ORIGINAL RESEARCH

In vitro anticholinesterase activity and molecular docking studies of coumarin derivatives isolated from roots of *Heptaptera cilicica*

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Abstract The chloroform extract of the roots of *Heptaptera cilicica* (Boiss. & Bal.) Tutin (Apiaceae) was investigated in terms of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory effects by Ellman method. Afterwards, a new furocoumarin: trichoclin angelate with five known coumarin derivatives: umbelliprenin, badrakemone, badrakemin, badrakemin acetate and prunate were isolated from this extract. Their structures were identified by means of spectroscopic methods (1D, 2D-NMR and HRESIMS). The next step of our study was determining AChE and BuChE inhibitory activities of the compounds by molecular docking and *in vitro* methods. According to the results, prunate was found to be the most potent compound, which exhibited significant inhibitory potency against acetylcholinesterase (IC$_{50}$ = 1.76 ± 0.003 μM) and butyrylcholinesterase (IC$_{50}$ = 0.21 ± 0.002 μM) as compared with the reference compound, galantamine hydrobromide.

Introduction

Alzheimer's disease is a neurodegenerative disorder that characterized by progressive loss of cognitive functions (Moreira et al. 2017). Plants contain active compounds have become new sources to investigate for the pharmaceutical industry. Numerous plants have been used to treat neurodegenerative diseases (Adewusi et al. 2010). *Heptaptera* (Apiaceae) is represented with four species in Turkey (Herrnstadt and Heyn 1972). These species are known to be rich in coumarin derivatives (Appendino et al. 1993, 1992a, 1992b). Several coumarins have showed inhibition against acetylcholinesterase (AChE). AChE inhibitory effect of *Heptaptera cilicica* root extract has been stated as more effective than fruit and aerial part extracts in a previous study. This led us to study on root extract to yield active coumarins (Şenol et al. 2010).

In a continuation of the previous research, in vitro butyrylcholinesterase (BuChE) inhibitory activity was studied in addition to AChE inhibitory activity of *Heptaptera cilicica* root extract by Ellman method. Afterwards coumarin derivatives were isolated from chloroform extract. Finally, AChE and BuChE inhibitory activities of the secondary metabolites were evaluated by molecular docking studies and in vitro Ellman method.

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Materials and methods

General experimental procedures

Optical rotation was obtained on Rudolph-Research Analytical Autopol IV automatic polarimeter. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury Plus 400 MHz for proton and 100 MHz for carbon by using Tetramethylsilane as the internal standard. The solvent was $CDCl_3$. Silica gel 60 (0.063–0.200 mm, Merck) was used for open column chromatographic separations. TLC was carried out on pre-coated Kieselgel 60 F_{254} aluminum sheets (Merck) and compounds were detected under UV fluorescence and sprayed with 1% vanillin-H_2SO_4 reagent, followed by heating at 105 °C. HRESIMS was performed on Agilent 1200/6210 TOF. Infrared (IR) spectrum was run on a Perkin Elmer FT-IR Spectrum Bx. The absorbance of the reaction mixture was measured using a Bio-Tek ELx800 microplate reader.

For determination of AChE (E.C. 3.1.1.7) and BuChE (E.C. 3.1.1.8) inhibitory activities 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI) and butyrylthiocholine iodide (BTCI) were purchased from Sigma Aldrich as well.

Plant material

Heptaptera cilicica (Apiaceae) was collected from Mersin, between Tarsus-Çamlıyayla, Beylice Village, roadside of Kayabaşı (Turkey) on 10.06.2013 (540 m). A voucher specimen (AEF 26679) has been deposited in the herbarium of the Faculty of Pharmacy, Ankara University, Ankara, Turkey.

Extraction and isolation

Air-dried and powdered underground parts of the plant (462.5 g) were extracted three times with chloroform at 40 °C (3 × 2 L). The chloroform extract was concentrated in vacuo to give a residue (16.8 g) with the yield of 3.63%. The chloroform extract was submitted to column chromatography (CC) on silica gel using *n*-hexane with increasing amounts of ethyl acetate (100:0→0:100, v/v) to give four main fractions (Fr. A-D). Fr. A (2.2 g) was subjected to a silica gel column with a solvent gradient of *n*-hexane-ethyl acetate (100:0→91:9, v/v). Then, the subfraction was crystallized with the same solvent system to yield umbelliprenin (**2**) (408.4 mg). Fr. B (2.7 g) was seperated by silica gel CC using *n*-hexane-ethyl acetate (100:0→90:10, v/v) to four subfractions (Fr. B$_{1-4}$). Fr. B$_2$ was crystallized with the same solvent system to yield badrakemin acetate (**5**) (42.1 mg) and prunate (**6**) (183.6 mg). Fr. B$_4$ gave badrakemon (**3**)

(116.2 mg) after crystallization. Fr. C (1.3 g) was chromatographed over silica gel column and eluted with *n*-hexane-ethyl acetate mixture of increasing polarities (100:0→84:16, v/v) to afford four subfractions (Fr. C$_{1-5}$). Fr. C$_2$ and C$_4$ were crystallized with the same solvent system to yield trichoclin angelate (**1**) (12.8 mg) and badrakemin (**4**) (62 mg). The structure of the isolated compounds were identified by detailed spectral methods, such as ^1H NMR, ^{13}C NMR, COSY, HMQC, HMBC, IR, OR, ESI-MS and HRESIMS.

Trichoclin angelate (1)

White powder; $[\alpha]_D^{30}$ -12.1 (*c* 0.3, $CHCl_3$); IR (KBr) ν_{max} 1704, 1624, 1588 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): $\delta =$ 7.76 (1 H, d, $J = 9.6$ Hz, H-4), 7.68 (1 H, d, $J = 2.2$ Hz, H-2'), 7.36 (1H, s, H-5), 6.80 (1H, d, $J = 2.2$ Hz, H-3'), 6.36 (1H, $J = 9.6$ Hz, H-3), 6.06 (1H, ddd, $J = 14.5/7.2/1.3$ Hz, H-3‴), 5.86 (1H, t, $J = 7.0$ Hz, H-2″), 5.11 (2H, d, $J = 7.0$ Hz, H-1″), 4.72 (2H, s, H-4″), 1.95 (3H, dd, $J = 7.2/1.3$ Hz, H-4‴), 1.85 (3H, t, $J = 1.3$ Hz, H-5‴), 1.82 (3H, s, H-5″); ^{13}C NMR ($CDCl_3$,100 MHz): $\delta = 167.68$ (C, C-1‴), 160.45 (C, C-2), 148.27 (C, C-7), 146.69 (CH, C-2'), 144.28 (CH, C-4), 143.63 (C, C-9), 138.30 (CH, C-3‴), 136.74 (C, C-3″), 131.34 (C, C-8), 127.64 (C, C-2‴), 125.95 (C, C-6), 124.93 (CH, C-2″), 116.50 (C, C-10), 114.78 (CH, C-3), 113.35 (CH, C-5), 106.74 (CH, C-3'), 69.15 (CH$_2$, C-1″), 62.38 (CH$_2$, C-4″), 21.50 (CH$_3$, C-5″), 20.58 (CH$_3$, C-5‴), 15.78 (CH$_3$, C-4‴); HRESIMS *m/z* 369.13276 $[M + H]^+$ (calcd for $C_{21}H_{21}O_6$, 369.387).

Anticholinesterase activity

The microplate assay for anticholinesterase activity

Inhibitory activities of AChE and BuChE of the chloroform extract and the test compounds were evaluated against AChE and BuChE spectrophotometrically by Ellman's method (Ellman et al. 1961) with some modifications using commercially available galantamine bromide as the reference compound (Yerdelen et al. 2015). Stock solutions were dissolved in dimethylsulfoxide and then diluted in a 50 mM Tris buffer (pH 8.0) to provide a final concentration range. In a 96-well polystyrene photometric microplates, the assay medium in each well consisted of 50 µl of a Tris buffer, 125 µl of 3 mM DTNB, 25 µl of 0.2 U/ml enzyme (AChE or BuChE) and a 15 mM substrate ATCI or BTCI. The assay mixture containing the enzyme, buffer, DTNB and 25 µl of the inhibitor compound was preincubated for 15 min at 37 °C before the substrate was added to begin the reaction. All test compounds were prepared at ten different concentrations: 0.195, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml. The absorbance of the reaction mixture was then measured three times at 412 nm every 45 s using a

microplate reader. The IC$_{50}$ values of the compounds showing percentage inhibition, the measurements and the calculations were determined with GraphPad Prism 6.

Docking procedure

The docking study was performed using Surflex-Dock in Sybyl-X 2.0 by Tripos Associates. 3D structures of the compounds were constructed using the Sybyl sketcher module. The structures were minimized using the conjugated gradient method until the gradient was 0.001 kcal/mol, max iterations: 1000 with the Tripos force field with the Gasteiger Huckel charge. The simulation system was built on the crystal structures of 1ACJ and 1P0I, which were obtained from the Protein Data Bank. At the commencement of docking, all the water and ligands were removed and the random hydrogen atoms were added. Docking calculations using Surflex-Dock for 1ACJ and 1P0I were performed through protomol generation by ligand. The parameters used were threshold 0.5 and bloat 0.

Results and discussion

Isolation and structure identification

A new furocoumarin: trichoclin angelate (**1**) along with four known sesquiterpene coumarin ethers: umbelliprenin (**2**), badrakemone (**3**), badrakemin (**4**), badrakemin acetate (**5**) and a coumarin derivative: prunate (**6**) were isolated from the chloroform extract of *H. cilicica* roots (Fig. 1).

Compound **1** was obtained as white powder, $[\alpha]_D^{30}$ −12.1 (c 0.3, CHCl$_3$), with the molecular formula C$_{21}$H$_{20}$O$_6$, which was deduced by HRESIMS (*m/z* 369.13276 [M + H]$^+$, calcd for C$_{21}$H$_{21}$O$_6$, 369.387). The IR spectrum of **1** showed absorption band for C=O groups (1704 cm^{-1}) and −CH=CH− bonds (1624, 1588 cm^{-1}). The ^{1}H NMR spectrum of compound **1** displayed two couples of doublets in the downfield region, one at δ_H 6.36 and 7.76 (*J* = 9.6 Hz) attributed to the H-3 and H-4 protons of the coumarin nucleus and the other couple at δ_H 7.68 and 6.80 (*J* = 2.2 Hz) in accordance with the benzofuran moiety together with a single aromatic proton (δ_H 7.36) attributed to H-5. The ^{13}C NMR spectrum revealed the presence of 11 carbon signals for furocoumarin and ten carbon signals for the side chain. ^{1}H and ^{13}C NMR spectra of compound **1** showed characteristic signals of a 3-methyl-2-butene-4-ol at δ_H 5.11 (d, *J* = 7.0 Hz, H-1″), 5.86 (t, *J* = 7.0 Hz, H-2″), 4.72 (s, H-4″), 1.82 (s, H-5″) and δ_C 69.15 (C-1″), 124.93 (C-2″), 136.74 (C-3″), 62.38 (C-4″), 21.50 (C-5″). HMBC spectrum (Fig. 2) displayed the correlation of the proton signal at δ_H 5.11 (H-1″) with the carbon signal at δ_C 131.34 (C-8). Therefore, this structure was identified as trichoclin

(Miyakado et al. 1978). In addition the signals at δ_H 6.06 (ddd, *J* = 14.5/7.2/1.3 Hz, H-3‴), 1.95 (dd, *J* = 7.2/1.3 Hz, H-4‴), 1.85 (t, *J* = 1.3 Hz, H-5‴) and δ_C 167.68 (C-1‴), 127.64 (C-2‴), 138.30 (C-3‴), 15.78 (C-4‴), 20.58 (C-5‴) were attributed to an angelate moiety. HMBC correlation between H-4″ (δ_H 4.72) and C-1‴ (δ_C 167.68) suggested that the angelate moiety was attached to C-4″ of trichoclin. Thus, the structure of compound **1** was assigned as trichoclin angelate.

^{1}H NMR and ^{13}C NMR data of the known compounds agree with data given in the literature for umbelliprenin (**2**) (Tian et al. 2013), badrakemone (**3**) (Appendino et al. 1992a), badrakemin (**4**), badrakemin acetate (**5**) (Asghari et al. 2016) and prunate (**6**) (Jeong et al. 2006). In this study, badrakemin and badrakemin acetate were isolated from *Heptaptera* species for the first time.

In vitro anticholineterase activities

Anatolian *Heptaptera* species including *H. cilicia* root extract have indicated AChE inhibitory effect (Şenol et al. 2010). Further phytochemical characterization of the plant extract was made to investigate the source of anticholinesterase effect. Chloroform extract of *H. cilicica* roots showed 37 and 71% inhibition against AChE and BuChE at 50 µg/ml, respectively. The ChE inhibitory activities of the compounds (**1–6**) were evaluated and compared with galantamine hydrobromide as the reference inhibitor. The activities were measured in vitro by the spectrophotometric method developed by Ellman (Ellman et al. 1961), with slight modifications (Yerdelen et al. 2015). Their anticholinesterase potency was described as IC$_{50}$. The IC$_{50}$ values (µM) for ChE inhibition were summarized in Table 1. As shown in Table 1, the compounds (**1, 4,** and **6**) clearly showed moderate-to-good potent inhibitory activity against BuChE, with IC$_{50}$ values ranging from 0.21 µM to 2.53 µM concentrations. Among the compounds, **6** displayed the highest inhibition with IC$_{50}$ value of 0.21 µM and high selectivity (8.38) against BuChE. The other potent BuChE inhibitor in the series, compound **1** showed moderate inhibitory activity with IC$_{50}$ value of 1.10 µM. The comparison of **1, 6** and the other compounds demonstrated that 2-methyl-but-2-enoate structural moiety significantly increased both anti-ChE activity. The results indicated that, 2-methyl-but-2-enoate moiety provides a very important contribution to anti-BuChE activity for the compound **6**.

This same crucial effect seemed on BuChE inhibition for the second most potent inhibitor **1**, with IC$_{50}$ value of 1.10 µM and selectivity index value of 1.78 and the other effective compound **4** exhibited moderate BuChE inhibition (IC$_{50}$ = 2.53 µM) in series.

Compounds **1** and **6** exhibited the best AChE inhibition in the series with IC$_{50}$ values of 1.96 and 1.76 µM

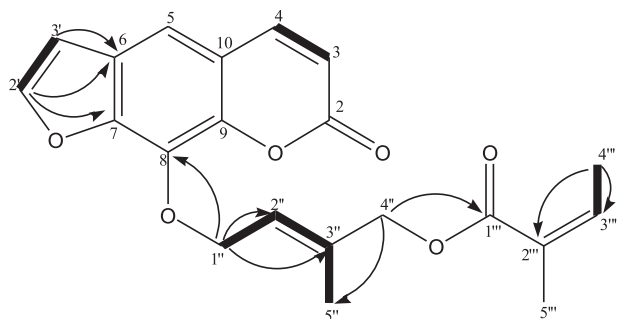Fig. 1 Chemical structures of compounds 1–6

Fig. 2 Key HMBC (→) and ^{1}H-^{1}H COSY (▬) correlations of compound 1

respectively (Table 1). The IC$_{50}$ values of the compounds 1 and 6 showed that these compounds were moderate AChE inhibitors. Despite the importance of the structure (2-methyl-but-2-enoate moiety) on both ChE inhibition, the results reveal that this possible pharmacophore structural part (Fig. 3) offers more positive contribution for BuChE inhibition than AChE inhibition.

Molecular docking studies

Docking studies helps to explore the target structure of the molecule for possible active sites (Amuthalakshmi and

Smith 2013). To support the ChE inhibition activity results, the most potent ChE inhibitors are docked at the binding site of AChE and BuChE enzymes in order to evaluate possible bonding interactions by utilizing the surflex-dock simulation programme. According to the simulation results, the most potent BuChE inhibitor **6** displayed multiple binding patterns with Human BuChE enzyme (1P0I) (Fig. 4). In the **1P0I-6** complex, all structural main moieties (2-methyl-but-2-enoate moiety, chromen-2-one ring and 2-hydroxy-2-methylpropyl chain) dominated several hydrogen bondings with amino acid residues Asp70, Tyr128, Gly116, Gly117, Ala199 and Glu197. The ester carbonyl group of but-2-enoate moiety exhibited three hydrogen bond interactions with NH groups of Gly116, Gly117 and Ala199 residues (distances 1.90, 2.12 and 2.23 Å, respectively). The other hydrogen bonding interaction occurred between the other oxygen atom of but-2-enoate moiety and NH group of Gly116 residue in oxyanion hole within the distance 1.96 Å. Also the carbonyl group at the C-2 position on the chromene ring created a hydrogen bond interaction with NH group of Asp70 (distance 2.07 Å). And the OH

group of the C-7 position of the chromene ring is bridged to OH group of Tyr128 residue via hydrogen bonding (2.66 Å). In the complex, OH group of the 2-hydroxy-2-methylpropyl chain is interacted with two hydrogen bonds with –CO groups of Glu197 residue (2.14 and 2.30 Å). On the other hand, the second most potent BuChE inhibitor **1** showed many hydrogen bonds with Thr120, Ala199, Trp82, Gly116 and Gly117 residues of butylcholinesterase enzyme (1P0I) (Fig. 5). Three hydrogen bond interactions occurred between the C=O group of but-2-enoate moiety of **1** and the NH groups of Gly116 (2.04 Å), Gly117 (1.80 Å), and Ala199 (2.20 Å). The oxygen atom in furano ring occurred a hydrogen bond with indole NH group of Trp82 (2.54 Å) of HuBuChE. In peripheral anionic site of 1P0I, carbonyl group at the C-2 position on the chromene ring created a hydrogen bond with OH group of Thr120 (2.63 Å) in PAS of HuBuChE. These several hydrogen bonding interactions support high anti-BuChE activities of the compounds **1** and **6**. And also, this molecular modeling study indicates the importance of the but-2-enoate structural moiety as a possible pharmacophore group.

Also the docking studies of two most active compounds (**1** and **6**) with AChE (PDB code: 1ACJ) were carried out. The results showed that compound **6** displayed multiple bindings with Torpedo californica (TcAChE) (Fig. 6). In the **1ACJ-6** complex, structural main moieties (chromen-2-one ring and 2-hydroxy-2-methylpropyl chain) exhibited several hydrogen bondings and π-π stacking interaction with residues Gly80, Trp84, Tyr130, and Tyr442. Compound **6** created two hydrogen bonds in CAS: one of them is interaction OH group of 2-hydroxy-2-methylpropyl moiety with N-H from Trp84 (2.54 Å) and the other one is carbonyl group at the C-2 position on the chromene ring with OH group of Tyr130 residue (2.09 Å). In the complex, carbonyl group of Gly80 is bridged to OH group of 2-hydroxy-2-methylpropyl moiety (2.31 Å). Another hydrogen bonding interactions occurred between OH groups of Tyr442 residue and 2-hydroxy-2-methylpropyl chain (1.78 and 2.75 Å). Also **6** created π–π stacking interaction with indole moiety of Trp84 in CAS (distance 3.05–3.39 Å). The other potent

Table 1 Anticholinesterase activity of the compounds (**1–6**)

Compound	IC$_{50}$ (μM) ± SD		Selectivity for BuChE^c
	AChE^{a,d}	BuChE^{b,d}	
1	1.96 ± 0.041	1.10 ± 0.067	1.78
2	≥100	≥100	-
3	≥100	≥100	-
4	≥100	2.53 ± 0.036	-
5	≥100	≥100	-
6	1.76 ± 0.003	0.21 ± 0.002	8.38
Galantamine HBr	0.18 ± 0.062	0.12 ± 0.187	1.50

^{a} 50% Inhibitory concentration of AChE

^{b} 50% Inhibitory concentration of BuChE

^{c} Selectivity for BuChE = IC$_{50}$ (AChE)/IC$_{50}$ (BuChE)

^{d} Values are expressed as mean ± standart error of the mean of three independent experiments. Each performed in triplicate (SD = standart deviation)
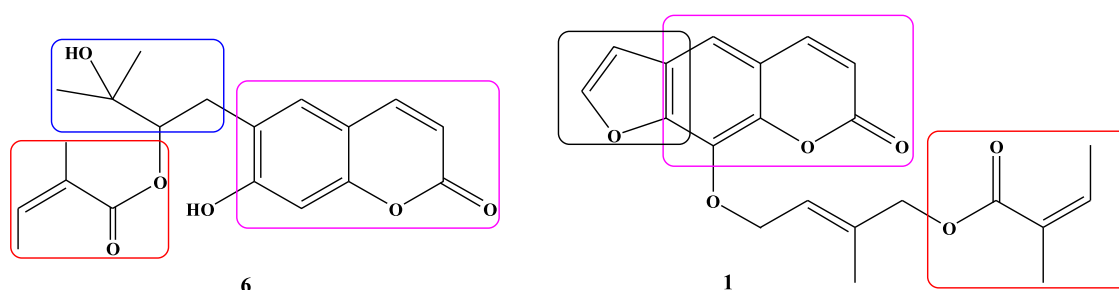

Fig. 3 The possible pharmacophore structures of the compounds **1** and **6**. **6** = 2-Methyl-but-2-enoic acid 2-hydroxy-1-(7-hydroxy-2-oxo-2H-chromen-6-ylmethyl)-2-methyl-propyl ester. **1** = 2-Methyl-but-2-enoic acid 2-methyl-4-(7-oxo-7H-furo[3,2-g]chromen-9-yloxy)-but-2-enyl ester
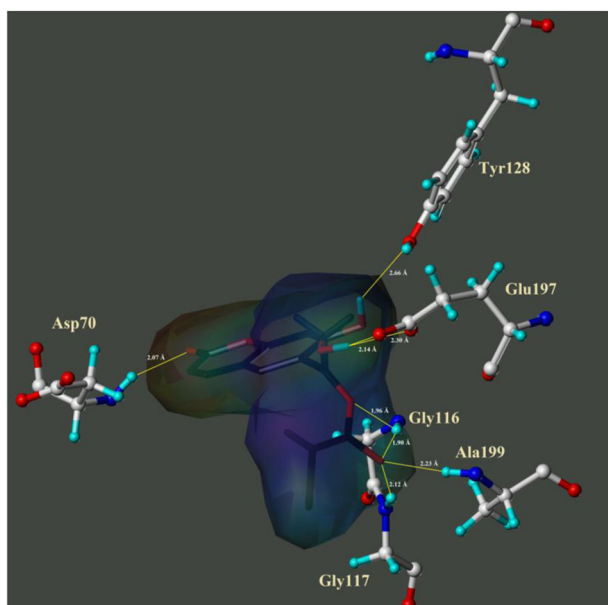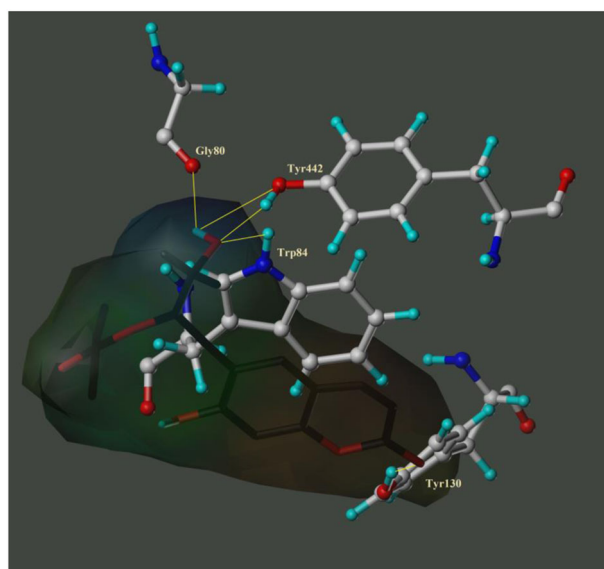
Fig. 4 Docking model of prunate and BuChE complex

Fig. 6 Docking model of prunate and AChE complex

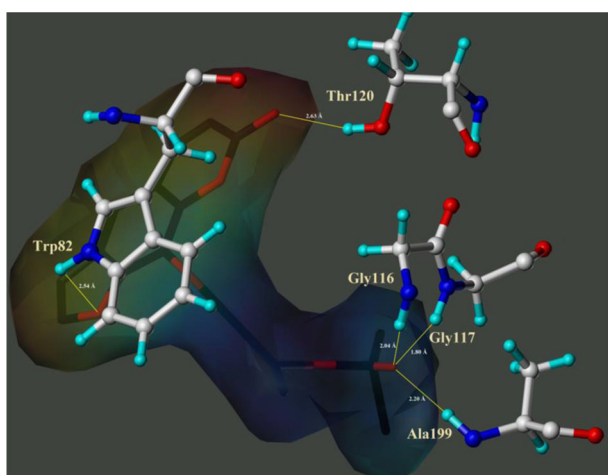**Fig. 5** Docking model of trichoclin angelate and BuChE complex

Fig. 7 Docking model of trichoclin angelate and AChE complex

AChE inhibitor, **1**, showed hydrogen bonding interaction with Ser122 residue (Fig. 7). In oxyanion hole, carbonyl group at the C-2 position on the chromene ring of ligand **1** is bridged to OH group of Ser122 via hydrogen bonding (1.97 Å). In catalytic anionic site of AChE, chromene ring of **1** created a π–π hydrophobic interaction with phenyl ring of Phe330 aminoacid residue (distance 3.21–4.00 Å).

A comparison study was done between the binding affinities and IC_{50} inhibition values of the isolated compounds by using some docking scores, which provide multiple approaches to evaluate inhibitor—enzyme interactions.

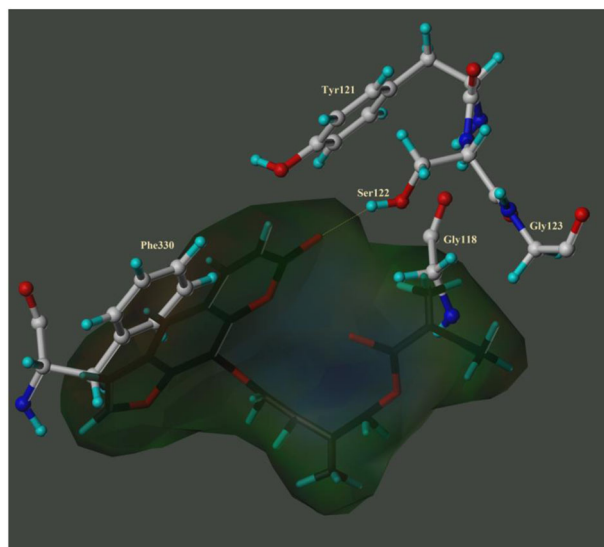The most potent AChE inhibitors (compounds **6** and **1**) exhibited the highest binding affinities in the series with the T_score values [5.68] and [4.99] in respectively. And the most potent AChE inhibitor **6** [IC_{50} (AChE) = 1.76 ± 0.003 μM] showed significant polarity interactions with AChE with high polarity_score [1.64]. At the same time, the highest Chem_score value of the compound **6** has indicated that the potent AChE inhibitory capacity is not only related with hydrogen bindings as well as lipophilic interactions have a significant effect on AChE inhibition.

A similar situation was seen in silico study on BuChE-**6** complex. The most potent BuChE inhibitor **6** (IC_{50} = 0.21 ± 0.002 μM) has better (T_, polarity, G_ and Chem_) scores

Table 2 Surflex-Dock scores (kcal/mol) of the compounds **1–6**

Compounds	T_score	Polarity_score	D_score	PMF_score	G_score	Chem_score	Cscore	Crash
AChE								
1	4.99	1.19	−146.120	−111.244	−206.808	−40.549	2	−0.51
2	3.70	1.07	−304.309	−93.517	−242.281	−47.855	5	−1.37
3	3.92	0.08	−244.314	−82.445	−231.483	−45.505	5	−1.83
4	3.20	1.41	−88.561	−103.086	−260.479	−52.226	4	−4.23
5	3.94	1.39	−187.116	−56.793	−253.000	−41.537	3	−2.93
6	5.68	1.64	−126.817	−67.799	−207.406	−29.180	3	−2.20
Galantamine HBr	4.73	1.05	71.938	−110.499	−156.987	−35.085	2	−0.48
BuChE								
1	4.99	2.20	−232.443	−68.728	−133.739	−29.218	4	−0.81
2	3.98	1.72	−455.101	−65.903	−184.705	−42.677	5	−0.32
3	3.81	4.46	−435.589	−33.262	−178.402	−39.598	3	−0.79
4	4.39	2.16	−222.149	−53.455	−156.768	−31.964	3	−1.45
5	3.45	0.22	−385.616	−77.304	−176.786	−40.366	4	−1.53
6	6.09	5.19	−352.480	−66.868	−207.059	−23.732	4	−2.00
Galantamine HBr	3.40	2.06	−124.512	−51.133	−165.444	−31.684	4	−0.71

than all other molecules in the series. These score values have indicated that increased interactions between the BuChE and **6** have resourced from the same parameters as seen in silico study of the compound **6** and AChE. As can be seen, high values of T_, polarity_ and Chem_score values parallels the experimental IC_{50} result of the compound **6** accurately. The potent inhibitor **6** was found as the most remarkable compound in the series with high T_score [6.09] and polarity score [5.19] values. These values indicate that compound **6** has a powerful binding affinity against BuChE. On the other hand, the lowest G_score value [-207.059] has supported the many hydrogen bonding interactions in **6-BuChE** complex. Also, it can be say that the lipophilic interactions may be the other important parameter at the potent inhibition of **6** on BuChE enzyme with the highest Chem_score value [−23.732]. All docking score values of the compounds are shown in Table 2.

Conclusion

The activities of the plants arrised from the presence of a wide range of bioactive compounds, thus it is very important to elucidate them. As a result, in series the compounds trichoclin angelate (**1**) and prunate (**6**) were identified as potent inhibitors against both AChE and BuChE enzymes at the low micromolar concentrations. Especially, prunate (**6**) was found as the most conspicuous ChE inhibitor in this study. Therefore, *Heptaptera cilicica* could be a potential source for anticholinesterase compounds.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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