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Acetylcholinesterase inhibitory activity and molecular docking study of steroidal alkaloids from *Holarrhena pubescens* barks

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ABSTRACT

An alkaloidal extract of the bark of *Holarrhena pubescens* showed several inhibition zones of acetylcholinesterase (AChE) inhibitor, using a bioautographic assay. Activity-guided fractionation afforded three new steroidal alkaloids, mokluangins A–C (**1**–**3**), together with three known compounds, antidysentericine (**4**), holaphyllamine (**5**), methylholaphyllamine (**6**). All structures were elucidated by analysis of NMR and MS spectroscopic data. Compound **2** showed moderate antibacterial activity against *Bacillus subtilis* and *Escherichia coli* with the MIC value of 16 μg/mL, while compound **3** exhibited moderate selective activity against *E. coli* with the MIC value of 16 μg/mL. In addition, compounds **1**–**4** also showed strong AChE inhibiting activity with IC_{50} values ranging from 1.44 to 23.22 μM. Molecular docking calculations were also performed and the results demonstrated that all compounds can bind at the aromatic gorge of AChE with estimated binding free energies correlated well with the *in vitro* inhibitory profiles. Hydrophobic and hydrogen bonding interactions contribute mainly to the binding of the alkaloids where the substituents at C-3 serving as key functional groups for the AChE inhibition. Our results will allow the development of new AChE-inhibitors based on steroidal alkaloid skeleton bearing the cyclic amide moiety.

1. Introduction

Alzheimer's disease (AD) is a common neurodegenerative disease that affects the elderly population and the primary symptom is a loss of memory [1]. The most remarkable biochemical change in AD patients is a reduction of acetylcholine (ACh) levels in the hippocampus and cortex of the brain [2]. Therefore, the use of acetylcholinesterase (AChE) inhibitors, the enzyme responsible for hydrolysis of ACh at the cholinergic synapse, is an effective clinical strategy for the treatment of AD [3]. However, only a few inhibitors, huperzine-A and galantamine, from plants have been a source of anti-AD drug. Until now, no drug of choice for the treatment of this disease has been decided. Thus, it is a promising approach to find new drug candidates from natural products. In the recent past, medicinal plants attracted attention due to their potential role for treating AD [4,5] and extensive reviews on their biological activities from various classes and species can be found elsewhere [6–10]. Steroidal alkaloids are an interesting class that has been extensively studied with potent AChE inhibitory activity [4,11–12].

The genus *Holarrhena* of the Apocynaceae family is comprised of 5 species and is known to accumulate structurally interesting and bioactive compounds [13,14]. *Holarrhena pubescens*, commonly known in Thai as "Mok Luang", is an important plant used for its anti-amoebic activity against *Entamoeba histolytica* [15]. The barks of this plant have astringent, anthelmintic, stomachic, febrifugal and tonic properties [16]. Phytochemical study on the isolation of the secondary metabolites of this species have identified steroidal alkaloids, *viz* conessine and conessimin [4,14,17] that exhibited antimalarial, antimicrobial and acetylcholinesterase inhibitory activities.

As part of our research program on the isolation of bioactive compounds from Thai medicinal plants, the alkaloidal extract of *H. pubescens* barks was examined for antimicrobial and acetylcholinesterase inhibitory activities. The results showed that this extract clearly exhibited several inhibition zones using a bioautographic assay [18] and inhibited moderate antibacterial activity against *Escherichia coli* TISTR 780 (32 μg/mL), *Bacillus cereus* TISTR 688 (64 μg/mL), and *Bacillus subtilis* TISTR 008 (32 μg/mL). The

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activities were further traced to the basic fraction derived from this extract. Subsequent fractionation of the bark extract led to the isolation of three new steroidal alkaloids, mokluangins A–C (**1**–**3**), together with three known compounds, antidysentericine (**4**) [19], holaphyllamine (**5**) [20], methylholaphyllamine (**6**) [21]. We report herein on the structure elucidation of these new steroidal alkaloids and their biological activities. Docking of the alkaloids into the active site gorge of AChE was also performed, demonstrating their unique binding mode and interactions relevant to the observed experimental inhibitory activities.

2. Results and discussion

A methanolic extract of the *H. pubescens* was evaporated, and partitioned between EtOAc and 1% HCl. The aqueous layer was adjusted to pH 8–9 with saturated NH_3, and the water soluble materials were extracted with EtOAc. The EtOAc soluble material was purified by chromatographic techniques and led to isolation of three steroidal alkaloids (**1**–**3**), together with three known compounds (**4**–**6**) (Fig. 1). All isolates were characterized by spectroscopic methods and compared with those previously published data.

Mokluangin A (**1**) had the molecular formula, $C_{22}H_{34}N_2O$, as determined by ^{13}C NMR spectroscopic data and a pseudomolecular ion peak at *m*/*z* 343.2760 $[M+H]^+$ (calcd. 343.2749) in the HR-ESI-MS, indicating seven degrees of unsaturation in the molecule. Four of these were accounted for by a pentacyclic structure related to conenine [22] and two were due to an endocyclic double bond and a five membered cyclic amide. This finding was confirmed by the absorption bands at 1710 and 1610 cm^{-1} in the IR spectrum [19]. Additionally, a secondary amino stretching absorption at 3415 cm^{-1} was also observed. The ^{13}C NMR and DEPT spectra of **1** showed 22 signals including three methyls (δ_C 29.9, 19.4 and 16.7), eight methylenes (δ_C 37.1, 34.9, 32.9, 32.2, 29.1, 24.9, 24.0 and 22.9), seven methines (δ_C 123.0, 67.9, 59.1, 55.3, 49.9, 48.1 and 33.4), three quaternary (δ_C 138.1, 66.2 and 36.9) and an amide carbonyl group (δ_C 172.2). The ^1H NMR data (Table 1) revealed the presence of a tri-substituted olefinic proton at δ_H 5.44 (1H, br s, H-6), one tertiary methyl at δ_H 1.08 (3H, s, Me-19) and one secondary methyl at δ_H 1.38 (3H, d, J = 6.5 Hz, Me-21). These signals were those expected for the conenine derivative and closely resembled antidysentericine (**4**) [12,14]. The connectivity of Me-21/H-20 and H-20/H-17 was assigned by the ^1H–^1H COSY spectrum. The placement of the amide carbonyl group at C-18 was fully supported by HMBC correlations of Me-21 with C-17 and C-20, of H-20 with C-16, C17, C-18 and C-21, and of H-17 with C-16, C-18 and C-21. Two protons were observed at δ_H 4.18 (1H, br t, J = 6.5 Hz, H-20) and 2.81 (1H, br t, J = 11.5 Hz, H-3), attributable to two α-amino protons, which were attached to two methine amino carbons resonating at δ_H 67.9 and 59.1, respectively, from HMQC experiment. In addition, the lower field methyl

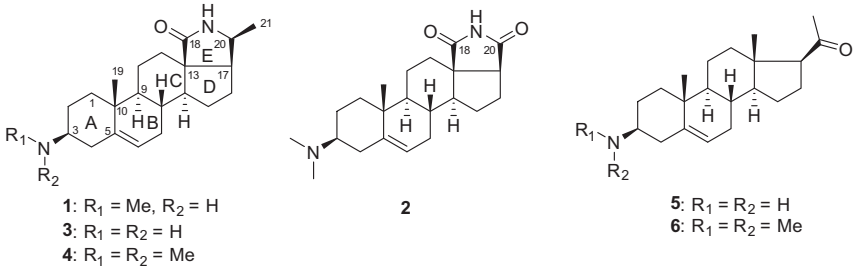

Fig. 1. Structures of isolated steroidal alkaloids (**1**–**6**) from *H. pubescens* barks.

Table 1
^1H and ^{13}C NMR (400 MHz, $CDCl_3$) spectroscopic data of mokluangins A–C (**1**–**3**).

No.	Mokluangin A		Mokluangin B		Mokluangin C	
	δ_C	δ_H (mult, J in Hz)	δ_C	δ_H (mult, J in Hz)	δ_C	δ_H (mult, J in Hz)
1	37.1	1.99 m, 1.12 m	37.8	2.00 m, 1.83 m	37.4	2.01 m, 1.35 m
2	24.9	2.13 m, 1.90 m	24.3	1.60 m	28.4	2.06 m
3	59.1	2.81 br t (11.5)	65.1	2.48 m	51.5	2.94 m
4	34.9	2.53 br d (11.8)	33.5	2.32 br d (7.0)	38.3	2.42 br d (7.1)
5	138.1		139.8		139.3	
6	123.0	5.44 br s	121.7	5.38 br d (5.4)	122.2	5.40 br d (4.6)
7	32.2	2.13 m	31.8	2.10 m, 1.60 m	32.3	2.13 m
8	33.4	1.96 m	33.6	1.33 m	33.5	1.64 m
9	49.9	1.20 m	49.6	0.98 m	50.1	1.21 m
10	36.9		36.7		36.7	
11	29.1	1.70 m, 1.24 m	22.0	1.94 m	29.1	1.68 m, 1.22 m
12	32.9	1.52 m	33.9	1.58 m	33.1	1.50 m
13	66.2		64.4		66.8	
14	55.3	1.35 m	55.4	1.17 m	55.2	1.39 m
15	22.9	1.42 m	23.9	1.62 m	22.9	1.55 m
16	24.0	1.91 m, 1.69 m	27.0	1.71 m	24.1	1.99 m, 1.50 m
17	48.1	2.13 m	53.3	2.02 m	48.4	2.13 m
18	172.2		177.6		170.0	
19	19.4	1.08 s	19.3	0.92 s	19.4	1.05 s
20	67.9	4.18 br t (6.5)	170.0		68.9	4.07 m
21	16.7	1.38 d (6.5)			17.0	1.35 d (7.2)
N*Me*	29.9	2.66 s	40.7	2.44 s		
*NH*CO		7.88 s				7.56 br d (2.9)
*NH*Me		9.50 br s				