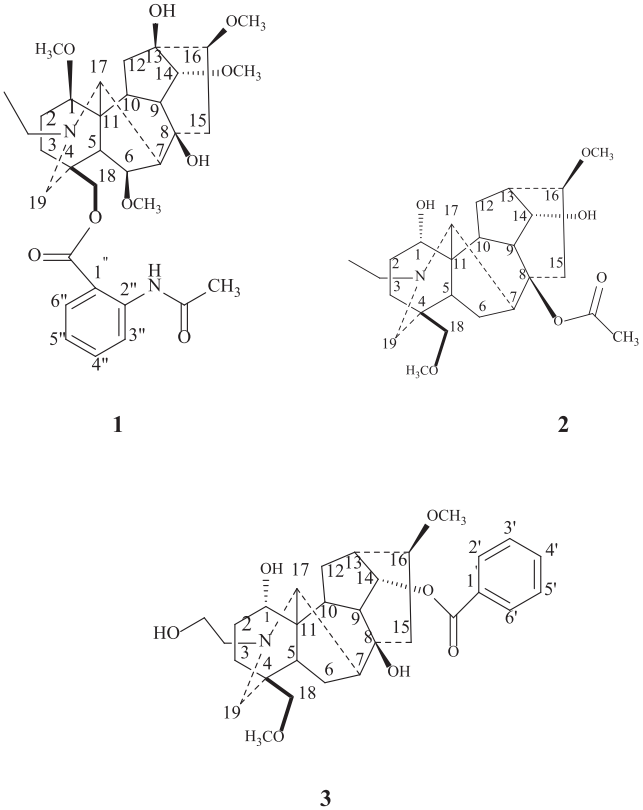
Uncinatine-D (**3**): White amorphous powder. IR; 3466 (OH); 1722 (ester); 1695 (C═O); 1620, 1595 (C═C) and 1080 cm^{-1} (simple ether bonds). HR-EIMS (*m*/*z*): $C_{30}H_{41}NO_7$ (*m*/*z* 527.6160, calcd. 527.6174. ^{1}H NMR ($CDCl_3$, 500 MHz): Table 1**:** ^{13}C NMR ($CDCl_3$, 125 MHz): Table 2.

2.4. Acetylcholinesterase inhibition assay

In vitro acetylcholinesterase (AChE) inhibition of compound **1–4** was determined by a modified method of Ellman as described elsewhere (Ellman et al., 1961; Elufioye et al., 2019). Acetylcholinesterase (Electric-eel EC 3.1.1.7), AChI, DTNB and galanthamine were obtained from Sigma Aldrich and used without any further processing. Stock solutions of the test samples (**1–4**) and the reference standard galanthamine was prepared. Solution was constituted by mixing Ellman reagent i.e DTNB (0.2 mM); sodium phosphate (62 mM) (pH 8.0, 880 µL), solution of the tested compound (40 µL) and AChE solution (40 µL). Reaction contents were incubated for 15 min at room temperature (25 °C). The hydrolysis of acetylcholine iodide (AChI) was marked spectrophotometrically at 412 nm. The experiment was conducted in triplicate by using spectrophotometer (BMS-USA). Compounds concentration that causes inhibition of ACh hydrolysis by 50 % was assessed by observing concentration (62.5–1000 µg/mL) effect of the tested compounds on inhibition values.

2.5. Molecular docking study

The docking simulation of the AChE enzymes and isolated natural products were accomplished though MOE (molecular operating Environment) software, to investigate the binding interaction of compound **1–4** (Bashir et al., 2021). For docking simulation, three-dimensional geometry of **1–4** was launched by means of MOE software tool. Through MOE default parameters (Force Field: MMFF94X, gradient: 0.05), the 3D protonation and energy minimization of **1–4** was conducted and was further saved as mdb file for later evaluation in docking analysis. 3D geometry of target enzyme (PDB ID: 1ACJ) was acquired from Protein Databank (PDB) and was opened, emitting water molecules from each protein. For the stability of protein, 3D protonation and energy minimization of enzyme carried by means of the default MOE parameters (Alam et al., 2016).



1 **2**

3

Fig. 1 Structures of compounds **1–3.**

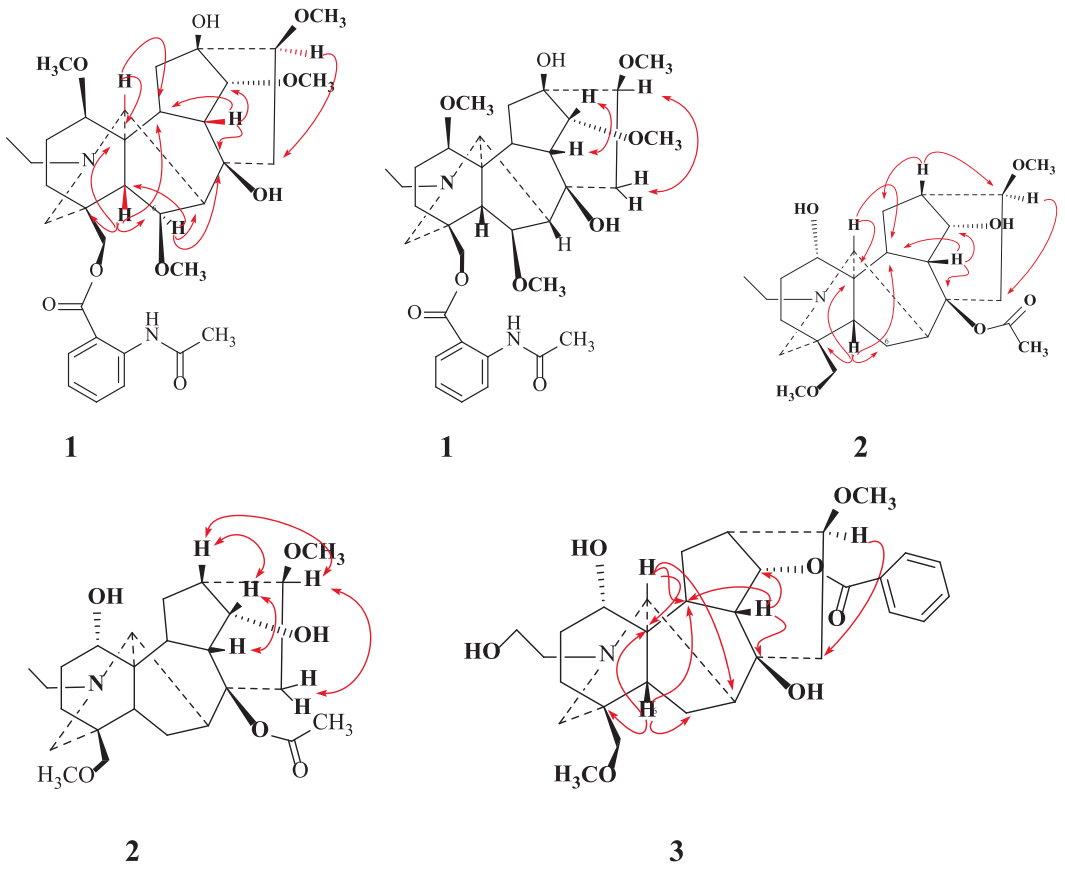


Fig. 2 HMBC and COSY correlations of compound **1**–**3.**

3. Results and discussion

3.1. Structure elucidation

Three unreported norditerpenoid alkaloids **1**–**3** (Fig. 1) together with known compound virescenine (**4**) were isolated from the chloroform fraction of *D. uncinatum* (aerial part). Structures of undescribed norditerpenoids **1**–**3** were established on the basis of spectral data interpretation, whereas structure for the known compound was confirmed by comparing their spectral data available in literature (Chen et al., 1999; Wada et al., 2016; Wangchuk et al., 2007) (Table 1).

Uncinatine-B **(1)** was isolated from basic (pH range = 8–10) chloroform fraction of *D. uncinatum as* white amorphous powder. Molecular formula $C_{34}H_{48}N_2O_9$ (*m*/*z* 628.7580, calcd. 628.7630) was established based on HR-EIMS. The IR spectrum of **1** showed bands at 3462 (OH), 3355 (NH), 1695 (C═O), 1620, 1595 (C═C) and 1080 cm^{-1} (simple ether bonds). A signal in the downfield region (δ_H 11.08, s, 1H) of the ^{1}H NMR spectrum (Table 1) was assigned to amide proton of **1**. Four other signals (δ_H = 3.03, 3.32, 3.34, 3.43 3H each, s) were assigned to the –OCH_3 at C-1, C-6, C-14, and C-16. The signal at δ_H = 3.47 (d, *J* = 4.3 Hz, 1H) was observed for *β*-oriented H-14 proton. Signal appeared upfield at δ_H = 1.15 (t, *J* = 7.1 Hz, 3H) was attributed to methyl of *N*-CH_2CH_3, a distinctive group of all norditerpenoids. The ^{13}C NMR spectrum (broad-band decoupled and DEPT]) (Table 2) of compound **1** displayed thirty-four signals for two methyl, four methoxy, seven methylene, thirteen methines and eight quaternary carbons atoms. HMQC spectrum was used to determine ^{1}H–^{13}C correlations whereas the long-range ^{1}H–^{13}C connectivity was confirmed by means of HMBC spectrum. In the ^{1}H–^{1}H COSY 45° spectrum (Fig. 2), H-16 (δ 3.22, m, 1H) displayed correlation with the C-15 methylene protons at [δ_H 2.42, m] whereas H-14 (δ_H δ 3.47, d, *J* = 4.3 Hz) exhibited COSY correlation with H-9 (δ_H 2.19, br s). The relative positions of the substituents in the basic skeleton of **1** were confirmed by means of HMBC spectrum. In the HMBC spectrum (Fig. 2), H-5 (δ_C 2.45) showed ^2J correlation with C-6 (δ_C 82.9), C-4 (δ_C 31.8), C-11 (δ_C 51.0), as well as ^3J correlation with C-7 (δ_C 49.8) and C-10 (δ_C 38.7). Similarly, H-6 (δ_H 3.32) showed interactions with C-7 (δ_C 49.8), C-5 (δ_C 48.5) and C-8 (δ_C 75.6). The structure of compound **1** was established from ^{1}H–^{1}H COSY, ^{1}H–^{13}C, HMBC, and HMQC data as 1*β*, 6*β*, 14*α*, 16*β*-tetramethoxy-8*β*, *13β*-dihydroxy, 18-acetamidobenzoate-*N*-ethyl aconitine (uncinatine-B) **1**.

Uncinatine-C **(2)** was isolated as white amorphous powder from basic (pH 8–10.) chloroform fraction of crude extract. The HR-EIMS of **2** showed molecular ion $[M]^+$ at *m*/*z* 449.2777 that corresponded to molecular formula $C_{26}H_{43}NO_6$ (calcd. 449.2783), marking the presence of seven degrees of unsaturation. The IR spectral data of **2** showed absorption signals at 3492 (OH groups), 1083 (ether bonds), 2900 cm^{-1} due to the C—H stretching. The ^{1}H NMR spectrum

of **2** displayed signals for *N*-ethyl, a characteristic group of all norditerpenoid alkaloids, two methoxy groups, eight methylene and several methines protons geminal to oxygen substituted groups. The spectrum exhibited signal at δ_H = 4.88 (t, J = 4.8, 1H) due to resonance of H-14 methine proton. A broad single at δ_H 3.76 was assigned to H-1β methine proton and a multiplet at δ_H 3.32 was due the H-16α methine proton geminal to methoxy substituent. The ^{13}C NMR spectrum of **2** showed twenty-five resonating signals, comprising two –CH_3, two –OCH_3, eight –CH_2-, nine methines and four quaternary carbons. ^{1}H–^{13}C correlations was established by applying HMQC and HMBC (long range) technique. In the ^{1}H–^{1}H COSY 45° spectrum (Fig. 2), H-16 (δ_H 3.32) showed correlations with the C-15 methylene protons at [δ_H 2.34 m], as well as H-13 (δ_H 2.66). The H-9 (δ_H 2.09, m) proton showed coupling with H-14 (δ_H 4.88, t, J = 4.8 Hz), and with H-13 (δ_H 2.66, m). In the HMBC spectrum (Fig. 2), H-5 (δ_H 1.88, m) showed ^2J correlation with C-6 (δ_C 25.0), C-4 (δ_C 36.5), C-11 (δ_C 48.9), as well as ^3J correlations with C-7 (δ_C 44.8) and C-10 (δ_C 43.3). Similarly, H-14 (δ_H 4.88) showed interactions with C-16 (δ_C 82.0), C-14 (δ_C 76.7) and C-9 (δ_C 45.5). The structure of **2** was established from ^{1}H–^{1}H COSY, ^{1}H–^{13}C, HMBC and HMQC was deduced as 16β, 18β- dimethoxy-1α, 14α-dihydroxy-8β-acetyl-*N*-ethyl aconitin (uncinatine-C) (**2**).

Uncinatine-D **(3)** was purified as white amorphous powder. The molecular formula $C_{30}H_{41}NO_7$ (m/z 527.6160, calcd. 527.6174) was deduced by means of HR-EIMS. IR spectrum of **3** exhibited absorption bands at 3466 (OH), 1722 (ester), 1695 (C=O), 1620, 1595 (C=C) and 1080 cm^{-1} (ether bonds). The ^{1}H NMR spectrum of **3** exhibited signal as triplet at δ_H 7.55 (J = 7.4 Hz, 1H), 7.15 (1H, t, J = 7.5 Hz) and a doublet at δ_H 7.73 (1H, d, J = 7.6 Hz) were assigned to aromatic protons. The methoxy protons present at C-18 and C-16 resonated as singlets at δ_H 3.33 and 3.39. The C-14 methine protons with β-orientation showed a triplet at δ_H 4.25 (J = 5.3 Hz). In the same spectrum a multiplet signal at δ_H 3.98 was due to the H-1 methine proton. The ^{13}C NMR spectrum of **3** displayed thirty signals for two –OCH_3, nine –CH_2-, fourteen methines and five quaternary carbons atoms. In the HMBC spectrum (Fig. 2), H-5 (δ_H 2.44, m) showed ^2J correlation with C-6 (δ 24.8), C-4 (δ 29.2), C-11 (δ 46.3), as well as ^3J correlations with C-7 (δ 44.7) and C-10 (δ 38.7). Similarly, H-14 (δ_H 4.25, t) showed interactions with C-9 (δ 45.9), C-13 (δ 42.2) and C-16 (δ 80.7). Furthermore, H-9 (δ_H 2.36, m) showed correlation with C-8 (δ 72.4), C-10 (δ 38.7), and C-14 (δ 75.2). While the H-17 (δ_H 5.28, d) showed interaction with C-10 (δ 38.7), C-7 ((δ 44.7) and C-11 (δ 46.3). The structure of **3** was established from ^{1}H–^{13}C NMR, HMBC and HMQC experiment. Thus, the structure of compound **3** was deduced as 16β, 18β-dimethoxy-1α, 8β-dihydroxy, 14-benzoyl-*N*-ethanol aconitine (uncinatine-D) (**3**).

3.2. In vitro acetylcholinesterase inhibitory potential

AD is a neurodegenerative disease which has not been able to be diagnosed and treated so far. AChE inhibitor helps a lot in its treatment and is regarded to be more contributive than BChE but it has been reported that both enzymes boost each other in one or the other (Ahmad et al., 2022; Islam et al., 2022; Luo et al., 2019). The synthetic drugs used for this disease have more negative effects than natural products that are regarded to be safe and low-cost remedy for AD (Mukherjee et al., 2007b; Oh et al., 2004).

To evaluate the potential role of target compounds (**1**–**4**) for the treatment of AD, natural products (**1**–**4**) were screened for *in vitro* acetylcholinesterase inhibitory activity, at different concentrations (62.5–1000 µg/mL) using modified spectrophotometric method reported by Ellman *et al* (Ellman et al., 1961). All the tested natural products were observed to be concentration dependent and specific inhibitor of AChE. The investigated IC_{50} value of **1, 2, 3,** and **4** against AChE was 188.10 ± 1.1, 94.33 ± 1.5, 367 ± 1.8 and 147.63 ± 1.0 respectively (Table 3). It is crystal clear from the experimental results that the isolated compounds (**1**–**4**) show promising inhibition against AChE. These inhibitory potential of the isolated natural products are attributed to the smaller active site of AChE enzyme that can fit in smaller group. As all the structural features were found to be actively involved in the inhibitory activity but the variation of various groups on the basic skeleton was really accountable for the change in inhibitory potential. Thus on the basis of aforesaid biological data, the structure–activity relationships (SARs) for the isolated natural compounds (1–4) can be briefly summarized. Among the isolated compounds, uncinatine-C (IC_{50} = 94.33 ± 1.5), was found to be properly accommodated in the active site of AChE as compared to other compounds. This high potency of compound **2** is attributed to the polar interaction of hydroxyl groups which makes the penetration process facile inside the pocket of AChE. The size and shape uncinatine-C also account for its strong hydrophilic interaction with receptor due to hydrogen bonding whereas other compounds demonstrate least inhibitory activity due to its hydrophobic nature and are unable to established strong hydrogen bonding with the target protein. These considerable results will obviously increase the importance of diterpenoids present in *Delphinium* species and hence encourage chemists for synthesis of such compounds.

3.3. Docking analysis of AChE

The isolated compounds (**1**–**4**) were docked into the binding pocket of AChE enzymes to find the interaction of these compounds with protein. On the basis of docking score, the top ranked conformation was chosen for analysis. Among different docking score, the lower scores represent suitable poses. All scoring functions were expressed in the unit of kcal/mol (Alam et al. 2016). All compounds were identified to fit firmly into the binding pocket of the AChE with the docking

Table 3 AChE inhibitory activity of norditerpenoids from *D. uncinatum*.

S.No	Compounds	AChE IC_{50} [µM] ± SEM[a]
1	Uncinatine B (**1**)	188.10 ± 1.1
2	Uncinatine C (**2**)	94.33 ± 1.5
3	Uncinatine D (**3**)	367 ± 1.8
4	virescenine (**4**)	147.63 ± 1.0
5	Galanthamine[b]	0.50 ± 1.5

[a] Standard error of mean of five assays.
[b] Positive control used in the assays.

Table 4 Binding interaction data for compound **1–4.**

Entry	ligand	enzyme	Binding energy (kcal)	Residue	Type of interaction	Ligand interacting moiety
1	1	*TcAChE*	−13.5322	Glu 292	Hydrogen	–OH
				Tyr 341	π-π interactions	Aromatic moiety
				Leu 76,	Hydrophobic	-NHCOR
				Trp 286	Hydrophobic	-NHCOR
				Phe 338	Hydrophobic	-NHCOR
2	2	*TcAChE*	−11.8173	Tyr 72	Hydrogen	-O-
				Tyr 341	Hydrogen	–OH
3	3	*TcAChE*	−12.4240	Tyr 341	Hydrogen	–OH
				Ser 293	Hydrogen	–OH
				Ser 293	Hydrogen	-O–H
				Trp 286	Arene-cation	–NHCH2
4	4	*TcAChE*	−8.9352	His 287	Hydrogen	–OH
				Trp 286	Hydrophobic	-OR
				Leu 76	Hydrophobic	-OR

score − 13.5322, −11.8173, −12.4240 and − 8.9352 respectively. Efficient interactions were observed for all the tested compounds with active site residues of the receptor protein Tyr 341, Glu 292, Leu 76, Trp 286, Ser and Phe 338.

In the skeleton of AChE, aromatic gorge with four loci has an important role in recognition of ligand and catalysis. Ligand interaction in the acyl binding locus is related to the acetyl specificity of AChE (Khalid et al., 2004). Intermolecular interactions like hydrogen bonding and hydrophobic interactions are recognized as basic and key players in the stabilization of energetically-favored ligands, that are usually observed in the open conformational environment of protein structures, but still it is poorly cleared that how binding parameters, relevant to these interactions can facilitate a drug to identify a specific target and enhance drug efficiency(Varma et al., 2010). By this docking study, we inspected the interactions between compounds uncinatine B-D (**1**–**3**) and virescenine (**4**) with the AChE. The interaction type, residues involved and ligand interacting group between enzymes and tested compound **1**–**4** tabulated in Table 4. In our sequential experiments, 3D docking simulations of the active compound **1**–**4** were anticipated. Docking scores of AChE-compounds complexes revealed that all the compounds were perfectly adhered in several pocket domains of AChE residues. Docking conformation of uncinatine-B (**1**) (−13.5322) showed that this compound formed hydrogen bond with Glu 292 and arene-arene or π-π interactions with Tyr 341. Furthermore, uncinatine-B (**1**) formed hydrophobic interactions with residues Leu 76, Trp 286 and Phe 338 (Fig. 3).

Docking conformation of uncinatine-C (**2**) (−11.8173), demonstrate that compound **2** exhibited three prominent polar interactions with the Tyr 72 and Tyr 341 residues (Fig. 4). Oxygen of the ether moiety of uncinatine-C (**2**) established hydrogen bonding with Tyr 72 whereas Tyr 341 also interact through hydrogen bonding with the hydrogen atom of the single bond OH moiety. All the binding interaction of the **2** was detected in the PAS of the AChE. The uncinatine-D (**3**) (docking score of − 12.4240) have two hydrogen bonds and one arene-cation interaction with the active side residues (Fig. 5). Tyr 341 formed hydrogen bond with OH moiety of this compound. Ser 293 established first hydrogen bond with OH moiety of uncinatine-D (**3**) and second hydrogen bond involved oxygen of Ser 293 and hydrogen of uncinatine-D (**3**). Trp 286 involved in arene-cation interaction with this compound. The viresce-

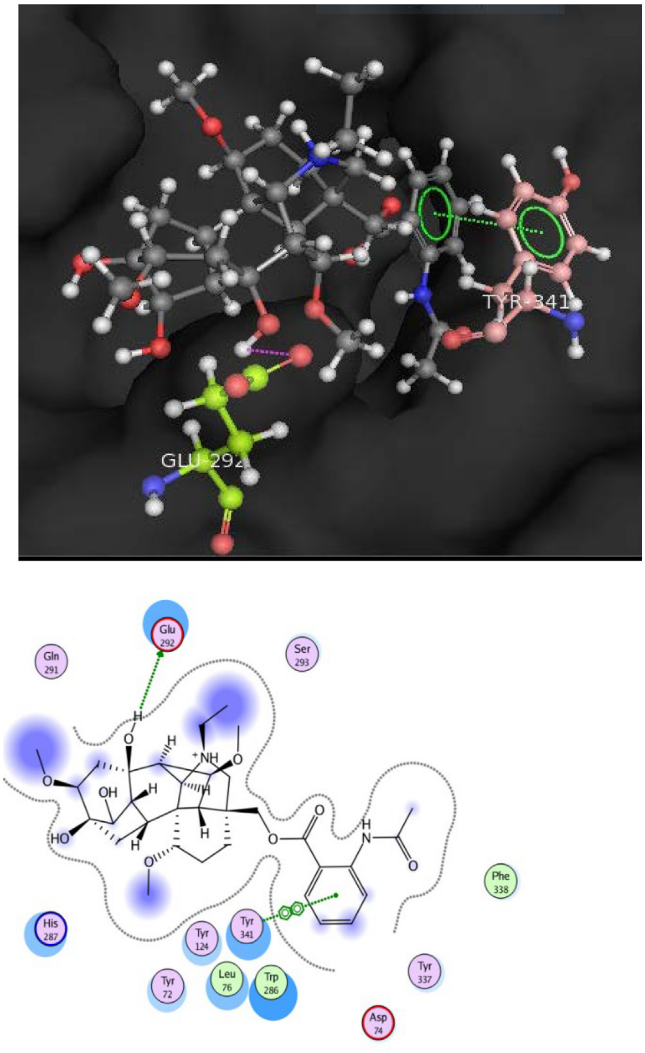
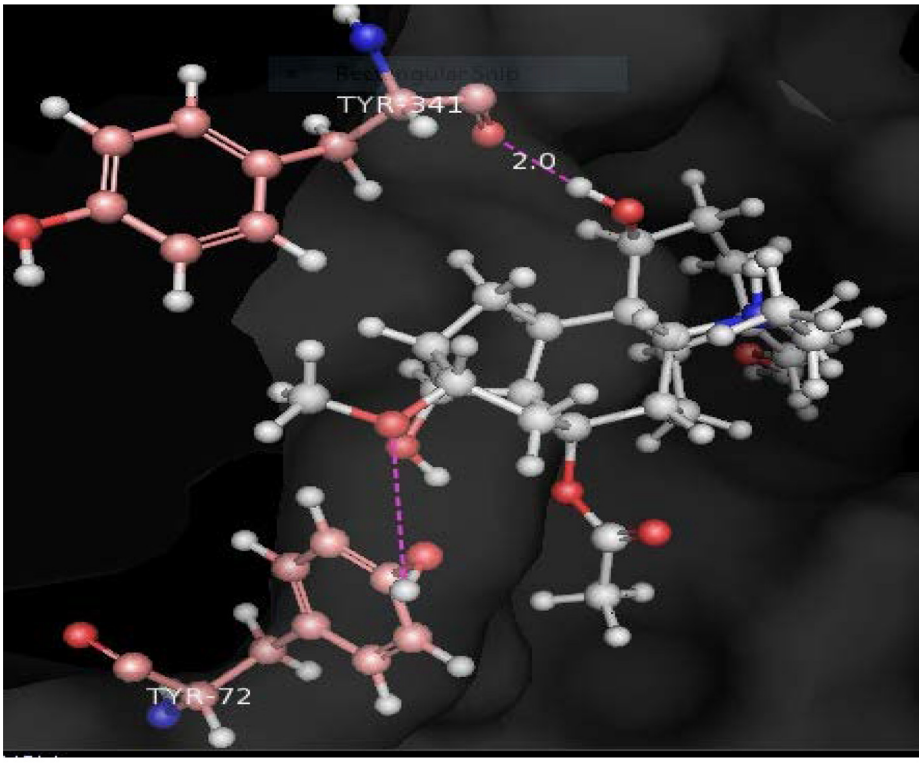


Fig. 3 Docking pose of compound **1** within cholinesterase target.



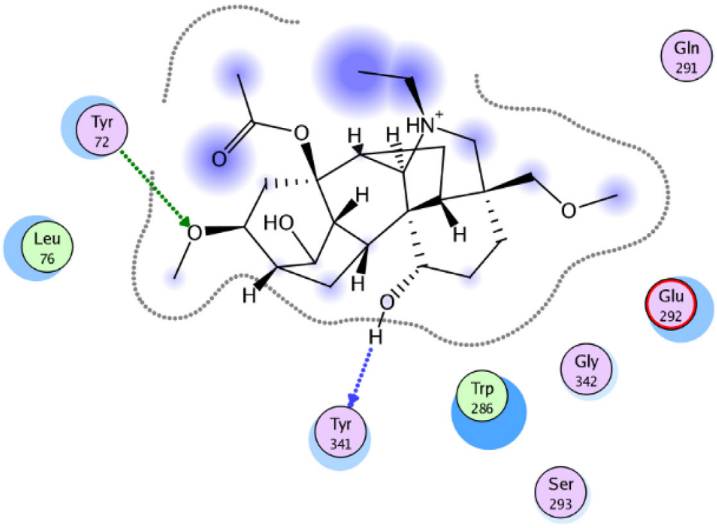
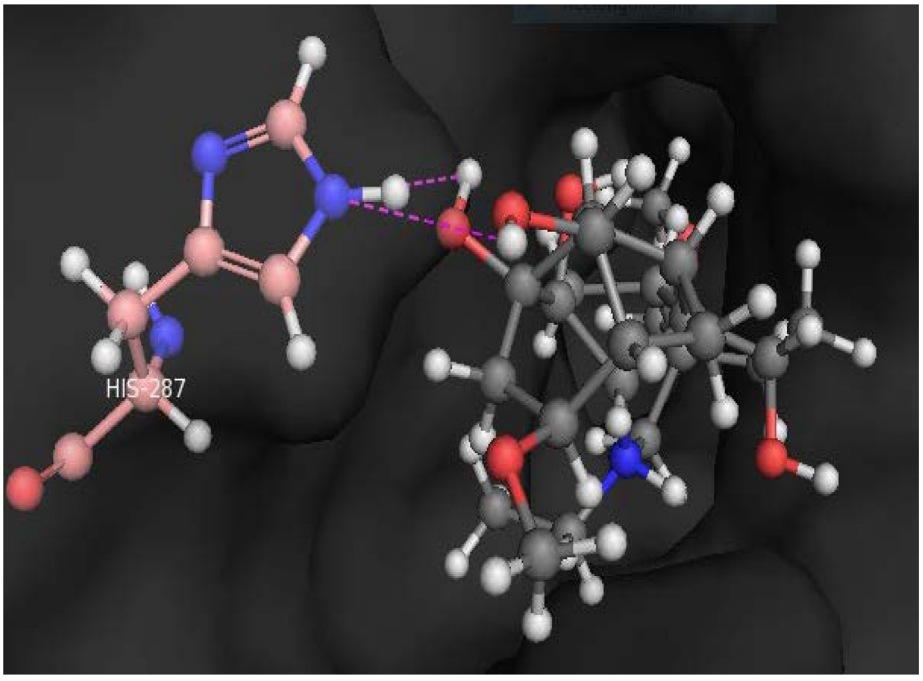


Fig. 4 Docking pose of compound **2** within cholinesterase target.

nine (**4**) (docking score of − 8.9352) showed only two hydrogen bonds with active site residue His 287 and forms hydrophobic interactions with residues Trp 286 and Leu 76 (Fig. 6).

4. Conclusion

The present research work, led to the identification of four natural alkaloids from *D. uncinatum*. The isolated compounds **1**–**4** were tested for AChE inhibitory activity. All the isolated natural products were found to possess promising acetylcholinesterase inhibitory potential. In silico assessment of **1**–**4** revealed binding modes and verified the experimental results. The negative binding energies of natural products **1**–**4** displayed suitable relationship to AChE enzyme. Therefore, the results obtained in our present study demonstrate the potential role of diterpenoids as possible alternative in drugs formulation. It may be concluded from our current investigation that diterpenoids present in *D. uncinatum* will be leading drugs for the treatment of AD or dementia-related neurovascular issues.



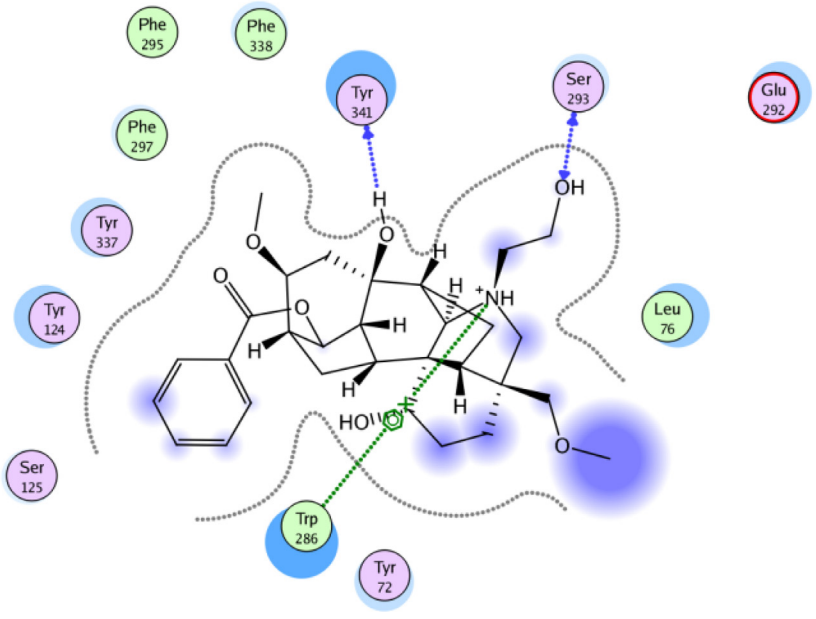


Fig. 5 Docking pose of compound **3** within cholinesterase target.

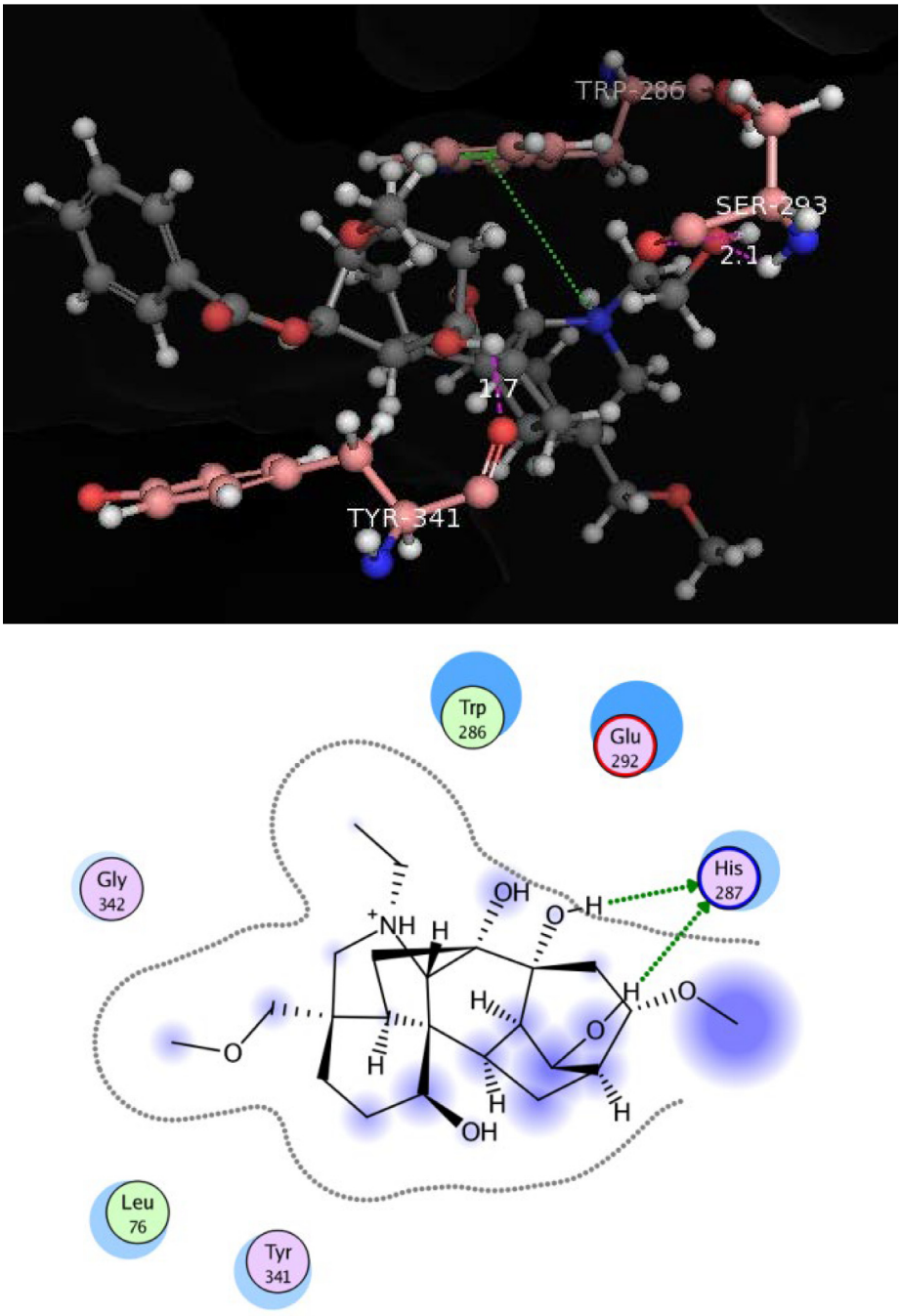


Fig. 6 Docking pose of compound **4** within cholinesterase target.

Acknowledgements

The authors would like to give thanks to Taif University, Taif, Saudi Arabia, for their support (Taif University Researchers Support Project number: TURSP-2020/80).

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