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Communication

Amyloid-β aggregation inhibitors from *Clerodendrum inerme* L. Gaertn

Thanavadee Kor-arnan [1,2], Thanawan Rojpitikul [1,2], Preecha Phuwapraisirisan [2], Surachai Pornpakakul [3] and Pattara Thiraphibundet [2,*]

[1] Program of Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[2] Center of Excellence in Natural Products Chemistry, Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[3] Research Centre for Bioorganic Chemistry, Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
* Corresponding author, e-mail: p_tiew@hotmail.com

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Abstract: *Clerodendrum inerme L.* (Verbenaceae) is a medicinal herb traditionally used in the treatment of neuropsychiatric disorders. This research investigates some of the chemical constituents of the leaves and roots of *C. inerme* and evaluates their possible inhibitory effect on the self-mediated amyloid-*β* (A*β*) aggregation, since A*β* aggregates in the human brain have been implicated as the vital cause of neurotoxicity in Alzheimer's disease. The dichloromethane extracts of the leaves and roots of *C. inerme* show a significant *in vitro* anti-A*β* aggregation activity, as determined by thioflavin-T fluorescence assay. Upon further fractionation of the extracts, three flavonoids (**2**–**4**), two steroids (**1** and **5**) and four triterpenoids (**6**–**9**) were obtained and their structures were established. Among them, the flavonoids show a higher inhibition than do the other two classes of constituents. The flavonoid acacetin (**3**) displays the highest anti-A*β* aggregation activity (29%), which is close to that of the curcumin standard (31%) at 20 μM. Substituent variation on the lupane skeleton (**7**–**9**) was also found to have a strong effect on the A*β* aggregation inhibition.

Keywords: *Clerodendrum inerme*, Amyloid-*β* aggregation, Alzheimer's disease

INTRODUCTION

Alzheimer's disease (AD) accounts for approximately 60–80% of dementia syndrome and mostly occurs in females older than 65 years. In 2010 there were 4.7 million individuals aged 65

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years or older with AD dementia and the total number of people with AD dementia in 2050 is predicted to increase to 13.8 million. Although the causes of AD are not yet fully understood, acetylcholinesterase (AChE) inhibitors (AChEIs) have become one of the main targets in the current therapy of AD [1]. Since AChE terminates nerve impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (ACh), an effective therapeutic approach is to stabilise and increase the availability of acetylcholine (ACh) within cholinergic synapses by preventing its hydrolysis. However, clinical AChEIs (donepezil, rivastigmine and galantamine) show only modest improvement in the behavioural symptoms and cognitive function but less progressive neurodegeneration prevention [2]. Besides the low level of ACh, amyloid-β (Aβ) aggregation in the brain is found to initiate the pathogenic cascade, ultimately leading to neuronal loss and dementia [3]. Several pieces of evidence indicate that the inhibition of Aβ accumulation is more effective in delaying the progressive neurodegeneration [4,5]. Thus, an Aβ self-aggregation inhibitor is currently a more attractive therapeutic drug for AD treatment.

Clerodendrum inerme Linn. Gaertn. is one of the medicinal plants in the Verbenaceae family. Previous studies reported that extracts of this plant have a variety of pharmacological effects, such as hepatoprotective, anti-inflammatory, anti-microbial, anti-bacterial, anti-oxidant and cytotoxic activities [6-10]. Furthermore, this plant has been used as a traditional Chinese medicine for treating neuropsychiatric disorders [11]. The phytochemical investigations on *C. inerme* have resulted in the isolation of a number of flavonoids, terpenoids and steroids [12-15], some of which have been reported to possess neuroprotective effects by reducing glutamate neurotoxicity [16]. However, the Aβ aggregation activity of the extracts has not been investigated. Therefore, the present study aims to identify the active components which can inhibit Aβ aggregation.

MATERIALS AND METHODS

General Experimental Procedures

Melting points were determined on a Fishers-Johns melting point apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were obtained on Varian model Mercury + 400 and Bruker AVANCE 400 spectrometers with tetramethylsilane as the internal standard. High-resolution electron ionisation mass spectrometry (EIMS) spectra were recorded on a Bruker micrOTOF model mass spectrometer. Thin-layer chromatography (TLC) was performed on pre-coated silica gel (Si-gel) 60 F_{254} plates (Merck) and spots were visualised by UV light (254 nm) and sprayed with 10% (v/v) H_2SO_4 followed by heating. Column chromatography (CC) was performed on Si-gel (70–230 mesh, Merck) and Sephadex LH-20 gel (GE Healthcare Bio-Sciences). All solvents for CC were distilled prior to use.

Plant Material

Roots and leaves of *C. inerme* were collected from Rayong province in Thailand on May 2012. A voucher specimen (herbarium number 013514 (BCU)) was deposited at the Department of Botany, Faculty of Science, Chulalongkorn University.

Extraction and Isolation

Air-dried leaves (3.8 kg) were macerated twice with dichloromethane (CH_2Cl_2). After filtration and solvent elimination, 57.4 g of CH_2Cl_2 extract was obtained. A portion of the CH_2Cl_2 extract (57.4 g) was fractionated by Si-gel CC using *n*-hexane: ethyl acetate (EtOAc) of increasing

polarity, followed by EtOAc: MeOH as eluents to afford seven fractions C1–7. Fraction C2 was further separated by Si-gel CC with *n*-hexane: EtOAc (10:90 → 0:100) as eluent to afford three fractions (C2.1–3). Fraction C2.1 was enriched by Si-gel CC with *n*-hexane: EtOAc (9:1) as eluent to obtain compound **1** (7.3 mg). Fraction C3 was refractionated by Si-gel CC with a gradient of *n*-hexane: EtOAc (10:90 → 0:100) as eluent to afford two fractions (C3.1 and C3.2). After repeated separation of fraction C3.1 by Si-gel CC with a gradient of *n*-hexane: CH_2Cl_2 (10:90 → 0:100) as eluent, compound **2** (46.5 mg) was obtained. Compounds **3** (25.4 mg) and **4** (20.5 mg) were obtained from fraction C3.2 after enrichment by Sephadex LH20 CC (n-hexane: CH_2Cl_2: MeOH = 7:2.5:0.5). Fraction C4 was separated by Si-gel CC with EtOAc: MeOH (100:0 → 10:90) as eluent to afford two fractions, and the first fraction was further separated by Si-gel CC with CH_2Cl_2: MeOH (9:1) as eluent to afford compound **5** (15.7 mg).

The CH_2Cl_2 extract from roots (4.5 kg) was obtained using the extraction method described above. A portion of the CH_2Cl_2 extract (60.5 g) of *C. inerme* roots was initially fractionated by Si-gel CC with *n*-hexane: EtOAc (90:10 → 0:100) followed by EtOAc: MeOH (100:0 → 90:10) as eluents to afford nine fractions (R1–9). Fraction R2 was separated by Si-gel CC with *n*-hexane: EtOAc (90:10 → 0:100) as eluent to obtain three fractions (R2.1–3). Fraction R2.3 was frationated by Si-gel CC with *n*-hexane: EtOAc (80:20 → 0:100) as eluent and further enriched by Si-gel CC with *n*-hexane: CH_2Cl_2 (9:1) as eluent to yield compounds **6** (8.8 mg) and **7** (9.4 mg). Fraction R4 was fractionated by Si-gel CC with *n*-hexane: EtOAc (80:20 → 0:100) as eluent to yield compounds **8** (35.6 mg) and **9** (13.8 mg).

Identification of the isolated compounds was performed by comparison of their ^{1}H- and ^{13}C-NMR spectroscopic data with those reported in the literature.

(3*β*,22*E*,24*S*)-Stigmasta-5,22,25-trien-3-ol (1)

$C_{29}H_{46}O$; white solid; mp. 147–149 ˚C; ^{1}H-NMR ($CDCl_3$,400 MHz) δ 0.69 (3H, *s*, H-18), 0.83 (3H, *t*, *J* = 7.4, H-29), 1.01 (3H, *s*, H-21), 1.02 (3H, *s*, H-19), 1.65 (3H, *s*, H-26), 3.52 (1H, *m*, H-3), 4.69 (2H, *m*, H-27), 5.20 (1H, *dd*, *J* = 15.2, 7.2, H-23), 5.24 (1H, *dd*, *J* = 15.4, 7.8, H-22), 5.35 (1H, *d*, *J* = 4.8, H-6); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 37.1 (C-1), 31.8 (C-2), 71.6 (C-3), 42.2 (C-4), 140.6 (C-5), 121.5 (C-6), 31.7 (C-7), 31.5 (C-8), 50.0 (C-9), 36.4 (C-10), 20.9 (C-11), 39.5 (C-12), 42.3 (C-13), 56.7 (C-14), 24.2 (C-15), 28.5 (C-16), 55.8 (C-17), 11.9 (C-18), 19.2 (C-19), 40.0 (C-20), 20.6 (C-21), 137.0 (C-22), 129.9 (C-23), 51.8 (C-24), 148.4 (C-25), 20.0 (C-26), 109.4 (C-27), 25.6 (C-28), 11.9 (C-29).

Pectolinarigenin (2)

$C_{17}H_{14}O_6$; yellow solid; mp. 210–211 ˚C; ^{1}H-NMR (acetone-d_6, 400 MHz) δ 3.72 (3H, *s*, -OCH_3), 3.76 (3H, *s*, -OCH_3), 6.48 (1H, *s*, H-8), 6.53 (1H, *s*, H-3), 6.97 (2H, *d*, *J* = 8.8, H-3′, 5′), 7.87 (2H, *d*, *J* = 8.8, H-2′, 6′), 9.31 (1H, *s*, -OH), 13.02 (1H, *s*, -OH); ^{13}C-NMR (acetone-*d6*, 100 MHz) δ 165.0 (C-2), 104.0 (C-3), 183.6 (C-4), 154.1 (C-5), 132.3 (C-6), 157.8 (C-7), 94.8 (C-8), 154.0 (C-9), 105.8 (C-10), 124.4 (C-1′), 129.1 (C-2′,6′), 115.4 (C-3′,5′), 163.8 (C-4′), 56.0 (-OCH_3), 60.7 (-OCH_3).

Acacetin (3)

$C_{16}H_{12}O_5$; yellow solid; mp. 284–289 ˚C; ^{1}H-NMR (acetone-d_6, 400 MHz) δ 3.83 (3H, *s*, -OCH_3), 6.17 (1H, *d*, *J* = 1.6, H-6), 6.46 (1H, *d*, *J* = 1.6, H-8), 6.59 (1H, *s*, H-3), 7.04 (2H, *d*, *J* = 8.8, H-3′, 5′), 7.93 (2H, *d*, *J* = 8.8, H-2′, 6′), 12.88 (1H, *s*, -OH); ^{13}C-NMR (acetone-*d6*, 100 MHz) δ

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165.1 (C-2), 104.5 (C-3), 183.1 (C-4), 163.7 (C-5), 99.8 (C-6), 164.8 (C-7), 94.7 (C-8), 158.8 (C-9), 104.6 (C-10), 124.3 (C-1′), 129.0 (C-2′,6′), 115.4 (C-3′,5′), 163.3 (C-4′), 56.0 (-OCH_3).

Galangustin (4)

$C_{17}H_{14}O_6$; yellow solid; mp. 209–215 ˚C; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 3.89 (3H, *s*, -OCH_3), 4.04 (3H, *s*, -OCH_3), 6.57 (1H, *s*, H-3), 6.59 (1H, *s*, H-6), 7.02 (2H, *d*, *J* = 8.8, H-3′,5′), 7.84 (2H, *d*, *J* = 8.8, H-2′,6′), 13.09 (1H, *s*, -OH); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 162.8 (C-2), 104.0 (C-3), 183.1 (C-4), 155.1 (C-5), 93.5 (C-6), 153.3 (C-7), 130.5 (C-8), 152.3 (C-9), 105.9 (C-10), 123.8 (C-1′), 114.7 (C-3′,5′), 128.2 (C-2′,6′), 164.3 (C-4′), 55.7 (-OCH_3), 61.0 (-OCH_3).

(3*β*, 22*E*, 24*S*)-Stigmasta-5,22,25-trien-3-yl-*O*-D-glucopyranoside (5)

$C_{35}H_{56}O_6$; white solid; mp. 259–261 ˚C; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.64 (3H, *s*, H-18), 0.78 (3H, *t*, *J* = 6.0, H-29), 0.93 (6H, *br s*, H-21, H-19), 1.59 (3H, *s*, H-26), 3.33 (1H, *m*, H-5′), 3.40 (3H, *m*, H-4′, 6′), 3.51 (1H, *m*, H-2′), 3.73 (1H, *m*, H-3′), 3.79 (1H, *m*, H-3), 4.35 (1H, *d*, *J* = 7.6, H-1′), 4.63 (2H, *s*, H-27), 5.12 (1H, *m*, H-23), 5.17 (1H, *m*, H-22), 5.31 (1H, *brd*, *J* = 4.4, H-6); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 38.8 (C-1), 31.9 (C-2), 76.4 (C-3), 37.3 (C-4), 143.4 (C-5), 122.2 (C-6), 31.9 (C-7), 35.6 (C-8), 49.3 (C-9), 36.8 (C-10), 20.8 (C-11), 40.2 (C-12), 42.3 (C-13), 56.9 (C-14), 28.7 (C-15), 29.7 (C-16), 55.9 (C-17), 12.1 (C-18), 21.1 (C-19), 39.7 (C-20), 20.2 (C-21), 140.3 (C-22), 137.2 (C-23), 52.0 (C-24), 148.7 (C-25), 19.3 (C-26), 109.5 (C-27), 25.7 (C-28), 11.8 (C-29), 100.8 (C-1′), 73.3 (C-2′), 79.0 (C-3′), 69.7 (C-4′), 75.4 (C-5′), 61.5 (C-6′).

***β*-Amyrin palmitate (6)**

$C_{46}H_{80}O_2$; white solid; mp. 79–82 ˚C; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.83 (3H, *s*, H-29), 0.87 (10 H, *s*, H-5, 24, 26 and 28), 0.88 (3H, *s*, H-23), 0.90 (3H, *m*, H-22′), 0.96 (3H, *s*, H-27), 0.97 (3H, *s*, H-25), 1.13 (3H, *s*, H-30), 1.25 (41H, *s*, H-7, 21-22, 4′-21′), 1.42 (2H, *m*, H-19), 1.57 (15H, *s*, H 1-2, 6, 9, 15-16, 18, 22 and 3′), 1.86 (2H, *m*, H-11), 2.29 (2H, *m*, H-2′), 4.50 (1H, *m*, H-3), 5.18 (1H, *t*, *J* = 3.4, H-12); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 38.5 (C-1), 22.8 (C-2), 80.8 (C-3), 37.9 (C-4), 55.5 (C-5), 18.5 (C-6), 32.7 (C-7), 41.9 (C-8), 47.8 (C-9), 37.3 (C-10), 23.7 (C-11), 121.9 (C-12), 145.4 (C-13), 47.0 (C-14), 25.3 (C-15), 25.2 (C-16), 33.5 (C-17), 47.5 (C-18), 40.0 (C-19), 32.8 (C-20), 35.0 (C-21), 37.1 (C-22), 27.1 (C-23), 17.0 (C-24), 15.7 (C-25), 16.9 (C-26), 23.9 (C-27), 26.3 (C-28), 34.9 (C-29), 23.8 (C-30), 173.8 (C-1′), 34.6 (C-2′), 26.1 (C-3′), 29.9 (C-4′), 31.2 (C-5′), 29.8 (C-6′), 29.8 (C-7′), 29.7 (C-8′), 29.7 (C-9′), 29.6 (C-10′), 29.5 (C-11′), 29.4 (C-12′), 29.3 (C-13′), 29.9 (C-14′), 29.3 (C-15′), 28.8 (C-16′), 28.6 (C-17′), 28.2 (C-18′), 28.2 (C-19′), 32.1 (C-20′), 23.7 (C-21′), 14.2 (C-22′); HREIMS *m/z*: 664.6144 $[M]^+$, calc. for, $C_{46}H_{80}O_2$, 664.6158.

Lupeol laurate (7)

$C_{42}H_{72}O_2$; white solid; mp. 214–217 ˚C; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.79 (3H, *s*, H-28), 0.84 (6H, *s*, H-23 and 25), 0.85 (3H, *s*, H-26), 0.88 (3H, *t*, *J* = 6.8, H-12′), 0.94 (3H, *s*, H-27), 1.03 (3H, *s*, H-24), 1.25 (18H, *brs*, H-3′-11′), 1.68 (3H, *brs*, H-30), 2.28 (2H, *t*, *J* = 7.4, H-2′), 2.37 (1H, *m*, H-19), 4.45 (1H, *dd*, *J* = 5.6, 10.4, H-3), 4.68 (1H, *brs*, H-29), 4.57 (1H, *brs*, H-29); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 40.2 (C-1), 23.9 (C-2), 80.8 (C-3), 38.6 (C-4), 55.6 (C-5), 18.2 (C-6), 34.4 (C-7), 43.2 (C-8), 50.6 (C-9), 38.3 (C-10), 22.8 (C-11), 28.1 (C-12), 38.0 (C-13), 43.0 (C-14), 29.3 (C-15), 35.0 (C-16), 41.1 (C-17), 48.5 (C-18), 48.2 (C-19), 151.1 (C-20), 29.5 (C-21), 37.3 (C-22), 29.4 (C-23), 16.2 (C-24), 16.7 (C-25), 16.3 (C-26), 14.7 (C-27), 18.4 (C-28), 109.5 (C-29), 21.1 (C-

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30), 173.8 (C-1′), 35.8 (C-2′), 27.6 (C-3′), 29.9 (C-4′), 29.8 (C-5′), 30.0 (C-6′), 29.8 (C-7′), 29.7 (C-8′), 29.6 (C-9′), 32.1 (C-10′), 25.3 (C-11′), 14.2 (C-12′); HREIMS *m/z*: 608.5532 $[M]^+$, calc. for, $C_{42}H_{72}O_2$, 608.5532.

Betulinic acid (8)

$C_{30}H_{48}O_3$; white solid; mp. 219–222 ˚C; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.75 (3H, *m*, H-1, 5), 0.83 (3H, *s*, H-24), 0.94 (3H, *s*, H-25), 0.97 (3H, *s*, H-26), 0.98 (3H, *s*, H-23), 1.25 (3H, *s*, H-27), 1.69 (3H, *s*, H-30), 3.00 (1H, *m*, H-3), 3.20 (1H, *m*, H-19), 4.61 (1H, *s*, H-29), 4.74 (1H, *s*, H-29); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 38.6 (C-1), 27.8 (C-2), 78.9 (C-3), 38.7 (C-4), 55.2 (C-5), 18.2 (C-6), 34.2 (C-7), 40.6 (C-8), 50.4 (C-9), 36.9 (C-10), 20.7 (C-11), 25.4 (C-12), 38.3 (C-13), 42.3 (C-14), 29.6 (C-15), 32.0 (C-16), 56.2 (C-17), 49.2 (C-18), 46.8 (C-19), 150.2 (C-20), 30.4 (C-21), 37.1 (C-22), 27.3 (C-23), 15.2 (C-24), 15.9 (C-25), 16.0 (C-26), 14.6 (C-27), 179.8 (C-28), 109.5 (C-29), 19.2 (C-30).

Betulin 3-acetate (9)

$C_{32}H_{52}O_3$; white solid; mp. 258–260 ˚C; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.82 (3H, *s*, H-23), 0.93 (3H, *s*, H-24), 0.96 (3H, *s*, H-25), 0.97 (3H, *s*, H-26), 1.37 (3H, *s*, H-27), 1.69 (3H, *s*, H-30), 3.19 (1H, *m*, H-18), 4.60 (1H, *m*, H-3), 4.69 (1H, *brs*, H-29), 4.74 (1H, *brs*, H-29); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 39.0 (C-1), 19.5 (C-2), 79.2 (C-3), 38.9 (C-4), 55.5 (C-5), 21.0 (C-6), 30.7 (C-7), 38.5 (C-8), 50.7 (C-9), 37.2 (C-10), 25.7 (C-11), 27.6 (C-12), 37.2 (C-13), 44.7 (C-14), 32.3 (C-15), 34.5 (C-16), 62.9 (C-17), 49.5 (C-18), 47.0 (C-19), 148.1 (C-20), 28.1 (C-21), 37.4 (C-22), 29.9 (C-23), 16.3 (C-24), 16.2 (C-25), 15.5 (C-26), 14.9 (C-27), 187.0 (C-28), 109.9 (C-29), 19.5 (C-30), 173.0 (C-2′), 18.5 (C-2′).

Measurement of Inhibition of Self-mediated A$\beta_{1\text{-}42}$ Aggregation

To investigate the inhibitory effect on the self-mediated A$\beta_{1–42}$ aggregation, a thioflavin-T fluorescence assay was performed as previously reported [17]. The A$\beta_{1–42}$ peptide (American Peptide Co.) was dissolved in phosphate buffer (pH 7.4) to obtain a 100 μM stock solution, which was mixed with the respective test compound (100 μM) and diluted with the buffer to give a final concentration of 20 μM each for both the peptide and test compound. In the case of the crude extracts, the final concentration of the respective extract was tested at 0.05, 0.5 and 2.0 mg/mL. Each mixture (80 μL) of the peptide and test compound was pipetted into a 96-well plate, incubated at 37°C for 48 hr and then 20 μL of thioflavin-T (5 μM in 50 mM glycine-NaOH buffer, pH 8.5) was added. Fluorescence intensities were measured at 485 nm (λem) with an excitation wavelength of 450 nm (λex) using a Cary eclipse fluorescence spectrophotometer (Agilent Technology, USA). Each test was run in triplicate. The percentage of A$\beta_{1–42}$ aggregation inhibition was calculated from: $(1\text{-}I_{Fi} / I_{Fc})\times100$, where I_{Fi} and I_{Fc} are fluorescence intensities, with the blank signal subtracted, obtained in the presence and absence of the test compound respectively. Curcumin (20 μM) was used as a reference standard.

RESULTS AND DISCUSSION

The CH_2Cl_2 extracts of *C. inerme* leaves and roots were separately examined for A$\beta_{1–42}$ peptide aggregation inhibition activities at the concentrations of 2, 0.5 and 0.05 mg/mL (Figure 1). Both extracts had a similar inhibition potency (close to 90% at 2 mg/mL) with a clear dose

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dependence. The extracts were further fractionated to afford the main active components. Five compounds from the CH_2Cl_2 leaf extract and four compounds from the CH_2Cl_2 root extract were isolated. Their chemical structures were elucidated based on NMR and mass spectrometric spectra. Compounds **1**–**5** isolated from the CH_2Cl_2 leaf extract were found to be (3*β*,22*E*,24*S*)-stigmasta-5,22,25-trien-3*β*-ol (**1**) [18], pectolinarigenin (**2**) [19], acacetin (**3**) [20], galangustin (**4**) [21] and (3*β*,22*E*,24*S*)-stigmasta-5,22,25-trien-3-yl-*O*-D-glucopyraoside (**5**) [22], while compounds **6**–**9** from the CH_2Cl_2 root extract were β-amyrin palmitate (**6**) [23], lupeol laurate (**7**) [24], betulinic acid (**8**) [25] and betulin 3-acetate (**9**) [26] (Figure 2).

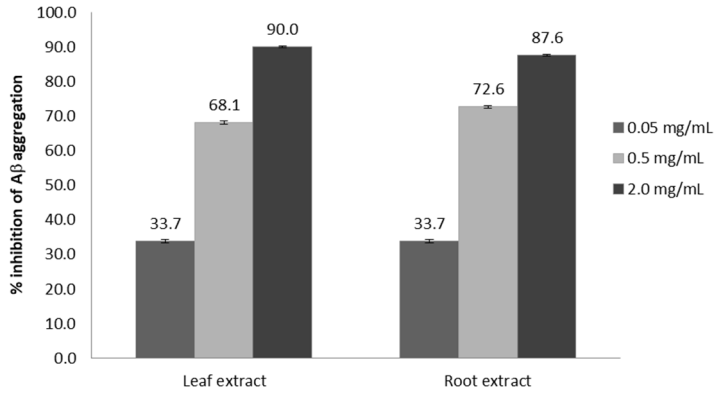

Figure 1. A$\beta_{1–42}$ aggregation inhibition activities of the CH_2Cl_2 extract of *C. inerme*. Data are shown as mean ± S.D. and are derived from three repeats.

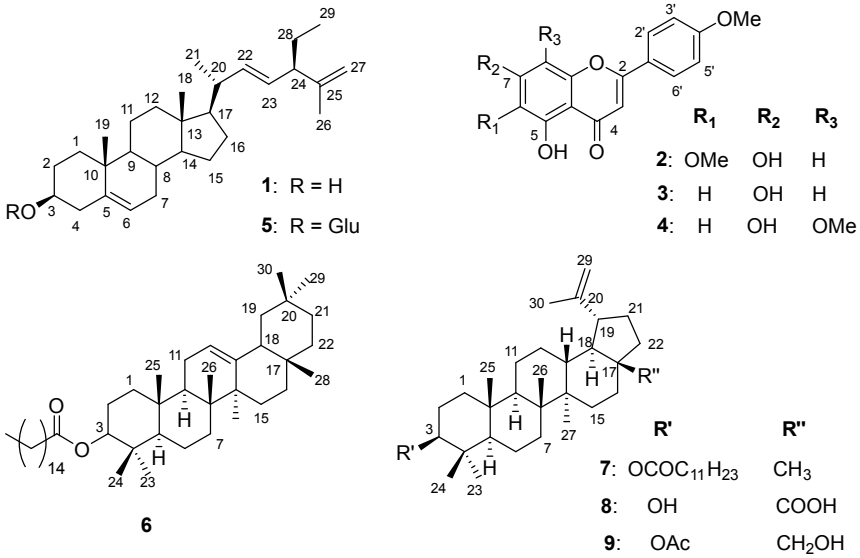

Figure 2. Chemical structures of compounds **1**–**9** from *C. inerme*

All isolated compounds were tested for their Aβ_{1-42} aggregation inhibition activities using the thioflavin T assay. These compounds showed percentages of inhibition ranging between 12–29% at 20 μM, while curcumin standard gave 31% inhibition (Figure 3). Among them, flavonoids **2**–**4** show higher A$\beta_{1–42}$ aggregation inhibition than steroids (**1** and **5**) and triterpenoids (**6**–**9**). The highest inhibitory activity of 29% at 20 μM was observed with acacetin (**3**). This activity of acacetin has not been reported, although it was previously noted as a potent neuroprotective agent. Acacetin was found to protect dopaminergic cells and inhibit production of inflammatory factors in

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in vitro and *in vivo* models of Parkinson's disease [27]. Moreover, acacetin derived from *C. inerme*, a traditional Chinese medicine, inhibits glutamate release from hippocampal synaptosomes by attenuating the voltage-dependent Ca^{2+} entry and effectively prevents kainic acid-induced *in vivo* excitotoxicity [16]. Acacetin has longevity promoting and neuroprotective effects in the *Caenorhabditis elegans* model system [28].

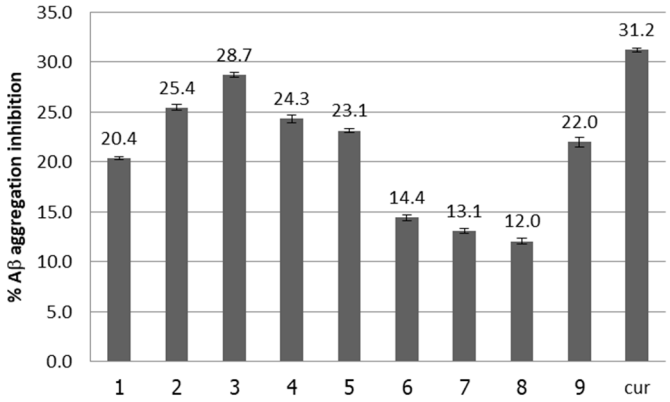

Figure 3. Inhibition of Aβ_{1-42} aggregation by compounds **1**–**9** compared to curcumin (cur) as reference standard. Data are shown as mean ± S.D. and are derived from three replicates.

Interestingly, betulin 3-acetate (**9**) showed a markedly higher inhibition than betulinic acid (**8**), which was previously reported to possess the unique property of promoting Aβ fibril formation and accelerate the clearance of neurotoxic soluble oligomers [29]. The Aβ fibril is not as toxic as the other soluble forms of Aβ aggregates, and so the progression of AD is consequently slowed down by treatment with this therapeutic agent. 3-*O*-Acetyl betulinic acid was previously reported to be cytotoxic against human lung carcinoma, ovary adenocarcinoma [26] and rat skeleton myoblast cells [30], but its neuroprotective property has hitherto not been reported. Triterpenoids **7**–**9** are lupane derivatives that differ in the substituents at positions 3 and 17, which suggests another interesting core structure for new potent Aβ inhibitors as AD drugs.

CONCLUSIONS

Nine compounds have been isolated and identified from CH_2Cl_2 extracts of *C. inerme* leaves and roots. Among them, the flavonoid acacetin (**3**) exhibits high vitro anti-Aβ_{1-42} aggregation activity, which is important for prevention of Alzheimer's disease (AD). This discovery provides another evidence for the neuroprotective effect of acacetin and *C. inerme* plant. Moreover, this work also shows that the substituents on the lupane skeleton have significant effects on the anti-Aβ_{1-42} aggregation activity and two lupanes may serve as promising lead structures for AD drug development.

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