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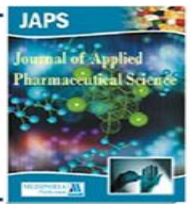

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Isolation and Elucidation of Pyrrolizidine alkaloids from tuber of *Gynura pseudo-china* (L.) DC

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are produced by numerous plant species throughout the world. Numerous intoxications in animals and humans caused by the consumption of certain plants were attributed from the middle of this century to compounds of vegetable origin, the pyrrolizidine alkaloids. Daun dewa (*Gynura pseudo-china* (L.) DC.) is one of Indonesian medicinal plant which also a native plant of India, Thailand and China. The tuber of this plant is commonly used in Indonesian traditional medicine such as for treatments of uterine hemorrhages, dysentery, and inflamed wounds. Although its medicinal value, it is reported that leaves of this plant and other Gynura species content pyrrolizidine alkaloids (PAs). There are concern about the present of PAs in medicinal plants because of the toxicity of this compounds to human and animal. It is reported that PAs are hepatotoxic, pneumotoxic, genotoxic, neurotoxic, and cytotoxic. The purpose of this study is to study the PAs content of *G. pseudo-china* tuber. The structures-were elucidated using spectral data of Infra Red, Liquid Chromatography-Mass Spectroscopy and a combination of one- and two dimensional ^{1}H and ^{13}C- Nuclear Magnetic Resonance spectroscopy. In this study, two pyrrolizidine alkaloids, senecionine (Compound 1) and senkirkine (Compound 2) were isolated from the tuber of *G. pseudo-china*. This results suggest that it should be a safety consideration in consuming this plant for traditional medicine because it is also contains PAs which are toxic compounds.

Keywords: Gynura pseudo-china (L.) DC., pyrrolizidine alkaloids, senecionine, senkirkine, Indonesian medicinal plant, toxicity.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are produced by numerous plant species throughout the world. Numerous intoxications in animals and humans caused by the consumption of certain plants were attributed from the middle of this century to compounds of vegetable origin, the pyrrolizidine alkaloids (Wiedenfeld, 1982; Roeder, 1999; Dominguez *et al.,* 2008; Roeder and Wiedenfeld, 2009). PAs are primarily found in members of three plant families: Asteraceae, Boraginaceae and Fabaceae. These alkaloids are especially found in plants of the genus *Senecio*, *Eupatorium*, *Ageratum* (Asteraceae); *Symphytum*, *Heliotropium*, *Cynoglossum* (Boraginaceae) and *Crotalaria*, *Chromolaena*, *Lototonis* (Fabaceae) (Roeder, 1995, Timbila *et al.*, 2007). Many literatures showed that some of the genus *Gynura* (Asteraceae) containing PAs, for example: *Gynura scandens* (Wiedenfeld, 1982), *G. segetum* (Liang and Roeder, 1984; Qi *et al.,* 2009), *G. sarmentosa* (Matheson and Robins, 1992), and *G. divaricata*.

Various Gynura species are reported to have medicinal value such as antioxidant (Puang pronpitag *et al.,* 2010; Wan *et al.,* 2010), antihypertensive (Kim *et al.,* 2006), antidiabetes (Li et al, 2009; Hassan *et al.,* 2011), antiplatelet aggregation (Lin *et al.,* 2000; Lin *et al.,* 2003). *Gynura pseudo-china* (L.) DC. is a native plant of India, Thailand and China and cultivated in Java (Perry and Metzger, 1980). In Indonesia, the plant was called *daun dewa* (Winarto, 2005). It's tuber used as a remedy for uterine hemorrhages, dysentery, and inflamed wounds. A decoction of the tuber may be drunk to combat fever. The tuberous roots were used both internally and externally for circulatory disturbances (Perry and Metzger, 1980). There were pyrrolizidine alkaloids (PAs) in the leaves (Windono, 2000; Windono *et al.,* 2001), but no information about the chemical compounds in the tuber. Thus, a phytochemical investigation of *G. pseudo-china* tuber was carried out.

MATERIAL AND METHODS

Plant material

G. pseudo-china tuber was collected during its flowering period (July to November 2005) in Surabaya, Indonesia, and was identified by Dr. Rugayah, Research Centre for Biology, Indonesian Institute of Science. A voucher specimen (No. 420/II.1.03/ If.Id/VI/2000) was deposited at the Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Surabaya, Indonesia.

General Methods

IR Spectra were recorded on a Shimadzu IR, melting point were determined using Fisher-John melting point apparatus Serial 50600158, and spectrometer on Liquid Chromatography–Mass Spectrometer Mariner Maldi TOF (LC-MS) were used for molecular weight determination. ^{1}H- and ^{13}C NMR and 1- and 2-D spectra were recorded on Nuclear Magnetic Resonance JEOL-500 using TMS as an internal Standard. Thin layer chromatography was carried out on silica gel 60 GF_{254} plates (Merck, Germany), chloroform-methanol-ammonia (80:10:1) as mobile phase and Dragendorf reagent as spot detection.

Extraction and isolation

The air dried and pulverized tuber (4 kg) was extracted with methanol in a Soxhlet apparatus for 75 hours. Residue after evaporating the solvent added with water was extracted with diethyl ether in a separating funnel to give diethyl ether fraction and aqueous phase I. Aqueous phase I was mixed and extracted with ethyl acetate give ethyl acetate fraction and aqueous phase II. After addition of NH_4OH until pH 10, aqueous phase II was extracted with $CHCl_3$, which give organic fraction and aqueous phase III. After solvent evaporation under reduced pressure, the $CHCl_3$ fraction as a yellow brownish solid residue (5.85 g) was separated on Silica Gel 60 H (pro TLC) with hexane-ethyl acetate (50:50) to methanol (100%) in a gradient eluting method by Dry Column Vacuum Chromatography (DCVC). The ethyl acetate-methanol fractions (10:90 to 1: 99) give 100 mg of white crystal named compound 1. Residue after evaporation of mother liquor was subjected to preparative TLC, which give crystal compound 2 (4 mg).

Compound. 1:

A white crystal, m.p. 242-244°C, Rf = 0.50, molecular formula $C_{18}H_{25}NO_5$. The IR spectrum: ν^{KBr}_{max} (cm^{-1}) 3456, 3060, 2963 – 2816, 1739 and 1714, 1662, 1163 – 1140. The ^{1}H-NMR spectrum: 6.1965 (1H, *d*, H-2), 0.9187 (3H, *d*, *J* = 6.7 Hz. CH_3-17), 1.3263 (3H, *s*, CH_3-16) and 1.8409 (3H, *d*, *J* = 1.85 and 4.85 Hz. CH_3-19). The complete 1H spectrum data were showed at Table 1.

Compound. 2:

A white crystal, m.p. 196-197°C, Rf = 0,43, molecular formula $C_{19}H_{27}NO6$. The IR spectrum: ν^{KBr}_{max} (cm^{-1}) 3445, 2919, 2849, 1734 and 1711, 1615, 1162 – 1145. The ^{1}H-NMR spectrum: 6.1309 (1H, *d*, H-2), 0.8669 (3H, *d*, *J* = 6.7 Hz, CH_3-17), 1.2929 (3H, *s*, CH_3-16), 1.8661 (3H, *d*, *J* = 6.70 Hz, CH_3-19) and 2.0568 (3H, *s*, CH3-N). The complete 1H spectrum data were showed at Table 1.

Table. 1: ^{1}H NMR data of compound 1 and 2 ($CDCl_3$, TMS).

	Compound 1 (δ)	Compound 2(δ)
C_{19}-H_3	0.9169, *d*, 3H	0.9052, *d*, 3H
	J = 4.85	*J* = 6.75
C_{18}-H_3	1.3263, *s*, 3H	1.3299, s, 3H
C_{21}-H_3	1.8452, *dd*,3H	1.8988, *dd*, 3H
	J = 4.85, *J* = 1.85	*J* = 1.85, *J* = 1.80
C_{13}-H	1.6767, *m*,1H	1.6750, *m*,1H
N-CH_3	-	2.0755, *s*, 3H
C_6-H_A	.1868, *m*,1H	2.3584, *m*,1H
C_6-H_B	2.3812, *m*,1H	2.5479, *m*,1H
C_{14}-H_A	1.7602, *m*,1H	1.7822, *m*,1H
C_{14}-H_B	2.1465, *m*,1H	2.2918, *m*,1H
C_5-H_A	2.5456, *m*,1H	2.7257, *m*,1H
C_5-H_B	3.2612, *t*,1H	2.8602, *m*,1H
	J = 8.55	
C_3-H_A	3.3975, *m*,1H	3.2171, *m*,1H
C_3-H_B	3.9408, *m*,1H	3.2537, *m*,1H
C_9-H_A	4.0526, *d*,1H	4.3464, *d*, 1H
	J = 11.60	*J* = 11.0
C_9-H_B	5.5010, *d*,1H	5.4110, *d*,1H
	J = 11.60	*J* = 11.0
C_8-H	4.2750, *d*,1H	----------
C_7-H	5.0225, *t*, 1H	4.9759, *t*, 1H
C_{20}-H	5.7167, *q*,1H	5.8590, *m*,1H
C_2-H	6.1965, *d*,1H	6.1309, t,1H
	J = 1.85	

δ Value in ppm; *J* in Hz

RESULTS

Two alkaloids were isolated from the tuber of *Gynura pseudo-china* as white crystal, and gave a positive orange spot on TLC-Dragendorf test, indicated that both of compounds were alkaloid (Wagner and Bladt, 1996). Molecular weight were deduced by LC-MS: [M+1] = 335.9680 (compound 1) and 366.1000 (compound 2), and these were correspond with molecular formula of $C_{18}H_{25}NO_5$ and $C_{19}H_{27}NO_6$, respectively. The ^{13}C-NMR spectral data and DEPT experiment of compound 1 showed the presence of 18 type of carbons as three methyls, five methylenes, five methines, three quarternary carbon atoms and two carbonyls, whereas compound 2 showed the presence of 19 type of carbons as

four methyls, five methylenes, four methines, three quarternary carbon atoms and three carbonyls, and these agree with molecular formula of both compounds. Besides hydroxyl group (OH) at wave numbers of 3456 cm^{-1}, the IR spectra of compound 1 displayed a split strong absorption bands at 1739 and 1714 cm^{-1} which were identified for conjugated and unconjugated carbonyls. The presence of wave number at 3060 and 1662 cm^{-1} indicated that compound 1 have unsaturated bond (C=C). The ^{1}H-NMR spectral data of compound 1 contained signal for one proton at 6.1655 ppm, *d*, defined that necine base of the pyrrolizidine alkaloid as unsaturated (Kostova, *et al.*, 2006). These data agreed with the IR spectrum data. Compound 1 have other signals at 1.3263 (3H, *d*, *J* = 6.7 Hz. CH_3-18), 0.9217 (3H, *s*, CH_3-19) and 1.8452 (3H, *d*, *J* = 1.85 and 4.85 Hz. CH_3-21) indicated the presence of three methyl groups. The ^{13}C-NMR spectrum of compound 1 showed signals for carbonyl group, *i.e.* at 178.3019 (unconjugated carbonyl, C-11) and 167.7239 ppm (conjugated carbonyl, C-16).

Table. 2: ^{13}C NMR data of compound 1 and 2 ($CDCl_3$, TMS).

Carbon No.	Compound 1	Compound 2	Carbon Type
8	-	190.00	C=O
	77.7584	-	CH
11	178.3019	178.1343	C=O
16	167.7239	166.5929	C=O
2	136.7720	137.2625	=CH
20	134.2825	137.1195	=CH
15	133.1951	131.9592	CH
1	131.5450	134.4678	=C
12	76.8523	76.7323	CH
7	75.0781	78.1631	CH
3	63.0503	58.8671	CH_2
9	60.7611	64.2753	CH_2
5	53.1972	53.4493	CH_2
N-CH_3	-	40.5919	CH_3
13	38.3746	38.7030	CH
14	38.5082	36.4234	CH
6	34.9408	37.7969	CH_2
18	25.0782	24.6531	CH_3
21	15.1774	15.3723	CH_3
19	11.2286	11.0323	CH_3

Besides hydroxyl group (OH) at wave numbers of 3445 cm-1, the IR spectra of compound 2 displayed a split strong absorption bands at 1734 and 1711 cm^{-1} which were identified for conjugated and unconjugated carbonyls. The presence of wave number at 2919 and 1615 cm^{-1} indicated that compound 2 have unsaturated bond (C=C). The ^{1}H-NMR spectral data of compound 2 contained signals for one proton at 6.1309 ppm, *d*, defined that necine base of the pyrrolizidine alkaloid as unsaturated (Kostova *et al.*, 2006). These phenomena agree with the IR spectrum data. Compound 2 have other signals at 0.8669 (3H, *d*, *J* = 6.7 Hz, CH_3-18), 1.2929 (3H, *s*, CH_3-19), 1.8661 (3H, *d*, *J* = 6.70 Hz, CH_3-21) and 2.0568 (3H, *s*, CH3-N) indicated the presence of four methyl groups. The ^{13}C-NMR spectrum of compound 2 showed signals for carbonyl group, *i.e.* at 178.1343 ppm (unconjugatedcarbonyl, C-11), 166.5929ppm (conjugated carbonyl, C-16) and 190.00 ppm (conjugated carbonyl,C-8).

The spectral data of ^{1}H and ^{13}C NMR of compound 1 and 2 are given in Table 1 and 2, respectively. Data of ^{1}H NMR (Table 1) showed differences between compound 1 and 2, *i.e.* N-CH_3 position of compound 2 have a proton signal for methyl group (2.0755, *s*, 3H), which wasn't in compound 1. Another difference, at C_8 position of compound 1 found a proton signal (4.2750, *d*,1H) which wasn't in compound 2, and as substitute for proton, there was a carbonyl group at 190,00 ppm (Table 2). The relationship between protons in compound 1 and 2 can be observed in ^{1}H-^{1}H Correlation spectroscopy (COSY) (Table 3 and 4). The correlations observed in ^{1}H-^{13}C HMQC and ^{1}H-^{13}C HMBC spectra of compound 1 and 2 are given in Fig 1 and Fig 2 as well as Table 3 and 4, respectively.

Table. 3: ^{1}H, ^{13}C, COSY, HMQC and HMBC NMR data of compound 1.

Proton	δ (J_{H-H} in H_Z)	COSY	Correlated carbon	
			HMQC	HMBC
1			C1 (131.5450)	-
2	6.1965, *d* (1.85)	-	C2 (136.7720)	C-8, C-3, C-9, C-1
3b	3.9408, *m*	H-3[a]	C3 (63.0503)	C-2, C-1, C-8, C-7, C-5
3[a]	3.3975, *m*	H-3b, H-2	C3 (63.0503	C-2, C-1, C-8, C-7, C-5
5b	3.2612,*t* (8.55)	H-5a, H-6a, H-6b	C5 (53.1972)	C-3, C-6
5[a]	2.5456, *m*	H-5b, H-6b	C5 (53,1972)	C-8, C-7, C-3, C-6
6b	2.3812, *m*	H-5a, H-5b, H-6[a]	C6 (34.9408)	C-8, C-7, C-5
6[a]	2.1868, *m*	H-5b, H-6b	C6 (34.9408)	
7	5.0225**,** *t*	H-8	C7 (75.0781)	C-5
8	4.2750, *d* (1.85)	H-2, H-7	C8 (77.7584)	C-2, C-1, C-9
9b	5.5010, *d* (11.60)	H-9[a]	C9 (60.7611)	C-10, C-2, C-1, C-8
9[a]	4.0526, *d* (11.60)	H-9b	C9 (60.7611)	C-10, C-2, C-1, C-8
11			C11 (178.3019)	
12			C12 (76.8523)	
13	1.6767, *m*		C13 (38.3746)	C-14, C-13, C-1
14b	2.1465, *m*		C14 (38.5082)	C-15, C-14, C-12, C-17
14[a]	1.7602, *m*		C14 (38.5082)	C-15, C-14, C-8, C-5
15			C15 (133.1951)	
16			C16 (167.7239)	
18	1.3263, *s*		C18 (25.0782)	C-10, C-11, C-12
19	0.9169, *d* (4.85)		C19 (11.2286)	C-11, C-13, C-16
20	5.7167, *q*		C20 (134.2825)	C-15, C-12, C-13, C-19
21	1.8452, *dd*		C21 (15.1774)	C-15, C-18, C-13

Table. 4: ^{1}H, ^{13}C, COSY, HMQC and HMBC NMR data of compound 2

Proton	δ (J_{H-H} in H_Z)	COSY	Correlated carbon	
			HMQC	HMBC
			C1 (134.4678)	
2	-	-	C2 (137.2625)	C-8, C-3, C-9, C-1
3b	3.3975, *m*	H-3[a]	C3 (63.0503)	C-2, C-1, C-8, C-7, C-5
3a	3.9408, *m*	H-3b, H-2	C3 (63.0503)	C-2, C-1, C-8, C-7, C-5
5b	2.5456, *m*	H-5a, H-6a, H-6b	C5 (53.1972)	C-3, C-6
5a	3.2612, *t* (8.55)	H-5b, H-6b	C5 (53,1972)	C-8, C-7, C-3, C-6
6b	2.1391, *m*	H-5a, H-5b, H-6[a]	C6 (34.9408)	C-8, C-7, C-5
6a	2.3848, *m*	H-5b, H-6b	C6 (34.9408)	
7	5.0225, *t*	H-8	C7 (75.0781)	C-5
8	4.2750, *d* (1.85)	H-2, H-7	C8 (77.7584)	C-2, C-1, C-9
9b	5.5010, *d* (11.60)	H-9[a]	C9 (60.7611)	C-10, C-2, C-1, C-8
9[a]	4.0526, *d* (11.60)	H-9b	C9 (60.7611)	C-10, C-2, C-1, C-8
11			C11(178.3019)	
12			C12 (76.8523)	
13	1.6635, *m*		C13 (38.5082)	C-14, C-13, C-1
14b	1.7602, *d* (2.45)		C14 (38.3746)	C-15, C-14, C-12, C-17
14a	2.1868, *m*		C14 (38.3746)	C-15, C-14, C-8, C-5
15			C15 (133.1951)	
16			C16 (167.7239)	
18	1.3263, *s*		C18 (25.0782)	C-10, C-11, C-12
19	0.9187, *d* (6.70)		C19 (11.2286)	C-11, C-13, C-16
20	5.7241, *q* (6.72)		C20 (134.2825)	C-15, C-12, C-13, C-19
21	1.8409, *dd* (1.22; 7.34)		C21 (15.1774)	C-15, C-18, C-13

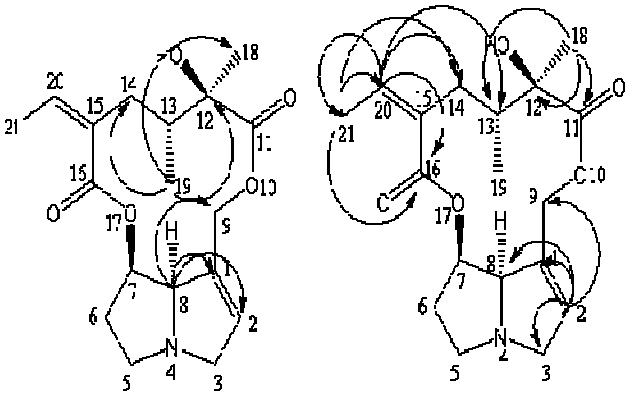

Fig.1: ^{1}H-^{13}C HMBC long-range correlation of compound 1.

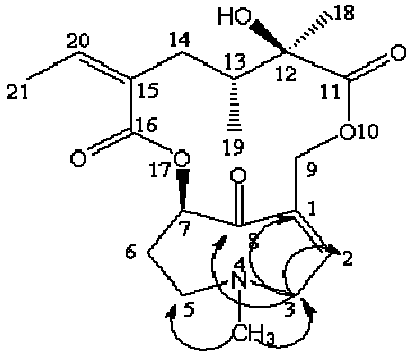

Fig.2: HMBC long-range correlation of compound 2.

DISCUSSION

Pyrrolizidine alkaloid consists of two parts, the necine base and the necic acid(s). The shift value for base ring protons are very distinctive and allow for immediate recognition of important features, particularly any unsaturation and oxygenation pattern. Usual oxygenation pattern occurred mono-oxygenation (C-9) or di-oxygentaion (C-7 and C-9). And proton shift values on C-2, C-6, C-7, C-8 and C-9. Proton shift at around δ 4.5 is an indication of hydroxylated C-7, while a shift at δ 3.5 is an indication of hydroxylated C-9. The C-1 resonance in compound 1 is observed at 131.1 ppm and C-15 at 133.1 ppm, correspondingly consistent with assignment for C-1, C-2, C-15 from retrosine. The shift position of the H-6 proton could be used as a preliminary guide to the stereochemistry of the necine base. Complete assignments for all protons of compound 1 was established by connectivity of previously published spectra (Segall and Dallas, 1982).

Compound 2 present interesting variation on the structure of normal pyrrolizidine alkaloid, which is being the seco-derivative whereas the C-8-N-4 bond of the heterocyclic ring is cleaved to give a C-8 carbonyl group and an N-4 methyl group. This environments in makes compound 2 molecule is identical to compound 1. Despite the identical molecules, the resonances for the macrocylcic diester moiety show little variation from compound 1; the downshift for C-18, and upfield shift for C-14. Intra molecular hydrogen bonding of C-12 hydroxyl group to the C-8 carbonyl in compound 2 produce entirely different relationship of C-14 an dC-18 to the ester carbonyl C-16 as compared to compound 1. Assignment of the resonances for the ester carbonyl group is made by observing the signal of α,β-unsaturated C-16 resonance which occurs at 166.9-167.5 ppm, whereas the unconjugated C-11 carbonyl occurs at lower field.

After interpretation of all data and comparison to published values, isolated alkaloid from tuber of *G. pseudo-china* (L.) DC are senecionine (compound 1) and senkirkine (compound 2), respectively. Both of the compounds isolated from *G. pseudo-china* are not a novel compound, but this is the first information of isolated PAs compounds from tuber of the plant. Previous reports showed that senecionine was isolated from *G. segetum* (Liang and Roeder, 1984), and some Senecio species (El-Shazly, 2002; Arciniegas *et al*., 2005, Kostova *et al*., 2006) while senkirkine was isolated from *Tussilago farfara* L. (Culvenor *et al*., 1976) and *G. sarmentosa* (Matheson and Robins, 1992). Hepatic veno-occlusive disease (HVOD) is a clinical syndrome characterized by hyper-bilirubinemia, painful, hepatomegaly and weight gain due to fluid retention. In 1920, Willmot and Robertson reported that HVOD is associated with the ingestion of *Senecio* tea, which contains PAs (Dai *et al.,* 2007). Result of this investigation showed that PAs was found in this plant so this phenomena was very dangerous for people who consume part or whole of *Gynura pseudo-china* and need to give a warning for another people who will consume the herbs.

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