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## Aporphine alkaloids from *Piper erecticaule* and acetylcholinesterase inhibitory activity

[Alcaloides de aporfina obtenida de *Piper erecticaule* y actividad inhibitoria de la acetilcolinesterasa]

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**Abstract:** Chemical constituents and biological activities of the aerial parts of *Piper erecticaule* C.DC. have been studied for the first time. Fractionation and purification of the extracts afforded aristolactam AII (1), aristolactam BII (2), piperolactam A (3), piperolactam C (4), piperolactam D (5), together with terpenoids of  $\beta$ -sitosterol,  $\beta$ -sitostenone, taraxerol, and lupeol. The structures of these compounds were obtained by analysis of their spectroscopic data, as well as the comparison with that of reported data. Acetylcholinesterase inhibitory activity revealed that compounds 1 and 3 showed strong AChE inhibitory effects with the percentage inhibition of 75.8% and 74.8%, respectively.

**Keywords:** Piperaceae; *Piper erecticaule*; acetylcholinesterase; aporphine alkaloids; aristolactam

**Resumen:** Se estudiaron por primera vez los constituyentes químicos y actividad biológica de las partes aéreas de *Piper erecticaule* C.DC. El fraccionamiento y la purificación de los extractos proporcionaron aristolactama AII (1), aristolactama BII (2), piperolactama A (3), piperolactama C (4), piperolactama D (5), junto con terpenoides de  $\beta$ -sitosterol,  $\beta$ -sitostenona, taraxerol, y el lupeol. Las estructuras de estos compuestos se obtuvieron mediante el análisis de sus datos espectroscópicos, así como mediante la comparación con datos ya informados. La actividad inhibitoria de la acetilcolinesterasa reveló que los compuestos 1 y 3 mostraron un potente efecto inhibitor de la AChE con un porcentaje de inhibición del 75.8% y 74.8%, respectivamente.

**Palabras clave:** Piperaceae; *Piper erecticaule*; Acetilcolinesterasa; Alcaloides de aporfina; Aristolactama

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## INTRODUCTION

Plants from the genus *Piper* have been used for a number of practical applications like remedies in many traditional medicinal systems such as traditional Chinese medicine, the Indian Ayurvedic system and folklore medicines of Latin America and West Indies (Parmar *et al.*, 1997). The chemistry of *Piper* species has been widely investigated and the phytochemical investigations from all parts have led to the isolation of a number of pharmacologically active compounds. They have been extensively investigated as a source of new natural products with potential antitumoral, antimicrobial, antifungal and insecticidal activities (Kato & Furlan, 2007).

*P. erecticaule* is commonly known as '*lada hutan*', is a shrubby, woody herb. The leaves are rather thin, and chartaceous, the underside of which is glaucous (Tawan *et al.*, 2002). The medicinal properties of this plant have not been studied. We have recently reported the chemical compositions and biological activities of the essential oils from this species (Salleh *et al.*, 2014a). Analysis of *P. erecticaule* essential oils resulted in the identification of 35 chemical components, and the most abundant group components were sesquiterpene hydrocarbons (63.4%).  $\beta$ -Caryophyllene (5.7%), spathulenol (5.1%),  $\beta$ -cadinene (3.8%) and  $\alpha$ -amorphene (3.8%) were identified as their major components. The essential oils also displayed strong activity on *Aspergillus niger* with Minimum Inhibitory Concentration value of 31.3  $\mu$ g/mL. In addition, screening on the anticholinesterase activity of the leaves extract has also been reported. The MeOH extract of *P. erecticaule* showed good activity against butyrylcholinesterase enzyme with inhibition of 70.9% (Salleh *et al.*, 2014b). In continuation of our phytochemical and bioactivity studies on this genus, herein we would like to report the detailed phytochemical study of *P. erecticaule* with their acetylcholinesterase inhibitory activity. To the best of our knowledge, there is no report on the constituents and bioactivity studies from this species.

## MATERIAL AND METHODS

### *Plant material*

*P. erecticaule* was collected from Borneo and identified by Mohizar Mohamad. The voucher specimen (UiTMKS-02/2012) was deposited at the Applied Sciences Faculty, UiTM Sarawak.

### *General experimental procedures*

Soxhlet extraction technique was applied to extract the phytochemicals from the dried sample using different polarity solvents (*n*-hexane, ethyl acetate, and methanol). Vacuum liquid chromatography (VLC) was performed on Merck silica gel 60 (230-400 mesh) while column chromatography (CC) on Merck silica gel 60 (70-230 mesh) as the stationary phase. Thin layer chromatography (TLC) analysis was performed on Merck pre-coated silica (SiO<sub>2</sub>) gel F254 plates (0.2 mm thickness) to detect and monitor the presence of compounds in the samples. The spots were visualized under UV light at 254 and 365 nm, included with spraying reagent vanillin-sulphuric acid in MeOH followed by heating. Melting points were measured using melting point apparatus equipped with a microscope, Leica Gallen III and were uncorrected. The <sup>1</sup>H (400 MHz) and <sup>13</sup>C NMR (100 MHz) spectra were recorded on a Bruker Avance 400 Spectrophotometer. Chemical shifts were reported in ppm and CDCl<sub>3</sub> as the solvent. Residual solvent was used as an internal standard. The IR spectra were recorded on Perkin Elmer ATR and 1600 spectrophotometer series as KBr disc. Mass spectral data were obtained from Mass Spectrometry Service, National University of Singapore (NUS), Singapore.

### *Solvents and chemicals*

Analytical grade *n*-hexane, diethyl ether, ethyl acetate, chloroform, dichloromethane and methanol used for extraction and isolation were purchased from Merck (Germany). Acetylcholinesterase enzyme (Type-VI-S, EC3.1.1.7), acetylthiocholine iodide, 5,5'-dithio-bis (2-nitrobenzoic) acid (DTNB), and galantamine were purchased from Sigma-Aldrich (Germany).

### *Extraction and isolation*

The dried and powdered aerial part of *P. erecticaule* (1 kg) was extracted successively by Soxhlet extractor with *n*-hexane, EtOAc and MeOH. Evaporation of the respective solvents gave hexane (5.0 g), EtOAc (9.2 g) and MeOH (9.5 g) extracts. The hexane extract was subjected to vacuum liquid chromatography (VLC) on SiO<sub>2</sub> 60 (230-400 mesh) using *n*-hexane and CHCl<sub>3</sub> in 5% increasing polarity to give 8 fractions (PEH1–8). The combined fractions of PEH1-3 were purified by column chromatography on silica gel 70-230 mesh to afford  $\beta$ -sitosterol (*n*-

hexane:CHCl<sub>3</sub>, 70:30) and β-sitostenone (*n*-hexane:CHCl<sub>3</sub>, 60:40). The crude EtOAc was fractionated by VLC on SiO<sub>2</sub> 70–230 mesh, using *n*-hexane and EtOAc in 10% increasing polarity to give 15 fractions (PEE1–15). The combined fractions PEE2–5 were purified and recrystallized from hexane:CHCl<sub>3</sub> to yield compounds **1** (*n*-hexane:CHCl<sub>3</sub>, 50:50) and **2** (*n*-hexane:CHCl<sub>3</sub>, 45:55). The combined fractions PEE6–8 were purified and recrystallized from hexane:CHCl<sub>3</sub> to yield compounds **3** (*n*-hexane:CHCl<sub>3</sub>, 40:60), taraxerol (*n*-hexane:CHCl<sub>3</sub>, 70:30), and lupeol (*n*-hexane:CHCl<sub>3</sub>, 60:40). The crude MeOH was fractionated by VLC on SiO<sub>2</sub> 70–230 mesh, using CHCl<sub>3</sub>:MeOH in 10% increasing polarity to give fractions (PEM1–5). The combined fractions PEE2–3 were purified by column chromatography to yield compounds **4** (*n*-hexane:CHCl<sub>3</sub>, 20:80) and **5** (*n*-hexane:CHCl<sub>3</sub>, 25:75).

#### Acetylcholinesterase activity

AChE inhibitory activities were measured by slightly modifying the spectrophotometric method (Salleh *et al.*, 2014b). Electric eel AChE were used, while acetylthiocholine iodides were employed as substrates of the reaction. 5,5'-Dithio-bis(2-nitrobenzoic) acid (DTNB) was used for the measurement of the AChE activity. Briefly, 140 μL of sodium phosphate buffer (pH 8.0), 20 μL of DTNB, 20 μL of the compound (concentration of 1 mg/mL) and 20 μL of AChE solution were added by multichannel automatic pipette in a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated with the addition of 10 μL of acetylthiocholine iodide. Hydrolysis of acetylthiocholine iodide was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer). Percentage inhibition (I%) of AChE was determined by comparison of reaction rates of samples relative to blank sample (ethanol in phosphate buffer pH = 8) using the formula:

$$I\% = [ E - S / E ] \times 100;$$

where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample.

Galantamine was used as a reference. Analyses were run in triplicate and the result was expressed as means ± SD of triplicate. Data obtained from the acetylcholinesterase activity are expressed as mean values. Statistical analyses were carried out by employing one way ANOVA ( $p > 0.05$ ). A statistical package (SPSS version 11.0) was used for the data analysis.

#### RESULTS AND DISCUSSION

Phytochemical studies on the aerial parts of *P. erecticaule* species which has led to the isolation of nine compounds, which characterized as five aporphine alkaloids, two triterpenes, and two steroids. These metabolites were identified by analyzing their spectroscopic data and comparing with the literature data. Five aporphine alkaloids elucidated as aristolactam AII (**1**), aristolactam BII (**2**), piperolactam A (**3**), piperolactam C (**4**), piperolactam D (**5**), together with terpenoids of β-sitosterol, β-sitostenone, taraxerol, and lupeol. Aporphine alkaloids broadly exist in nature and have a distinctive biological activity. Many of these isolated aporphine alkaloids were isolated previously from *Piper* genus. Compound (**1**) and (**2**) have been isolated from *P. officinarum* (Salleh *et al.*, 2014c) and *P. betle* (Lin *et al.*, 2013), whilst the compound (**3**), (**4**), and (**5**) have been isolated from *P. betle* (Amin *et al.*, 2017), *P. taiwanense* (Chen *et al.*, 2004), and *P. nigrum* (Ee *et al.*, 2008), respectively. The isolated terpenoids were also reported most of the *Piper* species. They were readily identified by comparison of physical and spectroscopic data and mass spectrometry data with values found in the literature (Salleh *et al.*, 2016a; Salleh *et al.*, 2016b).

Aristolactams, which also found in this species is a minor group of fused phenanthrene lactam alkaloids structurally and biogenetically related to aporphines (Michle *et al.*, 2014). The richest source of this class of alkaloids is undoubtedly plants of the family Piperaceae and Aristolochiaceae (Tsai *et al.*, 2005; Levrier *et al.*, 2013). Aristolactams are frequently used as folk medicines in several countries. Meanwhile, these molecules show an interesting array of biological properties such as anti-inflammatory, antiplatelet, anti-mycobacterial, neuroprotective and anti-cancer activities (Kumar *et al.*, 2003; Kim *et al.*, 2004; Zhang *et al.*, 2007).

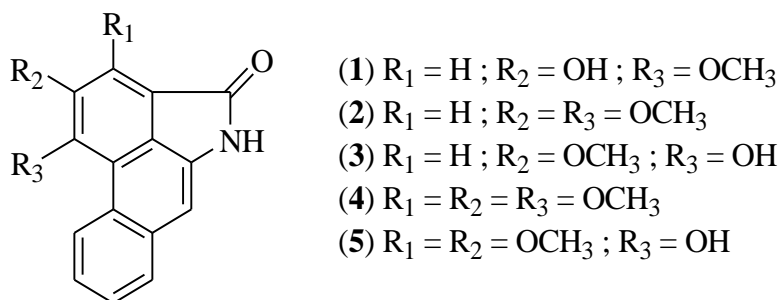


Figure N° 1  
 Aporphine alkaloids from *P. erecticaule*

**Aristolactam AII (1)** – Pale yellow needles (5.0 mg.); m.p. 269-272°C; IR (KBr)  $\nu_{\max}$  cm<sup>-1</sup>: 3335, 2918, 1717, 1597, 1498; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta_{\text{H}}$  4.11 (3H, s, 4-OCH<sub>3</sub>), 7.16 (1H, s, H-9), 7.59 (2H, ddd, *J*=7.2, 7.2, 2.0 Hz, H-6 and H-7), 7.92 (1H, dd, *J*=6.8, 2.0 Hz, H-8), 7.71 (1H, s, H-2), 9.77 (1H, br.s, N-H), 9.20 (1H, dd, *J*=6.8, 2.0 Hz, H-5); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz):  $\delta_{\text{C}}$  56.8 (4-OCH<sub>3</sub>), 104.3 (C-9), 107.9 (C-2), 124.9 (C-1a/4a), 126.7 (C-7/8), 127.9 (C-5/6), 128.5 (C-4b/9b), 134.7 (C-8a), 135.3 (C-9a), 148.1 (C-4), 149.1 (C-3), 168.6 (C-1); EIMS *m/z* 265 [M<sup>+</sup>, C<sub>16</sub>H<sub>11</sub>NO<sub>3</sub>] (Salleh *et al.*, 2014c).

**Aristolactam BII (2)** – Yellow powder (8.0 mg), m.p. 260-262°C; IR (KBr)  $\nu_{\max}$  cm<sup>-1</sup>: 3336, 2920, 1715, 1597, 1495; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta_{\text{H}}$  4.03 (3H, s, 4-OCH<sub>3</sub>), 4.12 (3H, s, 3-OCH<sub>3</sub>), 7.13 (1H, s, H-9), 7.56 (2H, m, H-6 and H-7), 7.85 (1H, s, H-2), 7.94 (1H, m, H-8), 9.11 (1H, m, H-5), 10.83 (1H, br s, NH); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz):  $\delta_{\text{C}}$  60.3 (4-OCH<sub>3</sub>), 60.6 (3-OCH<sub>3</sub>), 105.7 (C-9), 114.3 (C-2), 121.0 (C-4a), 124.3 (C-1a), 126.1 (C-7/8), 127.7 (C-9b), 129.2 (C-5/6), 131.0 (C-4b), 132.3 (C-8a), 134.4 (C-9a), 146.5 (C-4), 157.9 (C-3),  $\delta$  173.9 (C-1); EIMS *m/z* 279 [M<sup>+</sup>, C<sub>17</sub>H<sub>13</sub>NO<sub>3</sub>] (Salleh *et al.*, 2014c).

**Piperolactam A (3)** – Yellowish needle crystals (10 mg); m.p. 300-301°C; IR (KBr)  $\nu_{\max}$  cm<sup>-1</sup>: 3475, 3185, 1656, 1500, 1445; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta_{\text{H}}$  4.10 (3H, s, OCH<sub>3</sub>), 7.15 (1H, s, H-9), 7.25 (1H, m, H-7), 7.55 (1H, m, H-6), 7.75 (1H, s, H-2), 7.85 (1H, m, H-8), 9.30 (1H, m, H-5), 9.85 (1H, br.s, NH); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz):  $\delta_{\text{C}}$  57.5 (3-OCH<sub>3</sub>), 107.0 (C-9), 108.5 (C-2), 116.1 (C-4a), 116.5 (C-1), 126.5 (C-10a), 126.5 (C-6), 127.5 (C-7), 128.5 (C-4b), 129.0 (C-5), 129.5 (C-8), 135.8 (C-8a), 135.8

(C-10), 149.7.2 (C-3), 151.5 (C-4), 172.2 (C=O); EIMS *m/z* 265 [M<sup>+</sup>, C<sub>16</sub>H<sub>11</sub>NO<sub>3</sub>] (Amin *et al.*, 2017).

**Piperolactam C (4)** – Yellow crystalline crystals (5.0 mg); m.p. 250-252°C; IR (KBr)  $\nu_{\max}$  cm<sup>-1</sup>: 3265, 2928, 1678, 1615, 1485; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta_{\text{H}}$  4.00 (3H, s, 4-OCH<sub>3</sub>), 4.20 (3H, s, 3-OCH<sub>3</sub>), 4.50 (3H, s, 2-OCH<sub>3</sub>), 7.83 (1H, m, H-9), 7.50-7.55 (2H, m, H-6, H-7), 9.20 (1H, m, H-5); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz):  $\delta_{\text{C}}$  60.5 (4-OCH<sub>3</sub>), 61.5 (3-OCH<sub>3</sub>), 63.2 (2-OCH<sub>3</sub>), 106.0 (C-9), 116.8 (C-4a), 125.6 (C-6), 126.5 (C-4b/5), 126.5 (C-7), 128.6 (C-8), 133.5 (C-8a), 146.8 (C-4), 154.8 (C-3), 157.1 (C-2), 167.0 (C-1); EIMS *m/z* 309 [M<sup>+</sup>, C<sub>18</sub>H<sub>15</sub>NO<sub>4</sub>] (Chen *et al.*, 2004).

**Piperolactam D (5)** – Yellow crystalline solid (8.5 mg); m.p. 227-229°C; IR (KBr)  $\nu_{\max}$  cm<sup>-1</sup>: 3260, 2925, 1675, 1610, 1482; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta_{\text{H}}$  3.92 (3H, s, 3-OCH<sub>3</sub>), 4.45 (3H, s, 4-OCH<sub>3</sub>), 7.25 (1H, s, H-9), 7.49 (2H, m, H-6, H-7), 7.86 (1H, m, H-8), 9.26 (1H, m, H-5), 9.80 (1H, br.s, NH); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz):  $\delta_{\text{C}}$  61.6 (3-OCH<sub>3</sub>), 62.5 (4-OCH<sub>3</sub>), 105.5 (C-9), 106.5 (C-1), 112.0 (C-10a), 125.8 (C-7), 126.5 (C-6), 127.1 (C-4a), 127.8 (C-5a), 127.2 (C-5), 129.0 (C-8), 134.1 (C-9a), 135.6 (C-10), 140.2 (C-3), 153.5 (C-4), 154.6 (C-2), 167.2 (C=O); EIMS *m/z* 295 [M<sup>+</sup>, C<sub>17</sub>H<sub>13</sub>NO<sub>4</sub>] (Ee *et al.*, 2008).

**Acetylcholine (AChE)** is recognized as the most important neurotransmitter, have been studied for their inhibitory action on the progression of Alzheimer disease as they improve the cognitive function (Park, 2010). Previous studies revealed that most of the aporphine alkaloids exhibit acetylcholinesterase (AChE) inhibitory effects (Loizzo *et al.*, 2008). Thus, the AChE inhibitory

activities of compounds **1–5** were measured by slightly modifying the spectrophotometric method (Salleh *et al.*, 2014b). It was compared with that of galantamine, as a standard drug against Alzheimer's disease and the results were shown in Table 1. The results revealed that compounds **1** and **3** showed strong AChE inhibitory effects with the percentage inhibition of 75.8% and 74.8%, respectively.

Galanthamine was used as a standard, which exhibited 85.5% inhibition of AChE. By comparing the structure-activity relationship of compounds **1** and **3**, the hydroxyl at C-2 and C-3 position might play important roles in AChE inhibitory activity of aporphine alkaloids. Other exhibited potent AChE inhibitory activity with the percentage inhibition values ranging from 60.1-65.2%.

Table N° 1

Acetylcholinesterase inhibitory activity of aporphine alkaloids from *P. erecticaule*

Samples	AChE inhibition (I%)
Aristolactam AII ( <b>1</b> )	75.5%
Aristolactam BII ( <b>2</b> )	62.5%
Piperolactam A ( <b>3</b> )	74.8%
Piperolactam C ( <b>4</b> )	60.1%
Piperolactam D ( <b>5</b> )	65.2%
Galantamine	85.5%

## CONCLUSION

The present study is the first to report that aporphine alkaloids were isolated from *P. erecticaule*, which have much chemotaxonomic importance within the genus *Piper*. Additionally, compound **1** showed the highest inhibition among others, may be of interest to clarify the physiological role of this enzyme. In addition, to validate the above-mentioned activity, clinical trials should be carried out in order to ensure safe use of the compounds as therapeutic agents against neurodegenerative diseases such as Alzheimer. This study also provides valuable and useful information and indications for further exploring the potential nutraceutical and pharmaceutical applications of the Lauraceae species.

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