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Acetylcholinesterase Inhibitor from *Stephania suberosa* Forman

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ABSTRACT

Two acetylcholinesteraseacetyl (AChE) inhibitors, dicentrine and isolaureline, were isolated from the tuber of *Stephania suberosa* Forman (Menispermaceae). Their chemical structures were proved using spectroscopic techniques such as NMR, MS, and IR. Dicentrine showed the inhibitory activity against AChE in the same level as the well-known AChE inhibitor, galanthamine, while its demethoxy derivative, isolauretine, showed about ten fold lower activity.

Keywords: *Stephania suberosa,* Acetylcholinesterase inhibitor

INTRODUCTION

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases. It presents an enormous impact on individuals, families, the health care system, and society as a whole. The causes of Alzheimer's disease are still unclear. The cholinergic hypothesis suggests that the drugs potent central cholinergic function should improve cognition and perhaps even some of the behavioral problems in AD patient. Among cholinomimetic drugs, acetylcholinesterase (AChE) inhibitors have been the most successful drugs for enchancing cholinergic transmission. Currently, there are four AChE inhibitors in the market as drugs for treatment of AD; i.e. tacrine (Cognex®), donepezil (Aricept®), rivastigmine (Exelon®), and galanthamine (Reminyl®) (Moghul and Wilkinson, 2001; Scarpini *et al.*, 2003). However, there are limitations for these drugs in term of efficacy and side effects. The search of new leads for AChE inhibitors is still needed.

The success of an ethnobotany approach to drug discovery has been proved in many cases. Recently, we screened some Thai medicinal plants used as rejuvenating agents for AChE inhibitory activity using the *in vitro* Ellman method (Ellman *et al.*, 1961). The results showed that the extract from the tuber of a Thai medicinal plant, *Stephania suberosa* Forman exhibited high inhibitory activity on AChE (Ingkaninan *et al*., 2003). In the current study, the further isolation and structure elucidation of AChE inhibitors from *S. suberosa* tubers were conducted by using bioassay-guided fractionation. The AChE inhibitory activities of the compounds isolated were compared to that of the well-known AChE inhibitor, galanthamine.

MATERIALS AND METHODS

General experimental procedures- Liquid Chromatograph-Mass (LCT) sprectra were obtained using an LCT Spectrometer model KB 217 (Micromass, UK) using ESI positive mode. IR spectra were recorded on an FT-IR spectrometer GX series (Perkin-Elmer, USA). The ^{1}H, ^{13}C, DEPT-135, ^{1}H-^{1}H COSY, NOESY, HMQC, and HMBC NMR experiments were carried out on a Bruker av400 NMR spectrometer (Bruker, Switzerland), operating at 400 MHz for proton and 100 MHz for carbon. $CDCl_3$ and CD_3OD were used as solvents for NMR measurement with chemical shift values in ppm relative to the solvent peak.

Chemicals- All analytical grade organic solvents such as hexane, dichloromethane, chloroform, methanol, ethanol, and *n*-butanol were obtained from Labscan Asia (Bangkok, Thailand). The NMR solvents; $CDCl_3$ and CD_3OD, were bought from Aldrich, USA. Sephadex LH-20 was purchased from Amersham Biosciences, Sweden. Silica gel for column chromatography and TLC aluminum sheets, 20x20 centimeters silica gel 60 F_{254}, were bought from Merck, Germany.

The chemicals for the evaluation of the AChE inhibitory activity i.e. AChE from electric eel (type VI-S lyophilized powder, 480 U/mg, 530 U/mg protein), acetylthiocholine iodide (ACTI), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and bovine serum albumin (BSA) were purchased from Sigma, Germany.

Plant Material- The tubers of *S. suberosa* were collected from areas around Bangkok, Thailand. The plant materials were identified by Associate Professor Dr. Wongsatit Choakul, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. The voucher specimens are kept at the PBM herbarium, Bangkok, Thailand.

Extraction and Isolation- The tubers of *S. suberosa* were cut into small pieces and dried. The dried materials (53.92 g) were macerated with ethanol for 3 days. It was then filtered. The residue was macerated for another 3 days with ethanol and then filtered. The filtrates were pooled and evaporated in vacuum to dryness. The crude extract (**BR**) was dissolved in 72 mL of 10 percent CH_3COOH (adjusted to pH ≅3). **BR** solution was extracted with $CHCl_3$ (250 mL) and the $CHCl_3$ layer was evaporated under reduce pressure to dryness to yield fraction **BRA**. Then, the pH of the aqueous layer was adjusted to 10 with 37 percent NH_4OH (55 mL) and it was extracted with $CHCl_3$ (300 mL). The $CHCl_3$ layer was evaporated under reduced pressure to dryness to yield fraction **BRB.** The aqueous layer was extracted again with 300 mL of *n*-BuOH and the *n*-BuOH layer was evaporated in vacuum. The dried *n*-BuOH extract was named as **BRC**.

The **BRC** extract (3.11 g) was repeatedly subjected to silica gel column (ϕ 10 x 9 centimeters), using hexane, $CHCl_3$, acetone and ethanol as eluents. Thirty-one fractions (**BRC1-31**; 250 mL for each fraction) were obtained. **BRC1** fraction (1.85 g) was recrystallized with hot methanol and distilled water at the ratio of 100:1 (v/v). The crystals **BRC1C** (1.59 g) were obtained and purified on sephadex LH-20

column chromatography (ϕ1.5 x 150 centimeters) using methanol as eluent to yield **1** (440 mg) and **2** (720 mg).

dicentrine (**1**): MS: m/z (rel int) 340(100), 310(50), 309(88), 280(32), 279(95), 278(22), 251(5); IR: ν_{max} centimeters $^{-1}$(KBr) 3785, 3735, 3475, 3422, 2900, 2645, 2592, 2545, 1603, 1519, 1351, 1258, 1235, 932; ^{1}H-NMR and ^{13}C-NMR data (Table 1).

isolaureline (**2**): MS: m/z (rel int) 310(100), 309(60), 280(65), 279(98), 249(55), 237(21), 221 (3); IR: ν_{max} centimeters $^{-1}$(KBr) 3412, 2903, 2637, 2578, 1771, 1358, 1237, 964, 938; ^{1}H-NMR and ^{13}C-NMR data (Table 1).

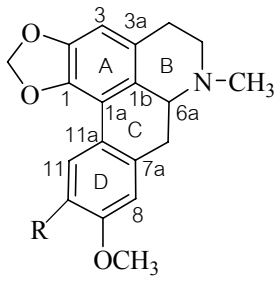
Microplate assay for AChE activity determination- The assay for measuring AChE activity was performed on a microplate as previously described (Ellman *et.al.*, 1961; Ingkaninan *et.al.*, 2003). AChE used in the assay was from the electric eel (type VI-S lyophilized powder, 480 U/mg solid, 530 U/mg protein, Sigma). Briefly, 125 µL of 3 mM DTNB, 25 µL of 1.5 mM ATCI, and 50 µL of 50 mM Tris-HCl buffer pH 8.0 and 25 µL of the respective sample were dissolved in this buffer containing not more than 10 percent methanol and were added to the wells followed by 25 µL of 0.28 U/mL AChE. The microplate was then read out at 405 nm every 5 sec for 2 min by a CERES UV 900C microplate reader (Bio-Tek Instrument, USA). The reaction rates were calculated. The enzyme activity was calculated as a percentage of the reaction rate of the samples compared to that of the blank. Inhibitory activity was calculated from one hundred percentage subtracted by the percentage of enzyme activity. Every experiment was done in triplicate. Stock solutions of samples in Tris-HCl buffer containing not more than 10 percent MeOH were diluted serially with Tris-HCl Buffer to obtain 8-9 different concentrations. The IC_{50} value, corresponding to the inhibitor concentration that caused 50 percent inhibitory activity, was calculated using the software package Prism (Graph Pad Inc, San Diego, USA).

Thin layer chromatography (TLC) assay for detection of AChE inhibitors-The TLC assay for the detection of AChE inhibitors was modified from the study of Rhee et al. (2001). A 2.5 mm silica gel plate F254 no. 5554 (Merck, Darmstadt, Germany) was used as a stationary phase. Three µL of samples (5 mg/mL in methanol) were applied to the plate. After the plate had been developed, it was dried at room temperature and then sprayed with 30 mM ACTI followed by 20 mM DTNB. The plate was dried at room temperature for 45 min, and then sprayed with 10.17 U/mL AChE. After 20 mins, the plate was observed under visible light. A positive spot indicating AChE inhibitor was a colorless spot on the yellow background.

RESULTS AND DISCUSSION

The dried tubers of *S. suberosa* were extracted with ethanol followed by acid-base fractionation. **BRC** were further fractionated by using silica gel column chromatography, gel filtration chromatography and crystallization. The fractions

with high AChE inhibitory activity were further purified to afford **1** and **2** (figure 1)



Compound **1**; R = OCH_3
Compound **2**; R = H

Figure 1 Structures of compounds **1** and **2**

Compounds **1** and **2** showed orange spots on TLC with Dragendolf spraying reagent. So both compounds were expected to be alkaloids (Stahl, 1967). The molecular structures of alkaloids were established by MS, ^{1}H NMR, ^{13}C NMR, DEPT-135, HMQC, HMBC, IR and MS. The ^{1}H and ^{13}C data of **1** and **2** were shown in Table 1.

Compound **1** was obtained as white powder. From the MS experiment, the molecular ion of 340 (MH^{+}) was found which indicated that the molecular weight of **1** was 339. The molecule formula of $C_{20}H_{21}NO_4$ was proposed.

The ^{1}H NMR spectrum showed three singlets at δ 6.56, 6.81 and 7.64 indicated the presence of three isolated aromatic protons. Two typical proton signals of methylenedioxy were observed as doublets at δ 6.16 and 6.00 with a coupling constant of 1.2 Hz. The attachments on the same carbon at δ 101.4 of these two protons were confirmed by HMQC. The singlet signals at δ 3.93 (3 protons) and 3.94 (3 protons) implied that there were two methoxy groups in the molecule. The broad singlet signal at δ 3.17 was likely to belong to an *N*- methyl group. The proton-proton correlations obtained from COSY indicated the presence of isolated CH_2-CH_2 and CH_2-CH systems. The chemical shift of one of the methylene carbon from the ethano group and methine C from CH_2-CH (δ 53.6 and 63.2) implied that both carbon atoms attached to the nitrogen atom. These data suggest that 1 may belong to aporphine class (Roblot *et al*. 1983; Bartley *et al.* 1994; Blanchfield *et al*. 2003).

All the methine, methylene, and methyl carbons were confirmed by DEPT 135. The ^{13}C-^{1}H directly coupling correlations and ^{1}H-^{13}C long range (2-3 bonds) correlations were confirmed by HMQC and HMBC experiments, respectively. In brief, the signals corresponding to C-1 (δ 142.9) and C-2 (δ 149.1) were identified by the three bond couplings to the methylenedioxy protons. C-1 and C-2 were

distinguished from each other by comparison with the previous reports (Roblot *et al*. 1983; Likkitwitayawuid *et al.* 1993; Bartley *et al*. 1994; Blanchfield *et al*. 2003). ^{13}C signal at δ 124.0 was assigned to position 3a on the basis of long range couplings to H-5 and H-4. Similarly, the long range couplings to the H-3, H-4 and H-6a also identified the ^{13}C signal at δ 119.4 as the signal belonging to C-1b. The ^{13}C signal for C-7a was identified at δ 124.8 by the presence of the 2-3 bonds coupling to this signal from H-7, H-8 and H-11. In the same manner, ^{13}C signals for C-1a and 11a were identified at δ 116.7 and 122.6 respectively. They were distinguished from each other by comparison with the data from the reports mentioned above. Moreover, the downfield signals corresponding to C-9 and C-10 (δ 148.4 and 149.4) were identified by the three-bond couplings to H-11 and H-8, respectively. The methoxy proton signal (δ 3.93) showed the long-range coupling to the carbon signals at 148.4 confirmed the presence of methoxy group on C-9 and implied that another methoxy group should be on C10. Selected HMBC correlations of 1 are shown in figure 2. All of the data led to the conclusion that this compound was an aporphine isoquinolines, dicentrine (figure 1). The IR data of **1** was in agreement with the proposed structure. Methylenedioxy peak at 939 centimeters $^{-1}$ was observed. In the mass spectra, the mass fragments at 340, 310, 309, 279 and 251 were observed (figure 3). They confirmed the structure elucidation of **1** as dicentrine.

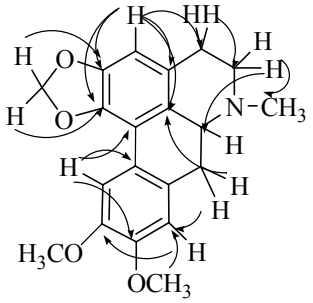


Figure 2 Selected HMBC correlations of **1**

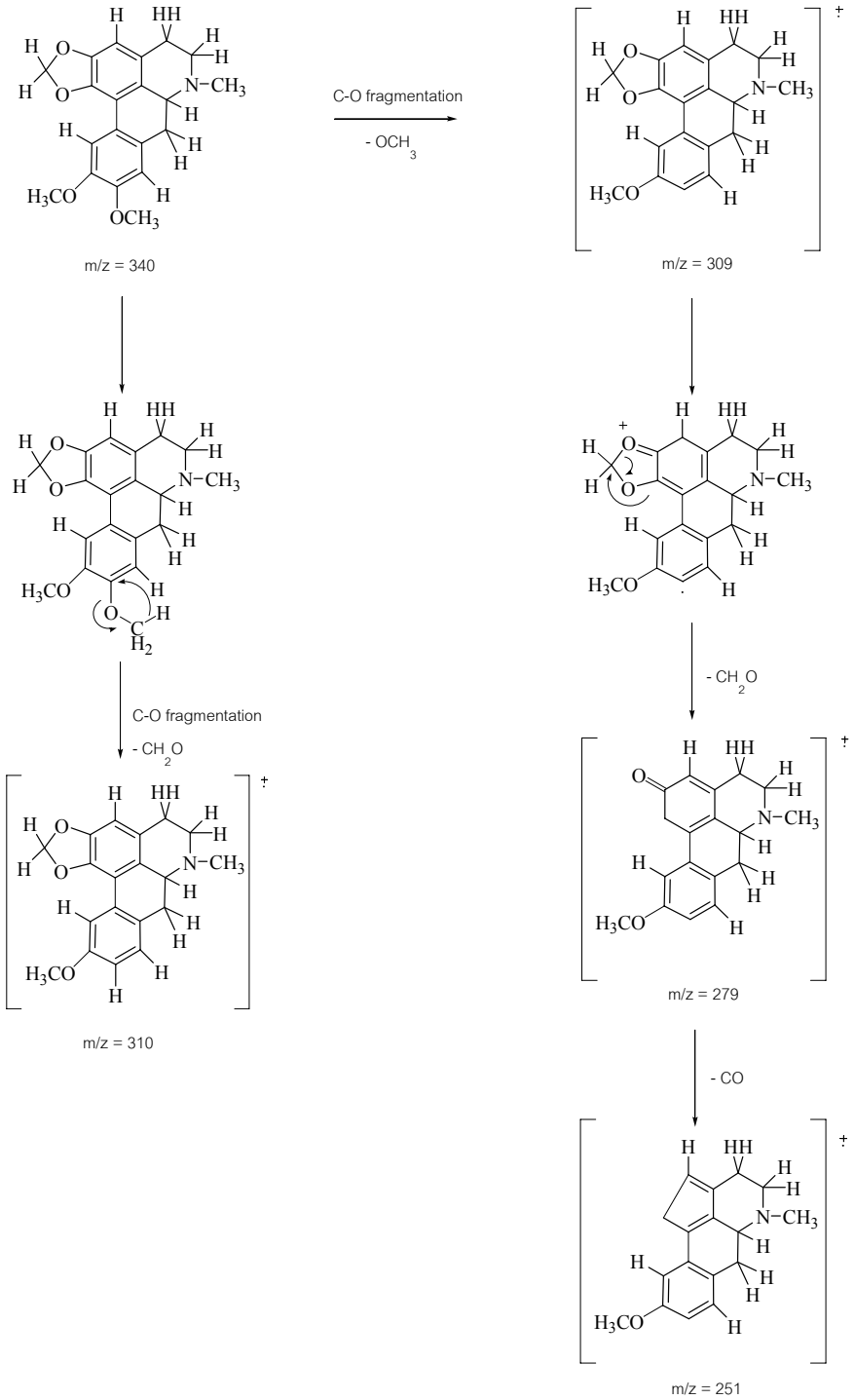


Figure 3 Proposed mass fragmentation ($[M+H]^+$) possibility of **1**

Compound **2** was obtained as white amorphous powder by repeated chromatographic techniques using sephadex LH- 20 with methanol as eluent. From MS experiment, m/z 310 (MH^+) was observed. The molecule formula of $C_{19}H_{19}NO_3$ was proposed.

^1H NMR and ^{13}C NMR spectra of compound **2** were much closed to that of compound **1**. The presence of methylenedioxy group was clear from the typical methylene carbon signal at δ 101.5 and the corresponding proton signals at δ 5.91 and 6.05. The isolated CH_2-CH_2 and CH_2-CH systems were still observed. The differences between 1 and 2 1H NMR spectra were the absence of one of the two methoxy peaks and an additional peak of aromatic proton. In aromatic region of ^1H NMR spectrum, four signals were present. Similar to that of **1**, the singlet at δ 6.56 belonged to H3. This assignment was later confirmed by HMBC experiment. Three proton signals showed ^1H-^1H correlation of aromatic protons of the benzene ring with *ortho*-and *para* substitutions. An *ortho*-coupling between H-10 (δ 6.82, dd, *J*=2.4, 8.8 Hz) and H-11 (δ 7.94, d, *J*=8.8 Hz) was observed. Moreover, H-10 showed meta-coupling with H-8 (δ 6.86). All ^1H and ^{13}C signals in the molecule were assigned using 1D and 2D NMR. The long range correlations of ^1H and ^{13}C in 2 were shown in figure 4. From the HMBC spectrum, the correlations of H-3 to C-1, 2, 4, 1b and the correlations of methylenedioxy protons to C-1 and C-2 confirmed the connection of ring A, B, and methylenedioxy. The connection between ring C and D was shown by the correlation of H-8 to C-7 and C-11a, H-7 to C-7a, and H-11 to C-1a. The correlations of H-11 to C-9, H-8 to C-9 and as well as methoxy proton at 9 to C-9 were also observed. In mass spectra, the fragment at 279 was observed as base peak. The proposed mass fragmentation of compound **2** is shown figure 5. From all spectroscopic data, **2** was unambiguously identified as an aprophine alkaloids, isolaureline.

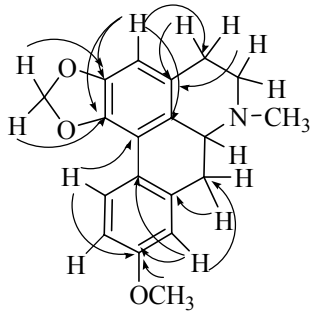


Figure 4 Selected HMBC correlations of **2**

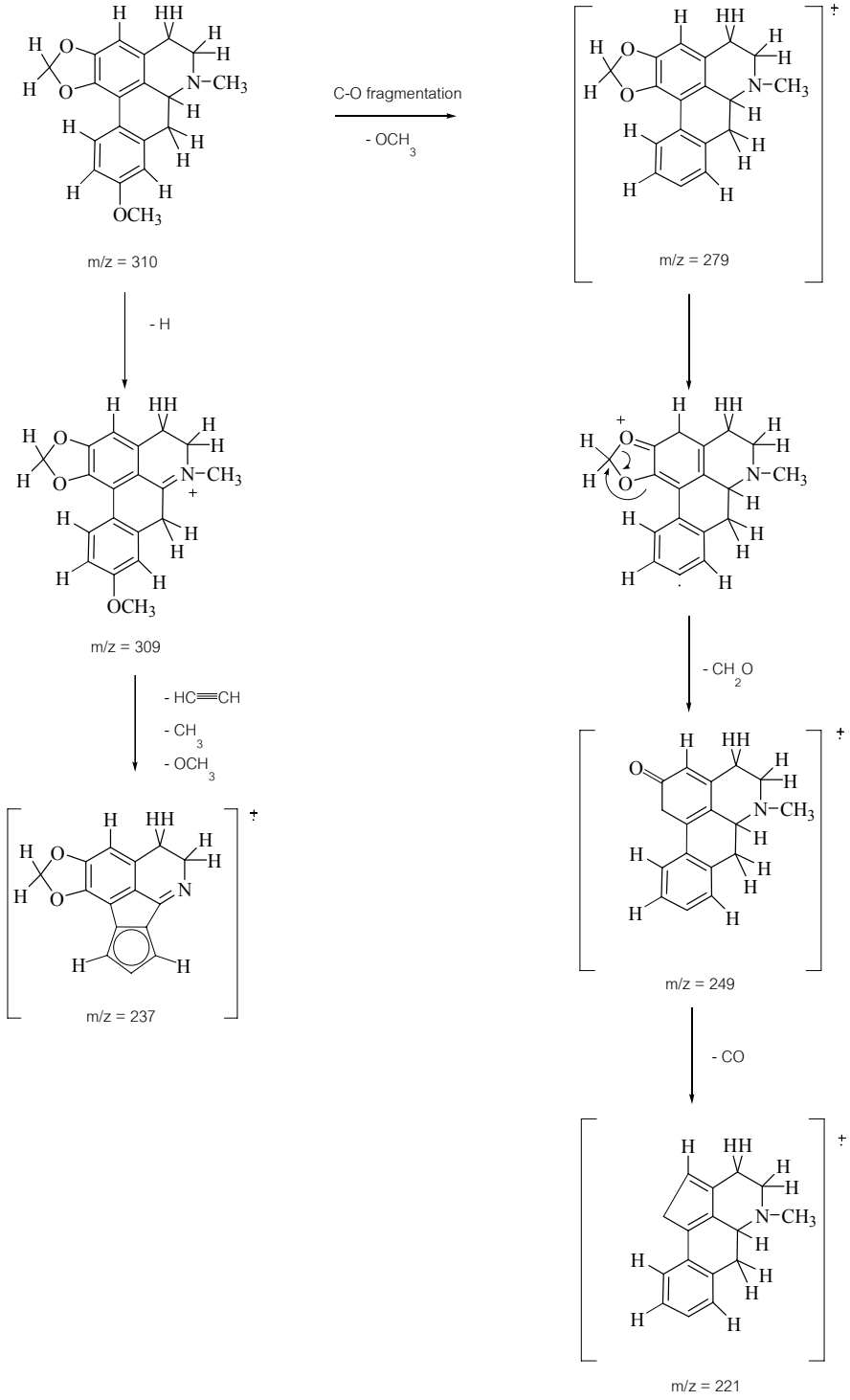


Figure 5 Proposed mass fragmentations ($[M+H]^+$) possibility of **2**

Table 1 Proton and carbon assignments of **1** and **2** (400 MHz, compound **1** was measured in $CDCl_3$ and **2** was measured in CD_3OD)

positions	1		2	
	δ ^{1}H (*J* in Hz)	**δ ^{13}C**	**δ ^{1}H (*J* in Hz)**	**δ ^{13}C**
1		142.9		142.9
1a		116.7		116.0
1b		119.4		119.7
2		149.1		149.1
3	6.56 s	106.8	6.58, s	106.3
3a		124.0		123.4
4	2.87, 3.63 m	26.1	2.94 dd (4.2, 17.0), 3.18 dd (5.2, 17.0)	25.7
5	3.24, 3.81 m	53.6	3.44 td (4.2, 10.2), 3.68 dd (5.2, 10.2)	54.5
6-*N*-CH_3	3.17 s	42.7	3.08 brs	40.6
6a	4.12 m	63.2	3.76 m	59.8
7	3.20 m	31.4	2.83 m, 2.90 m	31.4
7a		124.8		124.9
8	6.81 s	111.2	6.86 (overlapping)	113.6
9		149.4		159.7
10		148.4	6.82 dd (2.4, 8.8)	112.9
11	7.64 s	110.5	7.94 d (8.8)	128.4
11a		122.6		133.5
9-OCH_3	3.93 s	56.0	3.74, s	54.8
10-OCH_3	3.94 s	56.1		
methylenedioxy	6.16 d (1.2) 6.00 d (1.2)	101.4	6.05 d (1.2) 5.91 d (1.2)	101.5

Fractions and isolated compounds from *S. suberosa* were determined for AChE inhibitory activity using Ellman method on 96 well microplate (Ellman *et al.*, 1961; Ingkaninan *et al.*, 2001). At concentration of 0.01 mg/mL, the AChE inhibitory activity of the crude methanolic extract was 80.97 ± 1.29 while the activities of **1** and **2** were 94.39 ± 2.78 percent and 92.56 ± 0.29 percent, respectively. IC_{50} values against AChE of **1** and **2** were 0.67 ± 0.78 and 8.36 ± 1.19 μM, respectively while a positive control, ganlanthamine, showed an IC_{50} value of 0.56 ± 0.14 μM.

Both **1** and **2** have been found in the tuber of *S. pierrei* (Likhitwitayawuid *et al.*, 1993). The mild cytotoxicity and anti-malarial activity of these two compounds were reported. The authors discussed that 1,2-methylenedioxy group might involve with such activities. AChE inhibitory activity has been observed in some *Stephania spp.* The AChE inhibitory activity of some bisbenzylisoquinoline alkaloids in *S. tetrandra* was reported by Ogino and coworkers (1997). Our group also found that bisbenzylisoquinoline alkaloids isolated from *S. pierrei* showed anti-AChE activity (data not shown). Moreover, some protoberberine alkaloids isolated

from *S. venosa* were also expressed AChE inhibitory activity (Ingkaninan *et al.* 2006). However, this is the first time that acetylcholinesterase inhibitory activity of aporphine alkaloids in this genus has been reported. The AChE inhibitory activity of 1 was as potent as that of galanthamine. The methoxy substitution at C-10 might affect AChE inhibitory activity as compound **1** showed more than 10-fold higher potency than compound **2**.

The results from our experiments and from above mentioned reports suggest that the series of isoquinoline alkaloids from *Stephania spp*. show high potential as AChE inhibitors. Detailed studies of their structure-activity relationships and action mechanism against AChE can be very useful for in the future development.

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