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ORIGINAL ARTICLE

Two *Lycopodium* Alkaloids from the Aerial Parts of *Huperzia phlegmaria*

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ABSTRACT

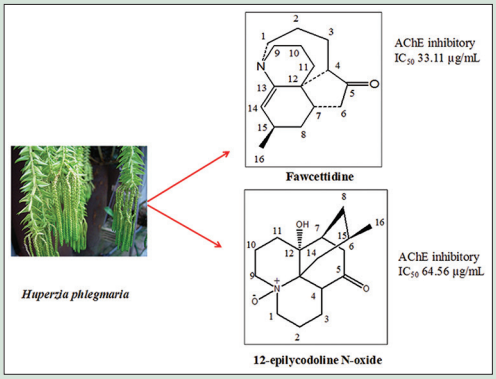
Background: *Huperzia phlegmaria* has been used to enhancing memory and alleviate brain disorders. It contains high amount of alkaloids, which are potent acetylcholinesterase (AChE) inhibitor. **Materials and Methods:** *Lycopodium* alkaloids from aerial parts of *H. phlegmaria* were isolated by chromatographic methods. Their structures were elucidated by spectroscopic methods, including mass spectrometry and nuclear magnetic resonance. AChE inhibitory effect of isolated compounds *in vitro* was evaluated using Ellman's assay. **Results:** These compounds were identified as fawcettidine and 12-epilycodoline N-oxide. Two compounds showed moderately AChE inhibitory effects with IC_{50} values of 33.11 μg/mL and 64.56 μg/mL, respectively. **Conclusion:** These isolated compounds could be promising drugs for the treatment of Alzheimer's disease.

Key words: 12-epilycodoline N-oxide, acetylcholinesterase, fawcettidine, *Huperzia phlegmaria*, lycopodium alkaloids

SUMMARY

- Two *Lycopodium* alkaloids were isolated from aerial parts of *Huperzia phlegmaria* by chromatographic methods. These compounds were identified as fawcettidine and 12-epilycodoline N-oxide. Two compounds showed moderately acetylcholinesterase inhibitory effects with IC_{50} values of 33.11 and 64.56 μg/mL, respectively.

Abbreviations Used: AChE: Acetylcholinesterase, Ach: Acetylcholine, AD: Alzheimer's disease.



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INTRODUCTION

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disease, which impairs memory and cognitive function.[1] According to the World Health Organization report in 2018, 50 million people worldwide have dementia and out of this population, 60%–70% is AD patients. The number of people with dementia is predicted to nearly triple to 152 million by 2050, whereby with the rise of dementia patients, the AD patients' cases is also expected to increase.[2] Cholinergic hypothesis explains that AD is caused by decreased the neurotransmitter acetylcholine (Ach). Many drugs used in the treatment of AD are based on cholinergic hypothesis.[3] Many studies showed lower level of neurotransmitters in cholinergic system is responsible for cognitive decline and memory loss in Alzheimer patients.[3-6] Acetylcholinesterase (AChE) is the main enzyme, which degrades the neurotransmitter Ach. Drugs such as galantamine, tacrine, donepezil, metrifonate, or rivastigmine are inhibitors of AChE, augment the level of ACh, and thereby improving cholinergic transmission. These drugs have been used to alleviate the symptoms of Alzheimer which are caused by degeneration of cholinergic neurons and injured transmission. However, AChE inhibitors have many side effects such as nausea, vomiting, diarrhea, abdominal pain, dyspepsia, and skin rash.[5] Therefore, it is important to find new AChE inhibitors with less adverse effects, which may be found in medicinal plant resources.

Medicinal plants may decrease the progress and symptoms of AD.[7] From the genus *Huperzia*, several alkaloids have been isolated. Thorroad *et al.* have isolated eleven *Lycopodium* alkaloids from the whole plants of *Huperzia carinata* and *Huperzia squarrosa* and these *Lycopodium* alkaloids compounds could moderate AChE inhibitory activity.[8] Nilsu *et al.* have isolated two *Lycopodium* alkaloids, squarrosine A, and pyrrolhuperzine A from the *H. squarrosa*.[9] In Vietnam, Chuong *et al.* have isolated six *Lycopodium* alkaloids, namely lycosquarosine a, acetylaposerratinine, huperzine A, huperzine B, 8α-hydrophlemariurine B and huperzinine, from Vietnamese *H. squarrosa*. These compounds lycosquarosine A and acetylaposerratinine have showed strong AChE inhibitory activity.[10] Among isolated compounds from the genus *Huperzia*, Huperzine A is the most important sesquiterpene alkaloid compounds found in *Huperzia serrata*. Huperzine A has been shown to strongly inhibit AChE and its mechanism was similar to rivastigmine, donepezil, and galantamine which have been used as drugs for the treatment of AD. Hirasawa *et al.* have isolated huperminone A, a novel C16N-type *Lycopodium* alkaloid consisting of a decahydroquinoline

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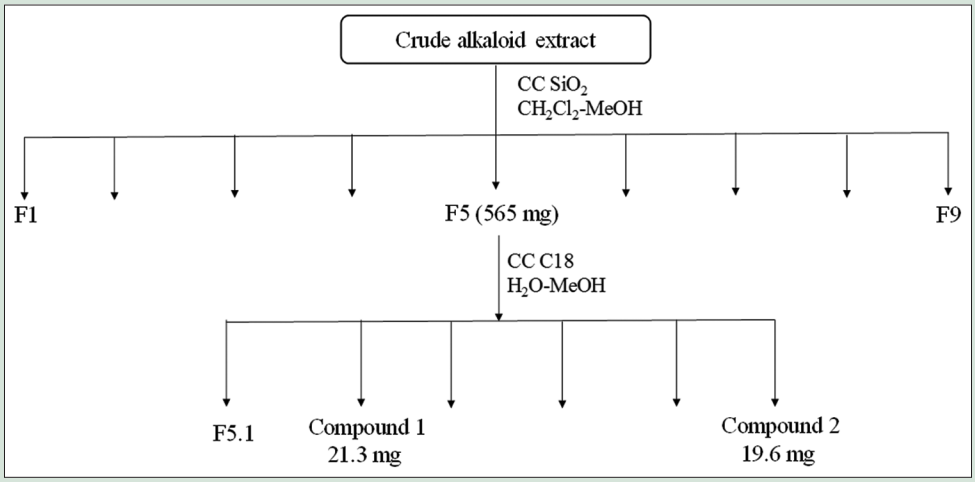

Figure 1: Scheme for extraction of alcaloid from *H. phlegmaria*

and a cyclohexanone, from the club moss of *Huperzia phlegmaria*[11] and hupermine A, novel $C_{16}N_2$-type *Lycopodium* alkaloid.[12] In this study, we report the two compounds isolated from *H. phlegmaria* and their AChE inhibitory activity.

MATERIALS AND METHODS

Plant material

The aerial parts of *H. phlegmaria* collected in Tam Dao, Vietnam, during 9/2018 and authenticated by the School of Medicine and Pharmacy (SMP), Vietnam National University, Hanoi, Vietnam. A voucher specimen has been deposited in the SMP.

General experimental procedures

The nuclear magnetic resonance (NMR) (^{1}H [500 MHz], ^{13}C [125 MHz] and Distortionless Enhancement by Polarization Transfer [DEPT]-90 and 135 MHz) spectra were recorded on an AVANCE spectrometer AV 500 (Brucker, Germany) in the Institute of Chemistry, Vietnam Academy of Science and Technology. Chemical shifts were reported in ppm downfield from Tetramethylsilane (TMS) with J in Hz. Electrospray Ionization Mass Spectra (ESI-MS) were recorded on a Varian Agilent 1100 liquid chromatography mass spectrometry (MS) D mass spectrometer. Column chromatography (CC) was performed on silica gel (70–230 and 230–400 mesh, Merck). Organic solvents were of analytical grade.

Extraction and isolation

The aerial dried plants of *H. phlegmaria* (1 kg) were pulverized and defatted with n-hexane using a Soxhlet extractor for 1 day. The plants were subsequently extracted with EtOH three times by reflux. The solvent was removed at reduced pressure to give a residue (52.6 g). This crude extract was suspended in 5% HCl solution and washed with CH_2Cl_2 the aqueous were obtained, basified with NH_4OH (pH = 11) and partitioned with CH_2Cl_2, to obtain the crude alkaloid extract after filtration under Na_2SO_4. The alkaloid extract was then subjected to CC over silica gel with CH_2Cl_2–MeOH (5:1–0:1) to yield nice fractions (F1-F9). Fraction F5 (565 mg) was chromatographed on C-18 silica gel with a MeOH–H_2O (0:1-1:0) to give compound 1 (21.3 mg) and compound 2 (19.6 mg) [Figure 1].

Acetylcholinesterase inhibitory activity assay

AChE inhibitory activity of isolated compounds was assayed by the spectrophotometric method developed by Ellman *et al.* with slightly modification.[13] Samples were dissolved in dimethyl sulfoxide. Reaction mixture consisted of 140 µL of 0.1 M sodium phosphate buffer (pH 8.0), 20 µL of samples, and 20 µL of AChE 0.25 IU/mL. Incubated the mixture for 15 min at 25°C. Added 10 µL of 5-5'-dithiobis-2-nitrobenzoic acid 2.5 mM và 10 µL acetylthiocholine iodide 2.0 mM and mixed well. Then incubate the mixture for 10 min at 25°C. The absorbance was measured at 412 nm. Each assay was repeated three times. Donepezil was used as positive control.

Percentage of AChE inhibition (% I) was calculated by followed formula:

$$\%\,I = \frac{Ac - At}{Ac - Ao} \times 100$$

Where I% is the percentage of AChE inhibition

A_c: Absorbance of control (without 20 µL sample)

A_t: Absorbance of sample

A_o: Absorbance of blank (200 µl of 0.1 M sodium phosphate buffer)–

Value IC_{50} was calculated using the graph of log (dose) versus % I.

RESULTS AND DISCUSSION

Chemical structure elucidation

Compound 1: Fawcettidine

ESI-MS (positive) m/z: 246.36 [M + H] + (calcd. 246.18 for $C_{16}H_{23}NO$).

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.71 (1H, d, J = 5.0 Hz, H-14); 2.72 (1H, dd, J = 7.5; 17.0 Hz); 1.04 (3H, d, J = 7.0 Hz, H-16); 3.14–2.98 (m, 4H), 2.34–2.24 (m, 2H), 2.20–2.05 (m, 3H), 1.79–1.59 (m, 3H), 1.41–1.34 (m, 2H), 1.28–1.21 (m, 2H), 1.99–1.93 (m, 1H), 1.91–1.83 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 218.8 (C-5); 145.7 (C-13); 127.3 (C-14); 60.3 (C-1); 56.2 (C-4); 51.9 (C-9); 46.1 (C-12); 44.0 (C-6); 39.1 (C-11); 37.3 (C-7); 34.1 (C-8); 31.2 (C-3); 29.1 (C-2); 27.7 (C-15); 23.7 (C-10); 20.8 (C-16).

Compound 1 was obtained as pale yellow oil. Its molecular formula was deduced to be $C_{16}H_{23}NO$ by high-resolution electrospray ionization-MS (HRESI-MS) data in conjunction with NMR data analysis, which contains eight degrees of unsaturation. The ^{1}H NMR spectrum of compound 1 measured in $CDCl_3$ showed typical one methyl group (δH 1.04 [3H, d, J = 7.0 Hz]). The characteristic of two signals of methine proton at (δ_H 5.71 [1H, d, J = 5.0 Hz, H-14]) and (δ_H 2.72 [1H, dd, J = 7.5; 17.0 Hz]) were observed. Moreover, the characteristic of multiplet signals at 3.14–2.98 (m, 4H), 2.34–2.24 (m, 2H), 2.20–2.05 (m, 3H), 1.79–1.59 (m, 3H), 1.41–1.34 (m, 2H), 1.28–1.21 (m, 2H), 1.99–1.93 (m, 1H), and 1.91–1.83 (m, 1H) were observed.

Analysis of the ^{13}C NMR (composite pulse decouplingand DEPT) spectra of compound 1 revealed sixty signals for one methyl, eight methylenes, four methines, and three non-hydrogenated carbons. Furthermore, the ^{13}C NMR spectrum contained signals corresponding to one ketone carbon δ_C 218,8 (C-5); one non-hydrogenated carbons δ_C 145.7 (C-13); and one methine nitrogenated carbon δ_C 127.3 (C-14); two nitrogenated carbon (δC 60.3 [C-1] and δ_C 56.2 [C-4]). By comparison with previously reported literature,[14] the structure of compound 1 was deduced as fawcettidine [Figure 2].

Compound 2: 12-epilycodoline N-oxide

ESI-MS m/z: 280.37 (M + H) + (calcd. 280.18 for $C_{16}H_{25}NO_3$).

^1H NMR (400 MHz, $CDCl_3$): δ_H 2.95–3.62 (2H, m, H-1); 1.83–1.90 (2H, m, H-2); 1.68–2.21 (2H, m, H-3); 2.84 (1H, dd, H-4); 2.43–2.62 (2H, dd, H-6); 2.09 (1H, overlap, H-7); 1.33–2.09 (2H, m, H-8); 3.07–4.05 (2H, m, H-9); 1.78–3.05 (2H, m, H-10); 1.65–2.25 (2H, m, H-11); 1.89–2.78 (2H, m, H-14); 1.49 (1H, m, H-15); 0.96 (3H, d, H-16).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 63.2 (C-1); 21.5 (C-2); 17.8 (C-3); 50.0(C-4); 207.0 (C-5); 44.1(C-6); 41.2(C-7); 35.2 (C-8); 59.7 (C-9); 16.4 (C-10); 29.6 (C-11); 71.0 (C-12); 72.8 (C-13); 29.6 (C-14); 24.8 (C-15); 22.5 (C-16).

Compound 2 was obtained as white oil. Its molecular formula was deduced to be $C_{16}H_{25}NO_3$ by HRESI-MS data in conjunction with NMR data analysis, which contains five degrees of unsaturation. The ^1H NMR spectrum of compound 2 measured in $CDCl_3$ showed typical one methyl group (δH 0.96 [3H, d, J = 6.0 Hz], H-16]. The characteristic of three signals of methine proton at 2.84 (1H, dd, H-4); 2.09 (1H, overlap, H-7); and 1.49 (1H, m, H-15) were observed. Moreover, the characteristic of multiplet signals at δ_H 2.95–3.62 (2H, m, H-1); 1.83–1.90 (2H, m, H-2); 1.68–2.21 (2H, m, H-3); 2; 2;43-2;62 (2H, dd, H-6); 1.33–2.09 (2H, m, H-8); 3.07–4.05 (2H, m, H-9); 1.78–3.05 (2H, m, H-10); 1.65–2.25 (2H, m, H-11); 1.89–2.78 (2H, m, H-14) were observed. The ^{13}C-NMR (DEPT) spectrum of compound 2 displayed 16 signals: One Me, nine CH2, and three CH groups and three quaternary C-atoms. Furthermore, the ^{13}C NMR spectrum contained signals corresponding to one ketone carbon δ_C 207.0 (C-5); one non-hydrogenated carbons δ_C 71.0 (C-12); and three nitrogenated carbon (δC 63.2 [C-1]; δC 59.7 [C-9]; and δ_C 72.8 [C-13]). By comparison with previously reported literature,[15,16] the structure of compound 2 was deduced as 12-epilycodoline N-oxide [Figure 3].

Acetylcholinesterase inhibitory activity

AD is a neurodegenerative disease characterized by progressive loss of neurons. The pathogenesis of AD is still not completely understood. Cholinergic deficits have been considered play an important role in the process of AD.[17,18] The cholinergic hypothesis of AD proposed that ACh deficits level induced AD is widely accepted.[19] AChE enzyme hydrolyze ACh to choline and decrease its level in the brain. Then, many intentions for increasing ACh levels in the brain have been studied for the treatment of AD by inhibiting AChE.[20] We have evaluated the AChE inhibitory effect of two isolated compounds using Ellman's assay.

Table 1 summarizes IC_{50} values of isolated compound 1, 2 and donepezil. Figure 4 presents the relationship between log (concentration) versus % inhibition of AChE activity. The AChE inhibitory activity of both compounds was dose-dependent manner. Compound 1 and compound 2 have activities with IC_{50} was 33.11 and 64.56 μg/mL, respectively, as compared with donepezil 2.57 μg/mL.

Several members from *Huperzia* species have been studied about the phytochemicals and its AChE inhibitory. Chuong *et al.* have isolated two compounds lycosquarosine A and acetylaposerratinine from Vietnamese *H. squarrosa* and showed that lycosquarosine A and acetylaposerratinine inhibit AChE with IC_{50} values of 54.3 and 15.2 μg/mL, respectively.[10] Hirasawa *et al.* showed the Hupercumines A and B, two *Lycopodium* alkaloids from *Huperzia cunninghamioides* inhibited AChE with IC_{50}, 41.9 and 92.3 μM, respectively.[21] Ohba *et al.* have showed that huperzine A, the main compound from *H. serrata*, has inhibited AChE with IC_{50} 87.17 nM.[22] Nguyen *et al.* have isolated two novel *Lycopodium* alkaloids, huperphlegmines A and B from the aerial parts of *H. phlegmaria* and showed they inhibited moderately AChE activity with IC_{50} values of 25.95 ± 0.67 and 29.14 ± 0.77 μg/mL, respectively.[23] In this study, we first time reported that fawcettidine and 12-epilycodoline N-oxide compounds were isolated from *H. phlegmaria,* and they showed moderately AChE inhibitory effects with IC_{50} values of 33.11 μg/mL and 64.56 μg/mL, respectively.

Table 1: Acetylcholinesterase inhibitory activity of of isolated compound 1 and compound 2 and donezepil

Sample	LogIC_{50} (μg/mL)	IC_{50} (μg/mL)
Compound 1	1.52	33.11
Compound 2	1.81	64.56
Donepezil	0.41	2.57

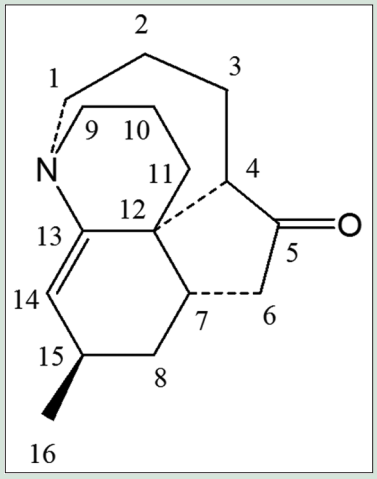

Figure 2: Compound 1

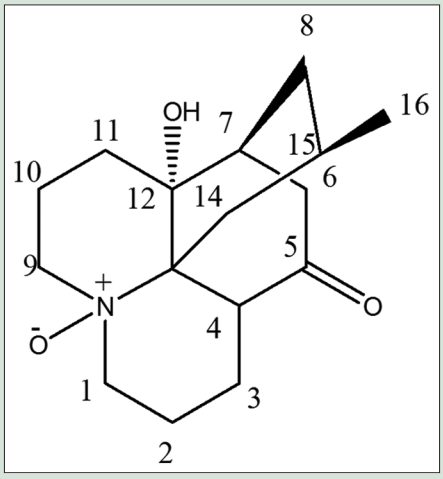

Figure 3: Compound 2

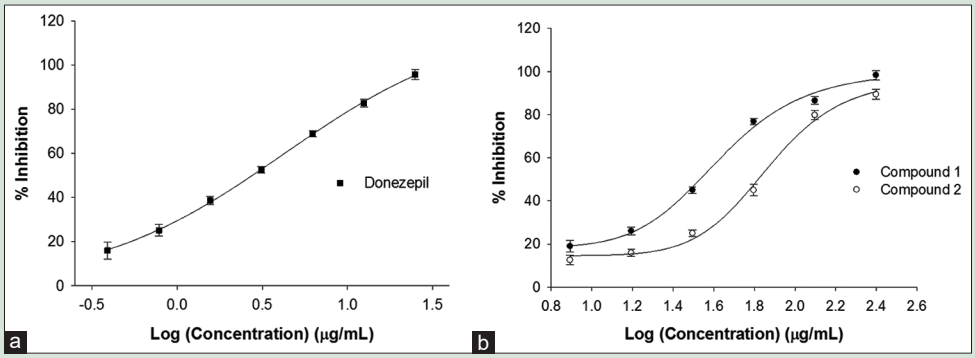

Figure 4: Acetylcholinesterase inhibitory activity on Ellman's assay. (a) Donezepil; (b) Compound 1 and compound 2

CONCLUSION

From the aerial parts of *H. phlegmaria* collected in Vietnam, two *Lycopodium* alkaloids were isolated by chromatographic methods. On the basis of spectroscopic analyses and by spectral comparison with the published literature, the isolated compounds were identified as fawcettidine and 12-epilycodoline N-oxide. Two compounds showed moderately AChE inhibitory effects, with IC_{50} values of 33.11 and 64.56 μg/mL, respectively.

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Conflicts of interest

There are no conflicts of interest.

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