Sesquiterpenoids from the Fruits of *Alpinia oxyphylla* and Their Anti-Acetylcholinesterase Activity

by **Ping Chen**, **Pei-Pei Wang**, **Ze-Zhao Jiao**, and **Lan Xiang***

School of Pharmaceutical Sciences, Shandong University, Jinan 250012, P. R. China (phone: +86-531-88382028; fax: +86-531-88382548; e-mail: xianglan02@sdu.edu.cn)

Fourteen sesquiterpenoids were isolated from the fruits of *Alpinia oxyphylla* MIQ. Their structures were elucidated based on NMR analyses (^{1}H, ^{13}C, DEPT, ^{1}H,^{1}H-COSY, HMQC, HMBC, and NOESY) and identified as 12-nornootkaton-6-en-11-one (**3**), (+)-(3*S*,4a*S*,5*R*)-2,3,4,4a,5,6-hexahydro-3-isopropenyl-4a,5-dimethyl-1,7-naphthoquinone (**5**), nootkatene (**6**), 9β-hydroxynootkatone (**7**), 2β-hydroxy-δ-cadinol (**8**), 4-isopropyl-6-methyl-1-tetralone (**11**), oxyphyllone E (**12**), oxyphyllone D (**13**), oxyphyllanene B (**15**), oxyphyllone A (**16**), oxyphyllol E (**17**), (9*E*)-humulene-2,3;6,7-diepoxide (**18**), mustakone (**20**), and pubescone (**21**). Among them, **3** was a new norsesquiterpenoid, **8** was a new natural product, and **5**, **6**, **11**, **20**, **21** were isolated from *A. oxyphylla* for the first time. Twenty sesquiterpenoids, **1**–**5** and **7**–**21**, were investigated for their *in vitro* acetylcholinesterase (AChE) inhibitory activities, including previously isolated seven sesquiterpenoids from *A. oxyphylla*, (11*S*)-12-chloronootkaton-11-ol (**1**), (11*R*)-12-chloronootkaton-11-ol (**2**), nootkatone (**4**), oxyphyllenodiol A (**9**), oxyphyllenodiol B (**10**), 7-epiteucrenone B (**14**), and alpinenone (**19**). TLC-Bioautographic assay indicated that **1**–**4**, **7**, **14**, **16**, **18**, **19**, and **21** displayed anti-AChE activities at 10 nmol. Microplate assay confirmed that **19**, **18**, **16**, and **21** displayed moderate-to-weak anti-AChE activities at the concentration of 100 μM, and **19** was the most potent inhibitor with an IC_{50} value of 81.6 ± 3.5 μM. The presence of anti-AChE sesquiterpenoids in *A. oxyphylla* may partially support the traditional use of this fruit for the treatment of dementia.

Introduction. – *Alzheimer*'s disease (AD), the most common form of dementia in elderly, is a chronic, progressive neurodegenerative disorder with characteristic cognition impairment [1]. According to cholinergic hypothesis, loss of cholinergic neurons in the AD brain leads to decreased acetylcholine (ACh) level and causes impairment in cognition function. Use of acetylcholinesterase inhibitor (AChEI) to improve the acetylcholine level is an important strategy for the treatment of AD. Medicinal plants have attracted attention for the discovery of AChEI due to their potential roles in the treatment of dementia and less side-effects compared with the synthetic AChEIs [2].

Previously, we screened anti-AChE activities of 48 traditional Chinese medicines which are commonly used for the treatment of dementia or related diseases. An EtOH extract (0.1 mg/ml) of the fruits of *Alpinia oxyphylla* MIQ. (Zingiberaceae) showed the highest activity (44.5 ± 3.7% inhibition) among the 48 tested plant species [3]. The fruits of *A. oxyphylla*, called '*Yi Zhi Ren*' in China, is used as a traditional medicine and also a medicinal food for the treatment of intestinal disorders, diuresis, and dementia [4]. Sesquiterpenoids are the major characteristic constituents of *A. oxyphylla*, which are divided into eremophilane, eudesmane, cadinane, guaiane, oplopanone, and humulane types. Several sesquiterpenoids showed potent inhibitory effects on nitric

oxide (NO) production [5–9]. To date, these sesquiterpenoids have not been investigated regarding their AChE activities. The discovery of anti-AChE constituents in *A. oxyphylla* will provide insight with respect to the functional mechanism of this plant for the treatment of dementia.

Herein, we report isolation and structure elucidation of 14 sesquiterpenoids and *in vitro* effects of 20 sesquiterpenoids on AChE activity.

Results and Discussion. – Previously, we screened three fractions from the EtOH extract of *A. oxyphylla* for their AChE activity, and found that AcOEt fraction displayed $30.4 \pm 2.6\%$ inhibition of the AChE activity at 0.1 mg/ml, higher than BuOH fraction ($9.1 \pm 4.2\%$) and aqueous fraction ($6.5 \pm 3.5\%$). Our previous phytochemical investigation on AcOEt fraction led to isolation of seven sesquiterpenoids, **1**, **2**, **4**, **9**, **10**, **14**, and **19** (*Fig. 1*), among which **1** and **2** were two new halogenated sesquiterpenoids [10]. In our continuous search for bioactive constituents of the AcOEt fraction of *A. oxyphylla*, fourteen sesquiterpenoids, **3**, **5**–**8**, **11**–**13**, **15**–**18**, **20**, and **21** (*Fig. 1*) were isolated, and they were identified as 12-nornootkaton-6-en-11-one (**3**), (+)-(3*S*,4a*S*,5*R*)-2,3,4,4a,5,6-hexahydro-3-isopropenyl-4a,5-dimethyl-1,7-naphthoquinone (**5**) [11], nootkatene (**6**) [12], 9β-hydroxynootkatone (**7**) [13], 2β-hydroxy-δ-cadinol (**8**) [14], 4-isopropyl-6-methyl-1-tetralone (**11**) [15], oxyphyllone E (**12**) [16], oxyphyllone D (**13**) [17], oxyphyllanene B (**15**) [7], oxyphyllone A (**16**) [18], oxyphyllol E (**17**) [9], (9*E*)-humulene-2,3;6,7-diepoxide (**18**) [19], mustakone (**20**) [20], and pubescone (**21**) [21] (*Fig. 1*). Among them, **3** was a new norsesquiterpenoid, 2β-hydroxy-δ-cadinol (**8**) was a new natural product, and **5**, **6**, **11**, **20**, **21** were isolated from *A. oxyphylla* for the first time.

Compound **3** was obtained as a white amorphous powder. Its molecular formula was deduced as $C_{14}H_{18}O_2$, with six degrees of unsaturation, based on the peak at *m*/*z* 219.1375 ($[M + H]^+$, $C_{14}H_{19}O_2^+$; calc. 219.1380) in HR-ESI-MS. The ^{1}H-NMR spectrum of **3** (*Table*) showed signals of two olefinic H-atoms at δ(H) 5.90 (br. *s*, 1 H) and 6.78 (br. *s*, 1 H), three Me groups at δ(H) 1.14 (*d*, $J = 6.6$, 3 H), 1.26 (*s*, 3 H), and 2.35 (*s*, 3 H). ^{13}C-NMR, DEPT, and HSQC spectra (*Table*) revealed the presence of 14 C-atoms, including three Me, three CH_2, and three CH groups, which include two olefinic C-atoms, and five quaternary C-atoms, which consist of two carbonyl and two olefinic C-atoms. These data suggested that **3** was a norsesquiterpenoid.

The ^{1}H,^{1}H-COSY correlations indicated the presence of –CH=C–CH_2–CH_2– and –CH_2–CH(Me)–C–CH = C– fragments (*Fig. 2*). HMBC Spectrum (*Table*, *Fig. 2*) also showed the correlations from H–C(1) (δ(H) 5.90) to C(3), C(5), and C(9), from H–C(6) (δ(H) 6.78) to C(5), C(7), C(8), C(10), C(11), and C(15), from Me(13) (δ(H) 2.35) to C(7) and C(11), from Me(14) (δ(H) 1.14) to C(3) and C(5), and from Me(15) (δ(H) 1.26) to C(4), C(6), and C(10). Based on these correlations, the planar structure of **3** was determined as a 12-noreremophilane sesquiterpenoid (*Fig. 2*). The ^{1}H- and ^{13}C-NMR assignments are compiled in the *Table*.

The relative configuration of **3** was determined by NOESY correlations (*Fig. 3*). The correlations Me(14)/H_α–C(3), H_α–C(8); H_β–C(8)/H_β–C(4), H_β–C(3); and Me(15)/H_α–C(3) indicated that Me(14) and Me(15) were α-oriented. Therefore, **3** was determined as a 12-noreremophilane and identified as 12-nornootkaton-6-en-11-one.

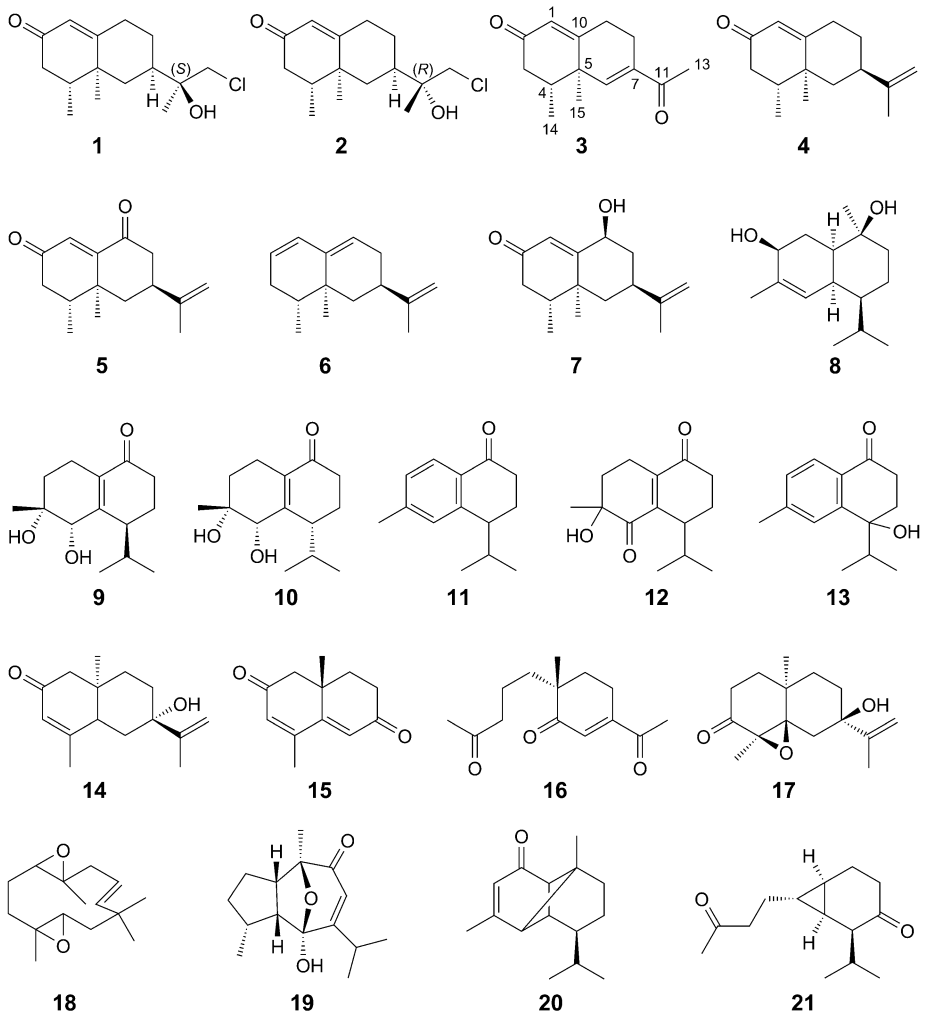

Fig. 1. *Sesquiterpenoids* **1**–**21** *isolated from* A. oxyphylla

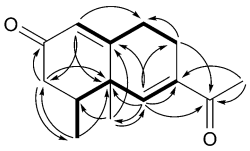

Fig. 2. *^{1}H,^{1}H-COSY* (—) *and key HMB* (H → C) *correlations of compound* **3**

Combined with the seven sesquiterpenoids, **1**, **2**, **4**, **9**, **10**, **14**, and **19**, previously isolated from *A. oxyphylla*, we evaluated the effects of 20 sesquiterpenoids (*Fig. 1*) on AChE activity. Although **6** was obtained in a relatively high amount, we found that it was easily converted to **4** when exposed to air or dissolved in MeOH; therefore, we did

Table. *^{1}H- and ^{13}C-NMR Data* (600 and 150 MHz, resp.; in $CDCl_3$) *of Compound* **3**. δ in ppm, J in Hz. Atom numbering as indicated in *Fig. 1*.

Position	δ(H)	δ(C)	HMBC (H → C)
1	5.90 (br. *s*)	124.9 (*d*)	3, 5, 9
2	–	198.3 (*s*)	
3	2.40–2.43 (*m*, H_α), 2.37–2.40 (*m*, H_β)	42.2 (*t*)	1, 2, 4, 5, 14
4	2.13–2.20	37.2 (*d*)	2, 3, 5, 6, 10, 15
5	–	40.7 (*s*)	
6	6.78 (br. *s*)	143.3 (*d*)	4, 5, 7, 8, 10, 11, 15
7	–	137.9 (*s*)	
8	2.13–2.20 (overlap, H_α), 2.8–2.86 (*m*, H_β)	24.7 (*t*)	6, 7, 9, 10, 11
9	2.50–2.55 (*m*)	29.9 (*t*)	1, 5, 7, 8, 10
10	–	168.0 (*s*)	
11	–	198.5 (*s*)	
13	2.35 (*s*)	25.4 (*q*)	6, 7, 11
14	1.14 (*d*, $J = 6.6$)	15.1 (*q*)	2, 3, 5
15	1.26 (*s*)	18.8 (*q*)	4, 6, 10

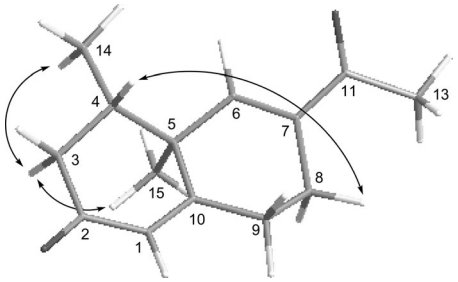

Fig. 3. *Key NOSEY* (H ↔ H) *correlations of compound* **3**

not test the activity of **6**. The methods frequently used for the determination of AChE activity are TLC bioautographic assay using Fast Blue B salt reagent, and 96-well microplate assay using *Ellman*'s reagent. Both of these two assays are considered suitable for the generation of new hits of AChE inhibitors. By comparison, the TLC assay is more suitable for the screening of crude extracts of plants as it allows separation and detection of active compounds at the same time, and the microplate assay is more appropriate for the screening of libraries of compounds, as it allows the possibility of automation and easy handling of large amounts of samples [22].

As shown in *Fig. 4*, the white spots in TLC autographic assay indicated that **1**–**4**, **7**, **14**, **16**, **18**, **19**, and **21** possessed anti-AChE activities at 10 nmol. The positive control galanthamine hydrobromide showed anti-AChE activity at 2 nmol. Nootkatone (**4**) is a major constituent of *A. oxyphylla* as shown in *Fig. 4*. This sesquiterpenoid was firstly isolated from heartwood of Alaska yellow cedar, and it was also detected in grapefruit (*Citrus paradisi*), pummelo (*Citrus grandis*), and vetiver (*Vetiveria zizanioides*) oil [23]. *Miyazawa et al.* evaluated the effect of nootkatone isolated from essential oils of *C. paradisi* on AChE from bovine erythrocytes using *Ellman* colorimetric assay and found that nootkatone showed 17.6% inhibition of AChE activity at the concentration

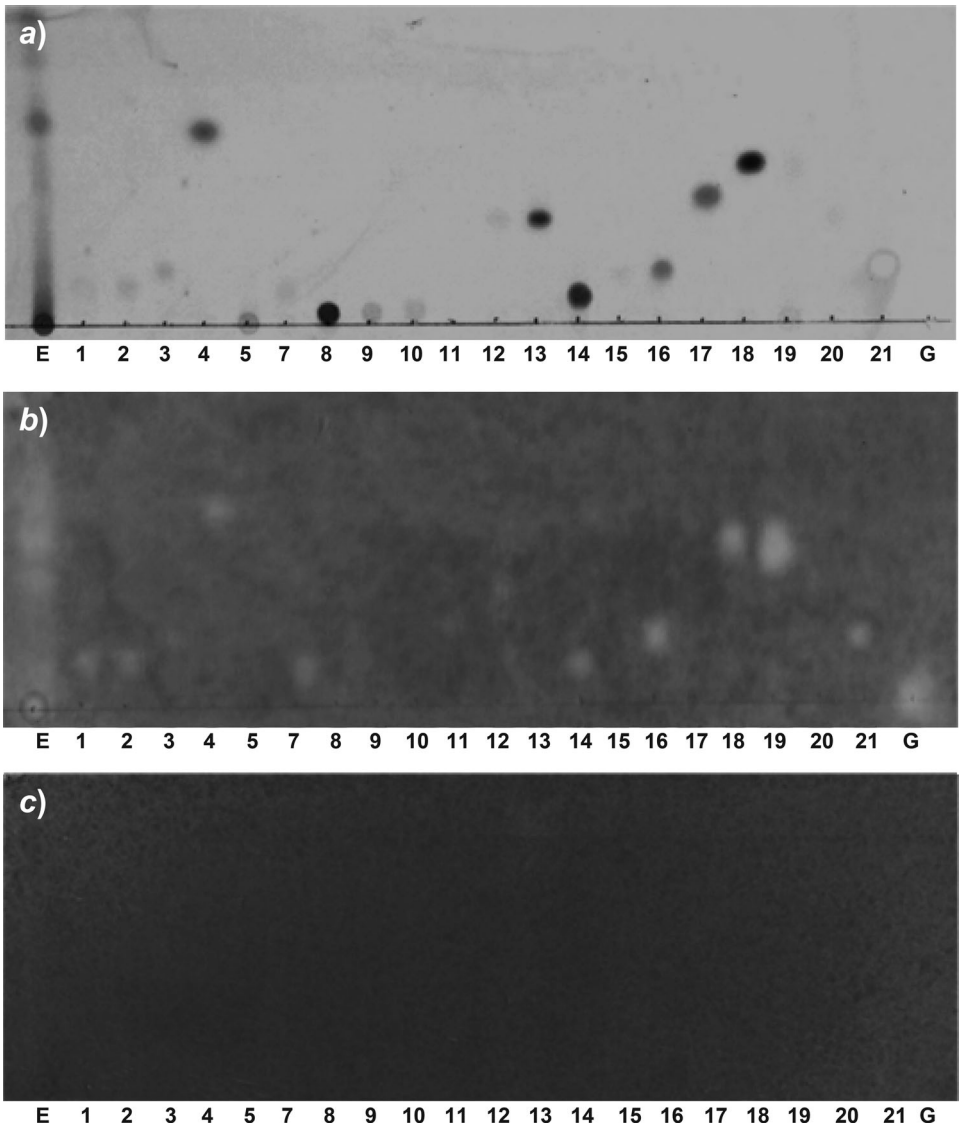

Fig. 4. *Thin layer chromatogram* (petroleum ether/AcOEt 3 : 1) *of the crude EtOH extract* (**E**, 10 mg/ml, 1 µl), *20 sesquiterpenoids* (**1**–**5** and **7**–**21**, 10 mM, 1 µl) *of* A. oxyphylla, *and the positive control galanthamine hydrobromide* (**G**, 1 mM, 2 µl). *a*) Reference chromatogram sprayed with vanillin-sulfuric acid. *b*) TLC Bioautographic assay; spots indicate inhibition. *c*) Absence of white spots indicate that there was no false-positive result.

of 1.62 µg/ml [24]. In contrast, *Anderson* and *Coats* found that nootkatone (ranging from 0 to 30,000 µM) did not inhibit the AChE in house fly (*Musca domestica*), yellow fever mosquito (*Aedes aegypti*), American dog tick (*Dermacentor variabilis*), and American cockroach (*Periplaneta americana*) [25]. *Ibrahim et al.*, on the other hand,

reported that the interference of nootkatone with AChE of worker termites fluctuated from slight inhibition to significant activation [26]. These contradictory results may be associated with the diversity in the sources of AChE. Compounds **1**–**3**, **5**, and **7** are derivatives of nootkatone (**4**). Concerning the structure–activity relationship of eremophilane-type sesquiterpenoid **1**–**7** observed by TLC autographic assay, it seems that halogenation at C(12) has no effect on AChE activity, as in the case of **1** and **2**. The white color produced by the new compound **3** was weaker than that observed for **4**, implying that the inhibition of AChE decreased when **4** was oxygenized into **3** at C(6) and C(11). When **4** was oxygenized to **7** at C(9), the activity was still retained, however, the inhibition disappeared when **7** was further oxygenized to **5**. None of the cadinane-typed sesquiterpenoids, **8**–**13**, exhibited anti-AChE activity.

However, when these 20 sesquiterpenoids were further evaluated by microplate assay at 100 μM (25 nmol in soln.), it was observed that only guaiane-type sesquiterpenoid **19**, humulane-type sesquiterpenoid **18**, 4,5-secoeudesmane-type sesquiterpenoid **16**, and carabrane sesquiterpenoid **21** showed anti-AChE activities, with the inhibition values of 56.1 ± 1.9, 22.2 ± 2.2, 16.2 ± 1.1, and $10.1 \pm 0.3\%$, respectively (*Fig. 5*). In a further microplate assay, the positive control galanthamine hydrobromide and alpinenone (**19**) showed anti-AChE activity in a concentration-dependent manner (*Fig. 6*). Their IC_{50} values were 1.3 ± 4.3 and 81.6 ± 3.5 μM, respectively, calculated according to the SIGMAPLOT logarithm equations, $Y = 11.879 \ln(X) + 46.687$ ($R^2 = 0.9530$) and $Y = 22.604 \ln(X) - 49.512$ ($R^2 = 0.9801$), respectively.

In our experiment, six sesquiterpenoids, **1**–**4**, **7**, and **14**, were positive in TLC bioautographic assay, while inactive in microplate assay. Only the results of **16**, **18**, **19**, and **21** in both assays were convergent. The reasons for the difference observed in these two different assays have been discussed in detail by *Reist* and co-workers in 2008, and might be explained by the interaction of either AChE or test compounds with the

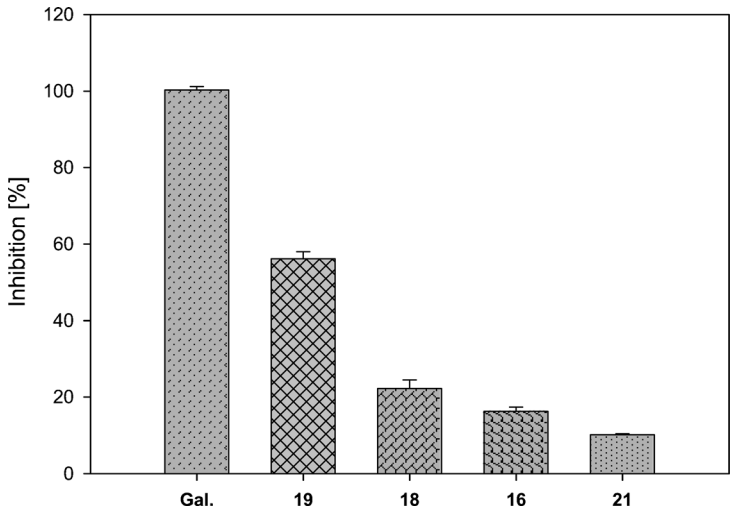

Fig. 5. *AChE Inhibitory activities of sesquiterpenoids* **19**, **18**, **16**, **21**, *and galanthamine hydrobromide* (100 μm) *in the 96-well microplate assay* ($n = 3$)

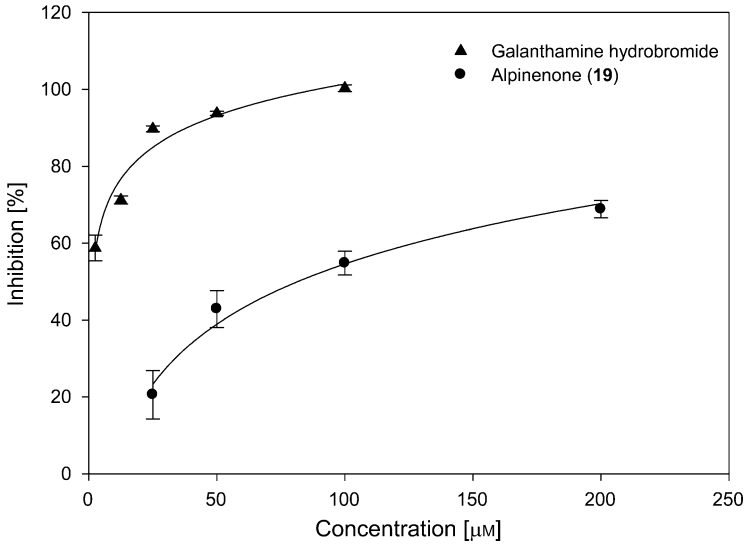

Fig. 6. *Concentration-dependent anti-AChE activities of alpinenone* (**19**) *and the positive control galanthamine hydrobromide* ($n = 3$)

silica of the TLC plates, resulting in an altered affinity of the enzyme for the compounds [22].

Although the activities of sesquiterpenoids tested in this experiment were lower than galanthamine hydrobromide, the possible synergic anti-AChE activities of these sesquiterpenoids present in *A. oxyphylla* may support the traditional use of this fruit for the treatment of dementia. Further *in vivo* experiment are needed to verify this hypothesis.

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 200–300 mesh; *Qingdao Haiyang Chemical Group Co., Ltd.*, P. R. China), *ODS-C18* (75 μm, *YMC Co.*, Japan), *MCI* gel (*CHP-20P*, 75–150 μm, *Mitsubishi Chemical Co.*, Japan), and *Sephadex LH-20* (*Pharmacia Fine Chemicals*, USA). Semi-prep. HPLC: *Shimadzu Prominence LC-20A* liquid chromatography, with *LC-20AT* pumps, *SPD-20A* UV detector (*Shimadzu Co.*, Japan) and a *YMC-Pack ODS-A* column (250 mm × 10 mm, 5 μm) (*YMC Co.*, Japan). TLC: *Silica Gel 60 GF254* plates (*Merck*, Germany); visualization by heating the plates sprayed with 10% H_2SO_4 or 5% vanillin-sulfuric acid reagent. UV Spectra: *UV-2450* spectrophotometer (*Shimadzu Co.*, Japan). Optical rotations: *Gyromat-Hp* digital automatic polarimeter (*Kernchen Co.*, Germany). NMR Spectra: *Bruker AV-600*; δ in ppm, with Me_4Si as an internal standard; J in Hz. ESI-MS: *API 4000* mass spectrometer (*Applied Biosystems Co.*, USA). HR-ESI-MS: *LTQ-Orbitrap* mass spectrometer (*Thermo Fisher Co.*, USA), in m/z. Microplate reader (model *680 UV*, *Bio-Rad*) was used in the experiment for anti-AChE assay.

Chemicals and Reagents. AChE from electric eel (type VI-S, lyophilized powder, 265 U/mg solid, 410 U/mg protein), acetylthiocholine iodide (ATCI), 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), 1-naphthyl acetate, naphthalen-1-ol and Fast Blue B salt were purchased from *Sigma*. Galanthamine hydrobromide was purchased from *Aladdin Chemistry*, China. Petroleum ether (PE), AcOEt, CH_2Cl_2, BuOH, EtOH, MeOH, and all other reagents were of anal. grade.

Plant Material. The fruits of *A. oxyphylla* were purchased from Shuyupingmin Pharmacy (Jinan, P. R. China) in March 2011 and authenticated by *L. X.* A voucher specimen (No. 090801) has been deposited with the Laboratory of Pharmacognosy, School of Pharmaceutical Sciences, Shandong University.

Extraction and Isolation. The dried fruits of *A. oxyphylla* (5 kg) were powdered and extracted with 95% EtOH (4 × 25 l) at r.t. The combined extracts were condensed to yield a dark brown crude extract (450 g). It was then suspended in 20% EtOH and partitioned with PE, AcOEt, and BuOH successively. The AcOEt extract (210 g) was chromatographed on a silica gel column (1.4 kg, 200–300 mesh, 10 × 75 cm) eluting with a gradient PE/AcOEt 100:1, 20:1, 10:1, 7:1, 5:1, 3:1, 2:1, 1:1 to yield twelve fractions, *Frs. 1–12*. *Fr. 2* (6.7 g) was subjected to CC (silica gel; PE/AcOEt 80:1 → 5:1) to yield six fractions, *Frs. 2.1–2.6*. *Fr. 2.3* (230 mg) was purified by semi-prep. HPLC (MeOH/H_2O 95:5) to obtain **11** (t_R 16.2 min; 4.2 mg). Crystallization (EtOH) of *Fr. 2.5* yielded compound **18** (40 mg). *Fr. 2.6* (1.0 g) was purified by *MCI* and silica-gel CC, and semi-prep. HPLC (MeOH/H_2O 75:25) to furnish **5** (t_R 23.0 min; 19.6 mg). *Fr. 4* (1.6 g) was separated by CC (*ODS-C18*; EtOH/H_2O 22:78 → 90:10) to afford three fractions, *Frs. 4.1–4.3*. *Fr. 4.2* (600 mg) was further purified by repeated CC (silica gel) and semi-prep. HPLC (MeOH/H_2O 85:15) to give **6** (t_R 30.6 min; 51.3 mg). *Fr. 5* (7.0 g) was subjected to CC (*MCI*; EtOH/H_2O 35:65 → 70:30) to afford three fractions, *Frs. 5.1–5.3*. *Fr. 5.1* (1.2 g) was subjected to CC (silica gel; petroleum ether (PE)/CH_2Cl_2 1:2 → 1:40) to yield five fractions, *Frs. 5.1.1–5.1.5*. *Fr. 5.1.2* (200 mg) was further purified by semi-prep. HPLC (MeOH/H_2O 60:40) to give **20** (t_R 19.5 min; 26.0 mg) and **12** (t_R 27.6 min; 35.6 mg). Besides, *Fr. 5.1.3* (120 mg) was further purified by semi-prep. HPLC (MeOH/H_2O 64:36) to afford **17** (t_R 32.1 min; 18.8 mg) and **13** (t_R 35.2 min; 5.3 mg). *Fr. 7* (5.8 g) was subjected to CC (*MCI*; EtOH/H_2O 20:80 → 100:0) to afford three fractions, *Frs. 7.1–7.3*. *Fr 7.2* (1.3 g) was then subjected to CC (silica gel; PE/CH_2Cl_2 1:4 → 0:1) to yield five fractions, *Frs. 7.2.1–7.2.5*. *Fr. 7.2.3* (50 mg) was further purified by semi-prep. HPLC (MeOH/H_2O 55:45) to yield **21** (t_R 40.5 min; 9.1 mg). *Fr. 7.2.4* (82 mg) was further purified by semi-prep. HPLC (MeOH/H_2O 52:48) to furnish **15** (t_R 20.1 min; 10.9 mg), **16** (t_R 32.5 min; 10.8 mg), and **3** (t_R 34.6 min; 7.0 mg). *Fr. 8* (11.1 g) was purified by CC (*ODS-C18*; EtOH/H_2O 20:80 → 100:0) to afford nine fractions, *Frs. 8.1–8.9*. *Fr. 8.5* (1.8 g) was subjected to CC (silica gel; CH_2Cl_2/AcOEt 20:1 → 4:1) to yield five fractions, *Frs. 8.5.1–8.5.5*. *Fr. 8.5.2* (80 mg) was further purified by semi-prep. HPLC (MeOH/H_2O 65:35) to provide **7** (t_R 37.5 min; 14.6 mg). *Fr. 9* (25 g) was separated by CC (silica gel; CH_2Cl_2/AcOEt (95:5 → 5:5) to afford ten fractions, *Frs. 9.1–9.10*. *Fr. 9.4* (5.1 g) was purified by repeated CC (silica gel) to yield twelve fractions, *Frs. 9.4.1–9.4.12*. *Fr. 9.4.4* (82 mg) was separated by CC (silica gel; CH_2Cl_2/MeOH 10:1 → 2:1) to afford six fractions, *Frs. 9.4.4.1–9.4.4.6*. *Fr. 9.4.4.3* (15 mg) was further purified by CC (*Sephadex LH-20*; EtOH) to afford **8** (2.3 mg).

12-Nornootkatone-6-ene-11-one (= *(4*R*,4a*R*)-6-Acetyl-4,4a-dimethyl-4,4a,7,8-tetrahydronaphthalen-2(3*H*)-one*; **3**). White amorphous powder. $[\alpha]_D^{24} = +14.6$ ($c = 0.1$, MeOH). UV (MeOH): 233 (4.01). ^{1}H- and ^{13}C-NMR: see the *Table*. HR-ESI-MS: 219.1375 ($[M+H]^+$, $C_{14}H_{19}O_2^+$; calc. 219.1380).

Sample Preparation for Anti-AChE Assay. The EtOH extract of *A. oxyphylla*, together with the AcOEt, BuOH, and residual aq. fractions, were dissolved in MeOH to obtain the stock solns. with a concentration of 10 mg/ml. Compounds were dissolved in MeOH or deionized H_2O to yield 10 mM stock solns.

TLC Bioautographic Assay for AChE Inhibition Activity. The stock solns. (10 mM) of 20 sesquiterpenoids, together with the positive control galanthamine hydrobromide, were applied onto silica-gel plate and separated by proper developing solvent, and then applied to TLC bioautographic assay as described in [27]. Briefly, the TLC plate was sprayed with the enzyme soln. (1 U/ml in buffer *A*) and 1-naphthyl acetate soln. (1.5 mg/ml; dissolved in 40% EtOH) subsequently. The TLC plate was quickly blown with cold air to remove EtOH, then it was put in a closed vessel containing some H_2O for humidity (*Note:* the TLC plate may not contact H_2O) at 37°. After incubation for 15 min, the TLC plate

was sprayed with Fast Blue B salt (0.5 mg/ml; dissolved in distilled H_2O). As reaction of 1-naphthyl acetate and AChE led to the production of naphthalen-1-ol, which showed purple color when reacted with Fast Blue B salt, the compound which had AChE inhibitory activity gives a white spot on the purple background. To eliminate the false-positive results, 1-naphthyl acetate was replaced by naphthalen-1-ol in above experiment. A white spot with this reagent would indicate a false positive-result.

Microplate Assay for AChE Inhibition Activity. Above stock solns. were diluted ten times with buffer *A* (50 mM *Tris*·HCl; pH 8), and the diluted solns. were used in the 96-well microplate assay. AChE Inhibition activity was measured based on *Ellman*'s method with slight modifications [28]. In the 96-well microplate, 25 µl of ATCI (15 mM in deionized H_2O), 125 µl of DTNB (3 mM in buffer *C* (50 mM *Tris*·HCl, pH 8, containing 0.1M NaCl and 0.02M $MgCl_2 \cdot 6\,H_2O$)), 50 µl of buffer *B* (50 mM *Tris*·HCl, pH 8, containing 0.1% bovine serum albumin), 25 µl of sample soln. or blank (MeOH or deionized H_2O diluted with buffer *A*) were added, and the absorbance was measured at 405 nm every 25 s for five times. Thereafter, 25 µl of 0.22 U/ml AChE (diluted with buffer *B*) was added, and the absorbance was measured again every 25 s for eight times. Galanthamine hydrobromide was used as the positive control. Percentage of inhibition was calculated by comparing the reaction rates for the sample to the blank. The results were expressed as mean ± SD.

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