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TRITERPENOID SAPONINS FROM THE ROOTS OF *POLYGALA TENUIFOLIA*: *IN VITRO* AND *IN SILICO* STUDY OF ACETYLCHOLINESTERASE INHIBITORY ACTIVITIES

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Summary

Triterpenoid Saponins from the Roots of *Polygala tenuifolia*: *In vitro* and *in silico* Study of Acetylcholinesterase Inhibitory Activities

Polygala tenuifolia, known as Yuan Zhi in traditional Chinese medicine and Viễn Chí in Vietnamese medicine, is renowned for its cognitive-enhancing and anti-inflammatory properties. Phytochemical investigations of its roots have led to the isolation of four oleanane-type triterpenoid saponins (**1–4**). Using sophisticated analytical techniques, such as electrospray ionization mass spectrometry (ESI-MS) and both 1D and 2D nuclear magnetic resonance (NMR) spectroscopy, the structures of these compounds were elucidated. The identified compounds were polygalasaponin XXVIII (**1**), onjisaponin B (**2**), desacylsenegasaponin B (**3**), and platycodin D (**4**). The acetylcholinesterase (AChE) inhibitory activities of these compounds were tested, revealing that compound **1** exhibited an IC_{50} value of 12.3 ± 1.6 μM, which is relatively higher than that of the positive control, tacrine (IC_{50} = 3.2 ± 1.8 μM). To further investigate the underlying mechanisms of AChE inhibition, molecular docking studies were performed. These findings validate the potential of *P. tenuifolia* as an AChE inhibitor through its triterpenoid saponin constituents. However, *in vivo* studies are crucial to confirm its therapeutic potential and explore its clinical applications.

Keywords: *Polygala tenuifolia*; *Triterpenoid saponin; Acetylcholinesterase inhibition.*

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly, a significant neurodegenerative disorder characterized by progressive cognitive decline, memory impairment, and behavioral changes [1],[2]. This cholinergic deficit has led to the exploration of various therapeutic strategies aimed at enhancing acetylcholine levels, particularly through the inhibition of acetylcholinesterase (AChE), the enzyme responsible for the degradation of acetylcholine in the synaptic cleft [3]. According to the World Health Organization (WHO), one person is diagnosed with this disease every three seconds, and the number of cases is expected to triple over the next 50 years [4]. In Vietnam, the number of people affected by Alzheimer's disease is increasing, particularly with the aging population.

In recent years, there has been increased interest in natural products and herbal medicines as potential sources of therapeutic agents for Alzheimer's disease (AD) management [5]. *Polygala tenuifolia* Willd, a prominent herb in traditional Asian medicine, has gained attention for its potential anti-inflammatory, neuroprotective effects, and cardiovascular benefits [6],[7]. The roots of *P. tenuifolia* have shown various pharmacological properties, including anti-inflammatory, anticancer, neuroprotective, antidepressant, and sedative-hypnotic actions, according to recent studies [8],[9]. Phytochemical investigations of the roots of *P. tenuifolia* have led to the isolation of various bioactive compounds, including xanthones, phenolics, oligosaccharide esters, and triterpenoid saponins, which have demonstrated promising biological activities, such as antioxidant, anti-inflammatory, and neuroprotective effects [6],[8],[9]. Additionally, *P. tenuifolia* is a key component in many Chinese and Vietnamese traditional remedies for heart and neurological diseases, including cardiovascular conditions, neurological disorders, and even COVID-19 [8]. However, there have been limited studies on triterpene saponins and their acetylcholinesterase inhibitory activities. Therefore, this study aimed to investigate bioactive compounds from the roots of *P. tenuifolia*.

2. Materials and methods

2.1. General experimental procedures

Analytical-grade solvents and reagents were used exactly as supplied, with no additional purification required. MS spectra were analyzed using a Shimadzu LCMS-8040 system (Kyoto, Japan) equipped with an electrospray ionization (ESI) source. NMR data were recorded using a

Bruker AM600 spectrometer. TMS was employed as an internal standard, with coupling constants (*J* in Hz) and chemical shift values (δ) expressed in ppm. For open column chromatography (CC), YMC*GEL (ODS-A, 12 nm S-150 mm, YMC) and *silica gel* (Kieselgel 60, 70-230 mesh, and 230-400 mesh, Merck, Darmstadt, Germany) were used. Thin-layer chromatography (TLC) was performed using Merck precoated *silica gel* $60F_{254}$ (1.05554.0001) and RP-C_{18} F_{254S} plates (1.15685.0001). Compounds on the TLC plates were identified under UV light (254 and 365 nm), and 10% (v/v) aqueous H_2SO_4 solution was sprayed to visualize them.

2.2. Plant material

Roots of *P. tenuifolia* were collected from Sapa in July 2022. The plant was authenticated by Dr. Nguyen Cao Cuong, and a voucher specimen (VC203) has been deposited at the herbarium of the Faculty of Medicine and Pharmacy, Yersin University of Da Lat, Lam Dong, Vietnam.

2.3. Extraction and isolation

The dried roots of *P. tenuifolia* (2.2 kg) were extracted three times with methanol (4 L) at room temperature. The combined filtrates were evaporated under reduced pressure to obtain a crude extract (360 g), corresponding to a yield of 16% based on the starting material. The MeOH extract was partitioned between water and *n*-hexane, dichloromethane, and ethyl acetate, yielding an *n*-hexane fraction (10 g), a dichloromethane fraction (D, 20.5 g), an ethyl acetate fraction (E, 30.8 g), and a water layer (W).

The water layer (W) was subjected to a Diaion HP-20 column and eluted with increasing concentrations of methanol in water (0%, 30, 70, and 100 %, v/v, 800 mL), resulting in four distinct fractions (W1–W4). Fraction W4 (20.6 g) was further separated using medium-pressure liquid chromatography (MPLC) on a *silica gel* column with a mobile phase of CH_2Cl_2-MeOH-H_2O (3:1:0.1, *v/v/v*, 400 mL), yielding ten subfractions (PTW4A–PTW4J).

Subfraction PTW4H (2 g) was purified using YMC RP-18 column chromatography with acetone-H_2O (1:1, v/v) as the eluent, successfully isolating compounds **2** (3.5 mg) and **4** (6.2 mg). Subfraction PTW4F (1.2 g) was further purified through chromatography using Sephadex LH-20 and RP-C18 column chromatography with a mixture of acetone-H_2O (2:1, *v/v*, 200 mL), yielding compounds **1** (10.5 mg) and **3** (2.5 mg).

Physical characteristics and key of spectroscopic data of isolated compounds:

Compound **1**. White amorphous powder, ^{1}H (600 MHz in MeOD-d_4) and ^{13}C-NMR (150 MHz in MeOD-d_4) see in **Table 1**; LC-MS *m/z* 1127.5 $[M+Na]^+$, and *m/z* 1103.7 $[M-H]^-$. The spectral data were consistent with previously reported values [10].

Compound **2**. White amorphous powder, ^{1}H (600 MHz in MeOD-d_4) δ_H 6.64 (d, J = 16.0 Hz), 7.13 (d, J = 9.0 Hz), 7.74 (d, J = 8.9 Hz), 7.88 (d, J = 16.0 Hz), and ^{13}C-NMR (150 MHz in MeOD-d_4): δ_C 44.3 (C-1), 70.3 (C-2), 85.5 (C-3), 52.2 (C-4), 52.3 (C-5), 20.5 (C-6), 33.5 (C-7), 40.9 (C-8), 49.3 (C-9), 36.6 (C-10), 24.3 (C-11), 128.5 (C-12), 138.9 (C-13), 47.2 (C-14), 24.3 (C-15), 23.5 (C-16), 43.9 (C-17), 41.8 (C-18), 45.5 (C-19), 30.6 (C-20), 33.9 (C-21), 32.2 (C-22), 180.3 (C-23), 13.3 (C-24), 17.5 (C-25), 18.3 (C-26), 64.2 (C-27), 177.8 (C-28), 32.5 (C-29), 23.4 (C-30), Cinnamoyl group 167.4 (C-α), 113.5 (C-β), 146.0 (C-γ), 127.6 (C-1‴‴‴), 130.1 (C-2‴‴‴), 114.4 (C-3‴‴‴), 162.1 (C-4‴‴‴), 114.4 (C-5‴‴‴), 130.1 (C-6‴‴‴), 55.6 (4-OCH_3, C-7), 105.0 (C-1′, Glc), 94.9 (C-1″, Fuc), 101.8 (C-1‴, Rha-1), 106.6 (C-1⁗, Xyl), 104.2 (C-1⁗, Gal), 104.5 (C-1⁗, Rha-2); LC-MS *m/z* 1595.7 $[M+Na]^+$, and *m/z* 1571.8 $[M-H]^-$. The spectral data were consistent with previously reported values [11].

Compound **3**. White amorphous powder, ^{1}H (600 MHz in MeOD-d_4) δ_H 4.93 (d, J = 7.8 Hz), 5.78 (d, J = 8.0 Hz), 6.23 (br, s), 4.77 (d, J = 7.5 Hz), 4.83 (d, J = 7.0 Hz), and ^{13}C-NMR (150 MHz in MeOD-d_4): δ_C 43.8 (C-1), 70.3 (C-2), 85.4 (C-3), 52.3 (C-4), 52.2 (C-5), 20.8 (C-6), 33.4 (C-7), 40.8 (C-8), 49.0 (C-9), 36.4 (C-10), 24.0 (C-11), 128.1 (C-12), 138.5 (C-13), 47.0 (C-14), 24.2 (C-15), 23.3 (C-16), 43.8 (C-17), 41.7 (C-18), 45.4 (C-19), 30.7 (C-20), 33.8 (C-21), 32.1 (C-22), 180.6 (C-23), 13.2 (C-24), 17.4 (C-25), 18.1 (C-26), 64.0 (C-27), 176.5 (C-28), 32.6 (C-29), 23.3 (C-30), 103.9 (C-1′, Glc), 94.2 (C-1″, Fuc), 100.9 (C-1‴, Rha-1), 106.3 (C-1⁗, Xyl), 103.3 (C-1⁗, Gal); LC-MS *m/z* 1289.5 $[M+Na]^+$, and *m/z* 1265.8 $[M-H]^-$. The spectral data were consistent with previously reported values [12].

Compound **4**. White amorphous powder, ^{1}H (600 MHz in C_5D_5N-d_5) δ_H 5.09 (d, J = 7.5 Hz), 5.67 (d, J = 3.7 Hz), δ_H 5.84 (d, J = 1.6 Hz), 6.26 (d, J = 2.4 Hz), 6.45 (d, J = 3.3 Hz), and ^{13}C-NMR (150 MHz in C_5D_5N-d_5): δ_C 43.8 (C-1), 70.3 (C-2), 85.4 (C-3), 52.3 (C-4), 52.2 (C-5), 20.8 (C-6), 33.4 (C-7), 40.8 (C-8), 49.0 (C-9), 36.4 (C-10), 24.0 (C-11), 123.0 (C-12), 144.1 (C-13), 47.0 (C-14), 24.2 (C-15), 74.0 (C-16), 50.8 (C-17), 40.6 (C-18), 45.4 (C-19), 30.7 (C-20), 33.8 (C-21),

31.2 (C-22), 63.1 (C-23), 67.1 (C-24), 17.4 (C-25), 18.1 (C-26), 64.0 (C-27), 175.8 (C-28), 32.6 (C-29), 23.3 (C-30), 106.1 (C-1′, Glc), 93.5 (C-1″, Ara), 101.0 (C-1‴, Rha), 106.6 (C-1⁗, Xyl), 111.0 Api (C-1⁗′); LC-MS *m/z* 1247.6 $[M+Na]^+$, and *m/z* 1223.7 $[M-H]^-$. The spectral data were consistent with previously reported values [13].

2.5. In vitro acetylcholinesterase enzyme assay

As previously described, with a few minor adjustments, AChE inhibition experiments were performed [14]. Briefly, a 96-well plate was prepared by adding 20 µL of MeOH or the test material dissolved in MeOH, followed by 130 µL of AChE (approximately 0.05 U/mL) in 50 mM phosphate buffer (pH 7.4). The mixture was then diluted with 25 µL of either butyrylthiocholine iodide (5 mM) or acetylthiocholine chloride (5 mM), followed by the addition of 25 µL of DTNB (1 mM). The product generation was recorded at 475 nm for 20 minutes using an UV-Vis spectrophotometer, following the initiation of the AChE reaction at 37°C. The % inhibition was calculated using the following formula:

Inhibitory activity % = [(Δcontrol – Δsample)/Δcontrol] × 100,

where the intensity of the wells containing the inhibitor and control after 20 minutes, respectively, served as the sample and control. The test compounds and the positive control, tacrine, were evaluated at concentrations of 10, 5, 2.5, 1.25 µM, 625, and 312.5 nM, respectively.

2.6. Molecular docking

The molecular docking simulation was conducted following a protocol similar to the one described in the previous report, with slight modifications [14]. Briefly, we used the crystal structure of the acetylcholine protein from the Protein Data Bank (PDB-ID: 6CQY). The protein preparation tool in Maestro v12.4 (Schrödinger, New York, NY, USA) and the 'Orthogonal Partial Least Squares 4' (OPLS4) potential energy function was employed to optimize the protein's structure until the average RMSD of non-hydrogen atoms reached 0.3 Å. The 2D structures were created using ChemDraw version 22.2 (PerkinElmer Informatics, Waltham, MA, USA). The LigPrep tool (Schrödinger) was then used to convert these 2D structures into 3D structures, yielding molecular models that were optimized at pH 7.0 ± 2.0. As the final step of LigPrep, OPLS4 was applied to minimize the energy of the 3D conformers after confirming the chirality of the ligands' 3D structures. Using the coordinates from the PDB file, we generated the docking grid for the receptor's active site with the Receptor Grid Generation tool in Maestro v12.4 (NM share, version 6.0.134; Schrödinger). The grid was created based on the location of the co-crystallized ligand, and the grid box dimensions were adjusted to match those of the co-crystallized ligand. Docking and calculations were performed using Glide in extra precision (XP) mode.

3. Results and discussion

After being suspended in water, a MeOH extract of *P. tenuifolia* roots was separated using dichloromethane and *n*-hexane. Four compounds (**1–4**) were obtained by repeating column chromatography over *silica gel*, RP-C_{18}, and Sephadex LH-20 to isolate the water-soluble residue portion.

Compound **1** was isolated as a white amorphous powder. The ^{13}C NMR spectrum, together with the molecular adduction peak at *m/z* 1127.5 ($[M+Na]^+$) in the ESI-MS spectrum, suggested the molecular formula of compound **1** as $C_{53}H_{84}O_{24}$. The ^1H and ^{13}C NMR spectra of compound **1** (**Table 1**) showed the presence of seven tertiary methyl groups at δ_H 0.71 (3H, s), 0.89 (3H, s), 0.92 (3H, s), 1.23 (3H, s), 1.36 (3H, s), 1.20 (3H, d, J = 6.6 Hz), and 1.27 (3H, d, J = 6.0 Hz), an olefinic proton at δ_H 5.61 (brs, H-12), and four anomeric proton signals at δ_H 5.44 (1H, d, J = 1.2 Hz), 5.33 (1H, d, J = 7.8 Hz), 4.43 (1H, d, J = 7.2 Hz), and 4.36 (1H, d, J = 7.2 Hz), suggesting that compound **1** possesses the Δ^{12}-oleanane triterpenoid skeleton. A doublet at δ_H 4.08 (1H, d, J = 3.6 Hz, H-3) was observed to have an HSQC correlation with a downfield-shifted oxymethine carbon at δ_C 85.4 (C-3), suggesting the location of a glycosidic linkage at C-3 of the pentacyclic triterpenoid nucleus. This was also confirmed by an HMBC correlation observed from δ_H 1.36 (s, H-24) to δ_C 85.4 (C-3) (Fig. 1). A COSY correlation observed between δ_H 4.08 (H-3) and δ_H 4.28 (brs, H-2) revealed that C-2 is an oxygenated methine group (Fig. 1). Additionally, carbon signals for an oxygenated methylene at δ_C 63.9 (C-27) and two carbonyl carbons at δ_C 183.5 (C-23) and 177.1 (C-28) were also observed in the ^{13}C NMR and HMBC spectra. These spectroscopic data suggest that compound **1** possesses the oleanane-type triterpene saponin, which is considered one of the major aglycones of triterpenoid saponins in the *Polygala* genus [10, 15]. The upfield chemical shift of C-28 (δ_C 177.1) indicated the location of a sugar moiety at the C-28 position of the aglycone, which was confirmed by the correlation between δ_H 5.33 (1H, d, J = 7.8

Hz) and C-28 (δ_C 177.1). In addition to the aglycone moiety, signals of four anomeric protons at δ_H 5.44 (1H, d, J = 1.2 Hz), 5.33 (1H, d, J = 7.8 Hz), 4.43 (1H, d, J = 7.2 Hz), and 4.36 (1H, d, J = 7.2 Hz) showed HSQC correlations with the respective carbon signals at δ_C 100.3, 94.3, 106.2, and 104.0, revealing that compound **1** has four sugar units. Finally, the locations of the sugar units were determined based on analyses of the HSQC, COSY, and HMBC spectra. Using COSY spectroscopic analysis, all the proton positions of the monosaccharides were assigned, starting from the anomeric protons. Subsequently, the positions of each monosaccharide were fully assigned through HMBC spectroscopic analysis. Accordingly, the location of the glucose at C-3 of the aglycone was confirmed by an HMBC correlation observed from δ_H 4.08 (H-1′) to δ_C 85.4 (C-3). The sugar sequence located at C-28 of the aglycone was established as *β*-D-xylopyranosyl(1→4)-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-fucopyranosyl through HMBC correlations (Fig. 2). Moreover, comparative analysis of the NMR data of compound **1** with those of the previously reported saponin, polygalasaponin XXVIII [10], showed that the structures of both compounds are closely related. Based on the spectroscopic evidence, the structure of compound **1** was established as 3-*O*-*β*-D-glucopyranosyl presenegenin 28-*O*-*β*-D-xylopyranosyl(→4)-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-fucopyranosyl ester.

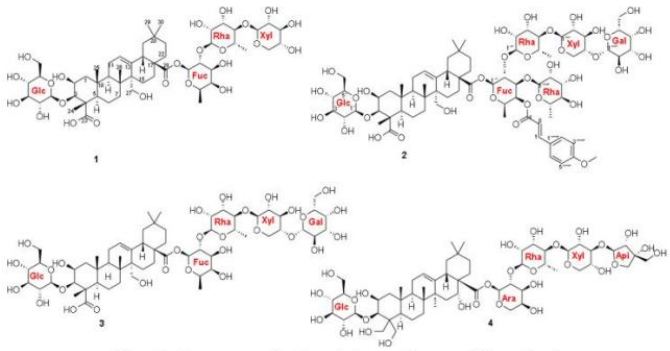

Fig. 1. Structures Isolated from *P. tenuifolia* (**1–4**)

Based on spectroscopic analysis and comparison with literature values, the remaining compounds were identified as onjisaponin B (**2**), desacylsenegasaponin B (**3**), and platycodin D (**4**), respectively.

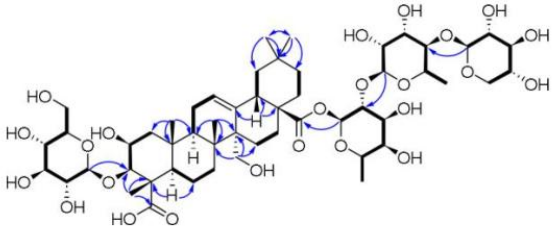

Fig. 2. Important HMBC and COSY correlations of **1**

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of **1** (^{1}H: 600 MHz; ^{13}C: 150 MHz; in MeOH-d_4).

Position	1				
	δ_H (J in Hz)	δ_C		δ_H (J in Hz)	δ_C
1	2.06 (2H, dd, 14.4, 2.4)	43.8	**C-3 sugar**		
2	4.28 (1H, brs)	70.3	**Glc-1**	4.36 (1H, d, 7.2)	104.0
3	4.08 (1H, d, 3.6)	85.4	**2**	3.70 (1H, m)	75.3
4	-	52.3	**3**	3.32 (1H, m)	77.4
5	1.62 (1H, m)	52.2	**4**	3.67 (1H, m)	71.8
6	1.60 (2H, m)	20.8	**5**	3.32 (1H, m)	77.4
7	1.18; 1.32 (m, each 1H)	33.4	**6**	3.66, 3.78 (2H, m)	61.5
8	-	40.8	**C-28 sugar**		
9	1.84 (1H, dd, 10.8, 6.6)	49.0	**Fuc-1**	5.33 (1H, d, 7.8)	94.3

Position	1				
	δ_H (*J* in Hz)	δ_C		δ_H (*J* in Hz)	δ_C
10	-	36.4	**2**	3.55 91H, m)	73.3
11	1.30 (2H, m)	24.0	**3**	3.24 (1H, m)	76.7
12	5.61 (1H, brs)	128.1	**4**	3.83 (1H, m)	73.1
13	-	138.5	**5**	3.67 (1H, m)	72.8
14	-	47.0	**6**	1.20 (3H, d, 6.6)	16.8
15	1.49 (2H, m)	24.2	**Rha-1**	5.44 (1H, d, 1.2)	100.3
16	1.94 (2H, m)	23.3	**2**	3.93 (1H, m)	71.5
17	-	43.8	**3**	3.83 (1H, m)	72.8
18	2.88 (1H, dd, 13.8, 4.8)	41.7	**4**	3.48 (1H, m)	83.7
19	1.17; 1.54 (m, each 1H)	45.4	**5**	3.80 (1H, m)	67.7
20	-	30.7	**6**	1.27 (3H, d, 6.0)	17.4
21	1.18 (2H, m)	33.8	**Xyl-1**	4.43 (1H, d, 7.2)	106.2
22	1.34 (2H, m)	32.1	**2**	3.69 (1H, m)	75.9
23	-	183.6	**3**	3.35 91H, m)	76.8
24	1.36 (3H, s)	13.2	**4**	4.28 (1H, m)	70.4
25	1.23 (3H, s)	17.4	**5**	3.17, 3.84 (2H, m)	66.3
26	0.71 (3H, s)	18.1			
27	3.46; 3.70 (m, each 1H)	64.0			
28	-	177.9			
29	0.89 (3H, s)	32.6			
30	0.92 (3H, s)	23.3			

Assignments were deduced by HSQC, COSY, HMBC, and NOESY experiments.

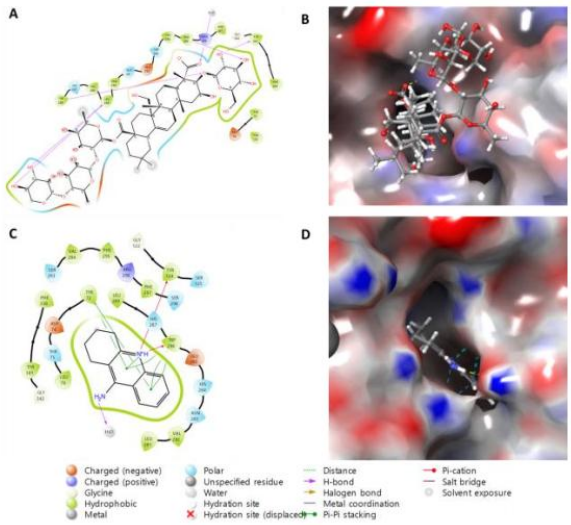

Fig. 3. Optimization and Molecular Docking of the AChE Receptor Complex (PDB ID: 6CQY) Using OPLS4 Potential Energy Function by Maestro version 12.4 (Schrödinger, New York, NY, USA). 2D (A) and 3D (B) docking interactions of **1** with AChE.

The *in vitro* AChE inhibitory activity was assessed based on our previous protocol with slight modifications [16]. The AChE conversion rate in the presence of compounds **1**–**4** was measured to evaluate their AChE inhibitory activity. Each compound was initially screened for anti-AChE activity at a concentration of 100 μM. Among the tested compounds, only compound **1** exhibited significant inhibition, with an IC_{50} value of 12.3 ± 1.6 μM, which was relatively higher compared to the positive control, tacrine (IC_{50} = 3.2 ± 1.8 μM). The other compounds are inactive (IC_{50} > 100 μM). Previously, saponins from the roots of *P. tenuifolia* have also been reported as AChE inhibitors [7]. Moreover, saponins from *P. tenuifolia*, such as onjisaponins Ng and J, as well as polygalasaponin XXXII, were reported to exhibit neuroprotective effects [15]. These findings suggest that polygalasaponin XXVIII (**1**) of *P. tenuifolia* may possess therapeutic potential for managing conditions such as Alzheimer's disease, where the regulation of acetylcholine levels is crucial.

Molecular docking simulations were carried out to identify the binding positions of substrate ligands on AChE [17],[18]. Compound **1** demonstrated strong binding to the catalytic site of AChE (PDB ID: 6CQY) [18] with a binding affinity of −10.482 kcal/mol, compared to the positive control, tacrine, which showed a binding affinity of −13.020 kcal/mol. These results were corroborated by *in vitro* experiments. Based on the high binding affinity of compound **1**, it is inferred that it exhibits strong interactions with the 6CQY target protein and may hold therapeutic potential. Compound **1** was associated with the AChE active site, as depicted in **Fig. 3**, forming hydrogen bonds with TRP286 (2.69 Å), LEU289 (1.87 Å, 1.97 Å), PHE295 (2.13 Å), and TYR341 (1.84 Å). These results suggest that polygalasaponin XXVIII (**1**) may possess significant AChE inhibitory activity.

4. Conclusion

In summary, our ongoing phytochemical investigation of *P. tenuifolia* led to the isolation of four oleanane triterpenoid saponins (**1**–**4**). The isolated compounds were identified as polygalasaponin XXVIII (**1**), onjisaponin B (**2**), desacylsenegasaponin B (**3**), and platycodin D (**4**), respectively. The AChE inhibitory activities of the isolated compounds were evaluated, with compound **1** exhibiting an IC_{50} value of 12.3 ± 1.6 μM, which is relatively higher compared to the positive control, tacrine (IC_{50} = 3.2 ± 1.8 μM). To further explore the underlying mechanisms, molecular docking simulations were performed, revealing that **1** interacts with the active site of AChE. Overall, *P. tenuifolia* emerges as a promising source of abundant saponins and effective natural agents in herbal medicine.

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