New Steroidal Alkaloids from *Fritillaria imperialis* and Their Cholinesterase Inhibiting Activities

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Two new cevanine steroidal alkaloids, impericine (1) and forticine (2) along with known bases delavine (3), persicanidine A (4), and imperialine (5) were isolated from the bulbs of *Fritillaria imperialis*. The structures of impericine (1) [(20*R*,22*S*,25*S*)-5α-cevanin-23-ene-3β,6β,16β-triol] and forticine (2) [(20*S*,22*S*,25*S*)-5α-cevanine-3β,6β-diol] were determined with the help of spectroscopic studies. These steroidal bases showed anti-acetylcholinesterase and anti-butyrylcholinesterase inhibitory activity.

Key words *Fritillaria imperialis*; impericine; forticine; cevanine-type; acetylcholinesterase; butyrylcholinesterase

The genus *Fritillaria* belongs to family Lilliaceae, which is an important steroidal alkaloid-bearing plant family. Bulbs of the genus *Fritillaria*, commonly known as "Bei-mu" or "Pei-mu" in Chinese and "Bai-mo" In Japanese, have long been known as one of the principal Chinese crude drugs.[1,2] The dried bulbs or a decoction of *Fritillaria* species are to be prescribed to treat coughs, asthma, bronchitis, scrofula, glands, tumors, hemoptysis and deficiency of milk.[3] Our previous work on *F. imperialis* have resulted in the isolation of a new anti-cholinergic alkaloid, ebeinone.[4] This paper describes the isolation and structure elucidation of two new cevanine-type alkaloids, impericine (**1**) and forticine (**2**) together with three known steroidal alkaloids, delavine (**3**), persicanidine A (**4**) and imperialine (**5**), and their cholinesterase inhibitory activity.

Results and Discussion

The main alkaloidal fraction of the bulbs of *F. imperialis* was extracted with chloroform at pH 3—5 and subjected to repeated column and thin layer chromatography over silica gel to obtain five steroidal bases including two new steroidal alkaloids, impericine (**1**) and forticine (**2**).

Impericine (**1**) was obtained as crystals of mp 195—197 °C. The IR spectrum showed absorptions at 3352—3365 (OH), 2775 (*trans*-quinolizidine moiety)[5] and 1608—1605 (C=C) cm^{-1}. The high resolution electron impact mass spectrum (HR-EI-MS) of **1** showed the $[M]^+$ at *m/z* 429.3717, suggesting the molecular formula $C_{27}H_{43}O_3N$ (Calcd 429.3719). The ^{1}H-NMR spectrum of **1** exhibited a tertiary methyl signal at δ 0.95 (H-19). Its downfield chemical shift as compared to C-10 methyl of 5α-cholestanol (δ 0.81), suggested a 1,3-diaxial interaction with the C-6β (axial) hydroxyl group.[6] Two secondary methyl signals appeared as doublet at δ 0.85 (J=6.2 Hz, H-21) and 1.02 (J=7.0 Hz, H-27). The chemical shift of C-20 methyl protons was in good agreement with the corresponding proton of the known compounds, delavine (**3**)[7] and ebeiedine (**6**).[8] Three downfield multiplets appeared at δ 3.50 ($W_{1/2}$=23.0 Hz, H-3α), 3.72 ($W_{1/2}$=8.0 Hz, H-6α), and 3.75 ($W_{1/2}$=7.6 Hz, H-16α) were assigned to methine protons geminal to the oxygen functions. The chemical shifts and half width of H-3α (δ 3.50) and H-6α (δ 3.72) were compatible to those of the cevanine-type alkaloids with β-equatorial hydroxy group at C-3 and β-axial hydroxy group at C-6.[9] The chemical shift of C-16 (δ 64.2) was comparable with the reported chemical shifts of C-16 (δ 64.8) in delafrine (**7**),[10] which was further supported by heteronuclear multiple bond connectivity (HMBC) experiments.

Two olefinic protons appeared as two distinct double doublets at δ 5.20 ($J_{24,25\alpha}$=8.5, $J_{24,23}$=15.3 Hz) and δ 5.50 ($J_{23,22\alpha}$=8.3, $J_{23,24}$=15.3 Hz) and were assigned to H-24 and H-23, respectively. The ^{1}H–^{1}H-correlation spectroscopy (COSY) spectrum was recorded to determine the connectivities around the double bond. The cross-peaks between vinylic H-23 and allylic H-22 (δ 2.80) were observed. Similarly, H-24 showed coupling with H-25 (δ 1.90). The double bond present in ring F causes dishielding of H-22 (δ 2.80) and H-25 (1.90) when compared with the corresponding protons of a saturated compound such as ebeinone (**8**) [H-22 (δ 2.52) and H-25 (δ 1.72)].[4] The position of a double bond between C-23 and C-24 was further confirmed by the HMBC correlation between H-23 and C-22 (δ 68.1), while H-24 (δ 5.20) displayed correlation with C-26 (δ 62.4).

The stereochemistries of the ring junctions and two secondary methyl groups are elucidated as follows. The ^{13}C-NMR data of impericine (**1**) are in good agreement those of with ebeiedine (**6**) and delavine (**3**) for the rings A, B, and C implying their stereochemical identity. The D/E *trans* ring junction was supported by the similarity of the data with those of delafrine (**7**).[10] Orientation of C-20 and C-25 methyl groups were assigned α (equatorial) and β (axial) from their ^{1}H-NMR chemical shifts, respectively. They are *trans* and *cis* to the lone-pair of nitrogen, respectively. Therefore, the structure **1** was elucidated as [(20*R*,22*S*,25*S*)-5α-cevanin-23-ene-3β,6β,16β triol]. Impericine (**1**) is the first example of the isolation of a 20-deoxy-5α-cevanine alkaloid, with a double bond in ring F from genus *Fritillaria*.

Forticine (**2**), a crystalline compound, showed the $[M]^+$ at *m/z* 415.3347 ($C_{27}H_{45}O_2N$, Calcd 415.3377) in the HR-EI-MS. The IR spectrum displayed intense absorptions at 3330—3438 (OH) and 2768 cm^{-1} (*trans*-quinolizidine moiety).[5] The base peak at *m/z* 111 is a characteristic ion of 5α-cevanine-type alkaloids lacking the hydroxyl function at C-

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Table 1. ^{13}C-NMR Data (δ) of Cevanine-Type Alkaloids (**1**—**10**)[a)]

C. No.	1	2	3	4	5	6	7	9	10
1	38.5	39.5	39.4	39.4	37.6	38.1	39.4	38.1	39.0
2	31.5	31.4	31.4	31.4	30.2	31.2	32.4	31.4	31.4
3	71.5	71.9	71.9	71.9	71.9	71.7	71.7	72.0	72.0
4	32.5	34.7	34.8	34.7	30.2	34.9	36.0	48.8	34.9
5	49.0	47.9	48.1	47.9	56.6	48.3	49.5	142.4	48.2
6	72.2	73.3	73.2	73.3	211.0	72.8	72.2	122.3	73.0
7	40.5	39.6	39.5	39.0	46.9	39.1	39.7	31.2	39.3
8	37.9	37.5	36.7	39.0	40.3	35.0	41.0	38.6	36.6
9	55.0	57.3	57.9	57.8	56.7	57.7	58.8	54.4	57.6
10	35.6	35.5	35.5	35.5	36.1	35.5	36.5	37.0	35.4
11	30.4	30.3	30.8	25.8	30.2	30.2	30.5	30.3	30.1
12	39.5	39.1	39.1	40.7	39.9	40.4	38.0	41.5	39.5
13	37.0	40.8	39.0	33.2	40.6	40.3	37.3	37.9	34.7
14	45.8	38.3	41.2	40.2	42.1	44.0	43.5	45.3	40.6
15	27.0	26.5	28.7	25.1	27.0	26.9	32.9	25.1	26.2
16	64.0	24.3	17.6	28.0	18.8	25.6	64.8	24.9	21.4
17	47.0	39.3	41.6	30.6	46.6	46.5	50.5	45.5	44.3
18	58.5	62.5	59.2	51.8	59.9	61.8	62.3	62.6	65.3
19	15.5	14.8	15.7	14.6	12.5	15.0	15.0	19.1	15.1
20	34.8	37.4	39.8	38.4	72.0	43.3	36.5	36.2	37.2
21	13.1	8.5	15.7	14.6	21.4	14.8	14.6	8.6	14.9
22	68.1	68.0	62.5	63.6	63.5	69.0	69.6	68.0	68.8
23	134.0	24.5	25.0	24.3	19.7	24.8	25.8	24.3	30.9
24	132.0	25.5	30.3	35.0	29.1	29.2	29.9	28.9	33.6
25	30.7	28.4	28.4	23.5	28.0	28.4	28.7	28.3	30.7
26	62.4	62.6	62.0	62.8	61.5	62.0	62.7	63.9	59.3
27	19.3	17.7	18.3	19.7	17.6	18.3	18.2	17.9	19.8

a) The data of ebeiedine (**6**), delafrine (**7**), shinonomenine (**9**), and tortifoline (**10**) are taken from refs., 8, 10, 12 and 14, respectively.

20.[11)]

The ^{1}H-NMR spectrum of **2** exhibited two secondary methyl doublets at δ 0.92 (J=6.9 Hz, H-21) and 1.09 (J=6.8 Hz, H-27). The downfield chemical shift of C-21 methyl (δ 0.92) suggested its 1,3-diaxial interaction with axially oriented lone pair of the nitrogen atom. The chemical shift of the H-27 methyl signal suggested its β-axial configuration (25*S*), as earlier reported in delavine (**3**).[7)] The hydroxyl group at C-3 was assigned to be equatorially oriented, since its geminal proton appeared as a broad multiplet at δ 3.50 ($W_{1/2}$=23.0 Hz). The presence of a β-oriented hydroxy group at C-6 was inferred from the downfield chemical shift of H-19 (δ 0.99), which is known to be deshielded due to a 1,3-diaxial interaction.

In the ^{13}C-NMR spectrum of **2** (Table 1), the chemical shifts of C-1 to C-16 were in good agreement with those reported for ebeiedine (**6**),[8)] while the chemical shifts of C-21 to C-27 corresponded well with those in shinonomenine (**9**).[12,13)] Apart from the stereochemistry of the two methyl groups (C-21, C-27), four stereochemical features concerning on the ring junction (13-H, 17-H, 22-H) are known for cevanine-type alkaloids, *i.e.* $\beta\alpha\alpha$ (ebeiedine, **6**),[8)] $\beta\beta\alpha$ (delavine, **3**),[7)] $\beta\beta\beta$ (tortifoline, **10**).[14)] and $\alpha\beta\alpha$ (persicanidine A, **4**).[15)] These are distinguishable by the ^{13}C-NMR chemical shifts of C-16, C-18 and C-22. Particularly, the chemical shifts of C-18, C-22 (carbon adjacent to nitrogen atom), and C-16 exhibited upfield shifts at δ 59.2, 62.5 and 17.6 when D/E has a *cis* ring junction as in delavine (**3**)[7)] (Fig. 1). The downfield chemical shifts of C-18, C-22 and C-16 (δ 62.2, 68.0 and 24.7, respectively) in compound **2** therefore indicated the presence of a D/E *trans* ring junction as in ebeiedine (**6**)[8)] and shinonomenine (**9**).[12)] The ^{13}C-NMR chemical shift of 20-methyl (δ 8.5) in compound **2** is closely resembled with 20-methyl (δ 8.6) of shinonomenine (**9**), which is the only example of 20β-methyl (axial) compound before **2**.

In the ^{13}C-NMR spectrum of **2**, carbon signals due to rings A, B and C corresponded to those of ebeiedine (**6**),[8)] suggesting the presence of A/B *trans* and B/C *trans* ring junctions, while the ^{13}C-NMR data of rings D, E and F found to be identical with shinonomenine (**9**), suggesting the presence D/E *trans* and E/F *trans* ring junctions. These evidence lead to the conclusion that compound **2** has the same stereochemistry for all rings with that of ebeiedine (**6**) and shinonomenine (**9**) (the structure of these have been settled by X-rays analyses) except those of two secondary methyl groups. The stereochemistry of the both C-20 and C-25 methyls are of β (axial) orientation (shinonomenine-type) as judged from the ^{1}H-NMR data. The above mentioned data indicated the structure of **2** to be [(20*S*,22*S*,25*S*)-5α-cevanine-3β,6β-diol].

Other known steroidal bases, delavine (**3**),[7)] persicanidine A (**4**),[15)] and imperialine (**5**)[16)] were also isolated from *F. imperialis* and identified on the basis of spectral data (^{1}H-, ^{13}C-NMR, IR, and mass spectra *etc.*). Compound **4** was earlier isolated from *F. persica*[15)] and this is the first time it was detected in *F. imperialis*. The reported mp of persicanidine A (**4**) is 208 °C, while our sample showed mp 228—230 °C.[17)]

Compounds **1**—**5** were screened for their cholinesterase inhibitory activity. Two forms of cholinesterase co-exist throughout the body, acetylchlinesterase (AChE) and butyrylcholinesterase (BChE). Inhibition of brain AChE can provide relief from the congnitive associated with Alzheimer's disease (AD). A few steroidal alkaloids isolated from the same plant showed anti-cholinergic activity.[4)] This paper de-

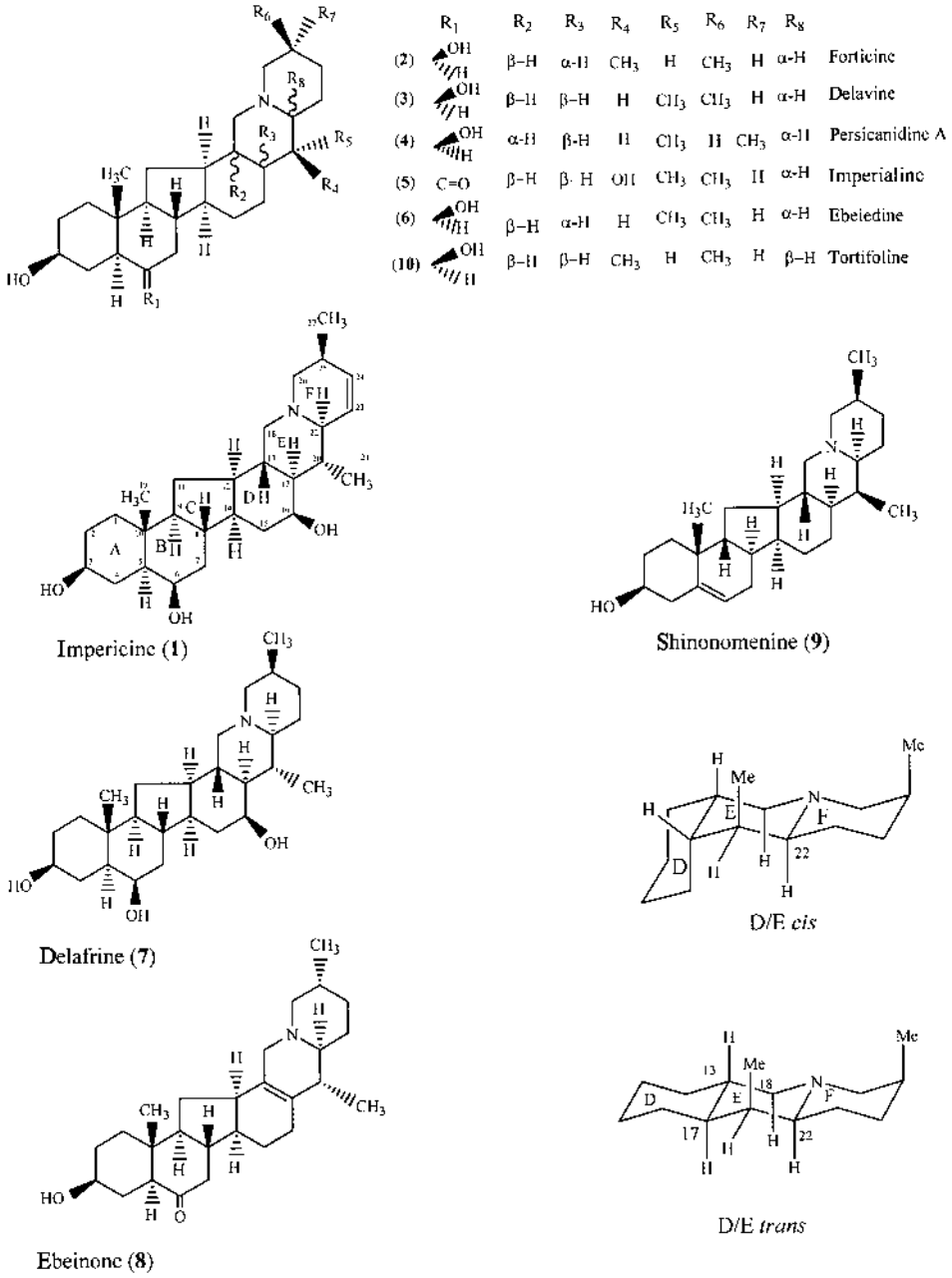

Fig. 1

Table 2. *In Vitro* Quantitative Inhibition of AChE and BChE by Compounds (**1**—**5**)

Compounds	AChE IC_{50} (μM)[a)]	BChE IC_{50} (μM)[a)]
Impericine (**1**)	67.97±2.46	1.607
Forticine (**2**)	>500	100.5±0.445
Delavine (**3**)	105.5±1.452	1.706±0.11
Persicanidine A (**4**)	352.2±4.036	4.245±0.079
Imperialine (**5**)	>500	121.5±6.612
Eserine (standard drug)	0.41±0.001	0.857±0.008

a) IC_{50} are the mean±standard mean error of three assays.

scribes the acetylcholinesterase and butyrylcholinesterase inhibition effects of compounds **1**—**5**. The concentration of compounds **1**—**5** that inhibits the enzyme by 50% (IC_{50}) are presented in Table 2. Eserine [(−)-physostigmine] was used as the standard inhibitor. As shown in the Table 2 these compounds were found to be more selective inhibitors of BChE.

Experimental

General Melting points were measured on a Yanaco micro melting point apparatus and are uncorrected. Optical rotations were measured on Jasco DIP-360 digital polarimeter by using 10 cm cell tube. IR spectra were recorded on a Jasco A-302 IR spectrophotometer. ^{1}H-NMR spectra were recorded on Bruker 300 or 400 MHz NMR spectrometers and chemical shifts were calculated from $CHCl_3$ in $CDCl_3$ (δ: 7.25). ^{13}C-NMR was recorded at 100 MHz on a Bruker AM 400 NMR spectrometer. Mass spectra (MS) were recorded on a Varian MAT 312 double focussing spectrometer connected to an IBM-AT compatible PC computer system. Column chromatography was carried out by silica gel (70—270 mesh, ASTM, Merck) and flash silica gel (230—400 mesh, ASTM, Merck). TLC was done on Merck precoated TLC cards with the solvent system of acetone : pet. ether : diethylamine (10—20% : 80—89.5% : 0—0.5%).

Extraction and Isolation The bulbs of *F. imperials* were collected (40 kg) at the flowering stage from Alanya, Turkey, Oct., 1997 by one of us

(B. Sener) and a voucher specimen was deposited at the herbarium of Department of Botany, Faculty of Pharmacy, Gazi University, Ankara, Turkey. The air-dried bulbs of *F. imperialis* (25 kg) were powdered and percolated in EtOH (25 l) at room temperature (2 weeks) to obtain crude extract (350 g). The crude extract was suspended in water (3 l) and extracted successively with hexane, chloroform, (pH 3—3.5), chloroform (pH 9—11) and ethyl acetate. The chloroform extract (pH 3—5, 75.2 g) was loaded on a silica gel column and eluted with increasing ratios of chloroform in petroleum ether. From $CHCl_3$/pet. ether, 76 fractions were collected. Fractions 14—45 (36.23 g) were combined and rechromatographed on flash silica gel by using acetone and hexane as eluent. After repeated column chromatography, impericine (**1**) (23.1 mg, *Rf* 0.70), imperialine (**5**) (210.9 mg, *Rf* 0.66), delavine (**3**) (30.1 mg, *Rf* 0.64), persicanidine A (**4**) (23.20 mg, *Rf* 0.45), and forticine (**2**) (16.90 mg *Rf* 0.41) were isolated.

Impericine (**1**): Needle-like crystals, mp 195—197 °C (decomp.). $[\alpha]_D^{25}$ −28° (*c*=0.5, $CHCl_3$), IR (KBr) cm^{-1} 3352—3365 (OH), 2775 (*trans*-quinolozidine moiety), 1608—1605 (C=C),. EI-MS *m/z* (rel. int., %): 429 $[M]^+$ (3.8), 414 (37), 400 (12), 386 (12), 289 (5), 111 (100), 109 (55), 96 (30). HR-EI-MS, 429.3717 (Calcd 429.3719 for $C_{27}H_{43}O_3N$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.85 (3H, d, *J*=6.2 Hz, H-21), 0.95 (3H, s, H-19), 1.02 (3H, d, *J*=7.0 Hz, H-27), 3.50 (1H, br m, $W_{1/2}$=23.0 Hz, H-3α), 3.72 (1H br d, $W_{1/2}$=8.0 Hz, H-6α), 3.75 (1H, m, $W_{1/2}$=7.6 Hz, H-16α), 5.20 (1H, dd, $J_{24,25\alpha}$=8.5 Hz, $J_{23,24}$=15.2 Hz, H-24), 5.50 (1H, dd, $J_{22\alpha,23}$=8.3 Hz, $J_{23,24}$=15.3 Hz, H-23). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: Table 1.

Forticine (**2**): Colorless needles, mp 221—223 °C, $[\alpha]_D^{23}$ −52° (*c*=0.50, $CHCl_3$), IR ($CHCl_3$) cm^{-1}: 3438—3430 (OH), 2768 (*trans*-quinolizidine moiety), EI-MS: *m/z* (rel. int., %) 415 $[M]^+$ (64), 400 (19), 386 (16), 358 (31), 330 (3), 218 (5), 179 (94), 111 (100), 98 (45), HR-EI-MS: *m/z* 415.3347 (Calcd 415.3377 for $C_{27}H_{45}O_2N$). ^{1}H-NMR (400 MHz, $CDCl_3$), δ: 0.92 (3H, d, *J*=6.9 Hz, H-21), 0.99 (3H, s, H-19), 1.09 (3H, d, *J*=6.8 Hz, H-27), 3.56 (1H, m, $W_{1/2}$=23.0 Hz, H-3α), 3.86 (IH, br d, $W_{1/2}$=8.0 Hz, H-6α). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: Table 1.

Delavine (**3**): Colorless needles from EtOH containing Et_2NH, mp 179—182 °C, $[\alpha]_D^{25}$ −17.2° (*c*=0.5, $CHCl_3$), (lit. 182—183 °C, $[\alpha]_D^{25}$ −20°, $CHCl_3$),[7] ^{13}C-NMR (100 MHz, $CDCl_3$) δ: Table 1.

Persicanidine A (**4**): Crystals from EtOH containing Et_2NH, mp 228—230 °C, $[\alpha]_D^{23}$ −9.7° (*c*=0.5, $CHCl_3$). (lit. 208 °C, $[\alpha]_D^{25}$ −7.2°, $CHCl_3$). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: Table 1.[15] Identity was confirmed by comparisons of ^{1}H- and ^{13}C-NMR data with those of authentic persicanidine A.

Imperialine (**5**): Colorless hexagonal-prisms from EtOH containing Et_2NH, mp 273—275 °C, $[\alpha]_D^{23}$ −32° (*c*=0.5, $CHCl_3$) (lit. 268—270, $[\alpha]_D^{23}$ −31.7°, $CHCl_3$).[16] ^{13}C-NMR (100 MHz, $CDCl_3$) δ: Table 1.

***In Vitro* Cholinesterase Inhibition Assay** Acetylcholinesterase and butyrylcholinesterase inhibiting activities were measured by the spectrophotometric method of Ellman *et al*.[18] Electric eel AChE (type VI-S, electric eel, Sigma Chemical Co.), and horse serum BChE (Sigma Chemical Co.) were used as sources of both the cholinesterases. Acetylthiocholine, iodide and butyrylthiocholine chloride (Sigma Chemical Co.) were used as substrates of the reaction, and 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) (Sigma Chemical Co.) were used for the measurement of the cholinesterase activity. All the other reagents and conditions were the same as described previously.[19] In this procedure; 140 μl of 0.1 mM sodium phosphate buffer pH 8.0, with 10 μl of DTNB, 20 μl of test compound solution, and 20 μl of acetylcholinesterase/butyrylcholinesterase solution were mixed and incubated for 15 min. at 25 °C. The reaction was then started by the addition of 10 μl of acetylthiocholine/butyrylthiocholine. The hydrolysis of acetylthiocholine and butyrylthiocholine was determined by monitoring the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine or butyrylthiocholine, at a wavelength of 412 nm. Test compounds was dissolved in 5% analytical grade ethanol. The control received the same volume of the solvent. All reactions were carried out in thrice.

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