SHORT COMMUNICATION

CHEMICAL CONSTITUENTS AND ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF *BEILSCHMIEDIA INSIGNIS* GAMBLE

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ABSTRACT. Secondary metabolites from natural products are potential sources of acetylcholinesterase inhibitors, which are key enzymes in the treatment of many neurodegenerative diseases. Inspired by the reported activities of alkaloids herein we report the chemical investigation on the chemical constituents from *Beilschmiedia insignis* and their acetylcholinesterase inhibitory activity. Isolation of the stem bark of *B. insignis* led to the isolation and identification of five aporphine alkaloids, namely isocorydine (**1**), norisocorydine (**2**), (+)-laurotetanine (**3**), (+)-*N*-methyllaurotetanine (**4**), and (+)-boldine (**5**), together with β-sitosterol (**6**), β-sitostenone (**7**), lupeol (**8**), and lupenone (**9**). The chemical 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 all isolated alkaloids were found to inhibit AChE with percentage inhibition values ranged from 44.9 to 74.5%. This is the first report on phytochemicals from *B. insignis.*

KEY WORDS: Lauraceae, *Beilschmiedia insignis*, Alkaloid, Acetylcholinesterase, Alzheimer

INTRODUCTION

Alzheimer's disease (AD) is mainly characterized by progressive neurodegenerative disorder, clinically demonstrated by cognitive and memory decline, progressive impairment of daily activities, and a variety of neuropsychiatric symptoms and behavioral disturbances. It is a main cause of dementia and has become a population aging-related concern for public health systems around the world by its both direct and indirect costs [1]. One treatment strategy to enhance the cholinergic function is to use acetylcholinesterase (AChE) inhibitors to increase the amount of acetylcholine, which is present in the synapses between cholinergic neurons [2]. Current drugs exhibit two action mechanisms, either prosthetic or acid-transferring. Prosthetic inhibitors have an affinity for the anionic site of acetylcholinesterase and prevent acetylcholine from accessing it (competitive inhibitors). Acid-transferring inhibitors react with the enzyme and form an intermediate compound. Depending on the stability of this product, the effects could be short-term and reversible or long-acting and irreversible [3]. Currently, several kinds of AChE inhibitors such as donepezil, galantamine, and rivastigmine have been approved by the Food and Drug Administration in the United States and are available for the treatment of mild-to-moderate AD patients [4]. However, these marketed medicines suffer from several drawbacks, which include gastrointestinal disturbances, insomnia, fatigue, and depression [5]. Until now, no drug of choice

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for the treatment of this disease has been decided. Therefore, the search for new AChE inhibitors is of great interest.

Beilschmiedia insignis belongs to the Lauraceae family, is a tree endemic to Peninsular Malaysia. It grows in the primary and secondary forests from lowlands to mountains at 30-350 m altitude [6]. Some species of the genus are used in traditional medicine for the treatment of several ailments such as uterine tumors, rheumatism, and pulmonary diseases in Africa. The genus produces several classes of compounds such as alkaloids, endiandric acid derivatives, lignans, neolignans, flavonoids, and terpenoids [7]. Alkaloids have been found in other *Beilschmiedia* species such as *B. glabra* [8], *B. brevipes* [9], *B. alloiophylla* [10], *B. kunstleri* [11], and *B. penangiana* [12]. Most of them possesses aporphine or benzylisoquinoline skeletons.

We have recently reported the chemical composition of the leaf oil of *B. insignis* [13]. Analysis of the essential oil revealed the presence of twenty-six components, accounting for 85.7%, and characterized by the presence of high concentrations of oxygenated sesquiterpenes (61.1%).The major components identified were (*E*)-nerolidol (32.4%), spathulenol (12.9%), bicyclogermacrene (5.2%), globulol (5.0%), and viridiflorol (4.4%). Besides, the methanolic bark extract of *B. insignis* demonstrated optimum inhibitory effects against α-amylase and α-glucosidase with IC_{50} values of 3.2 and 12.3 µg/mL, respectively. In addition, the extract also showed antioxidant potential which gave phenolic content of 420.3 mg GAE/g extract, IC_{50} value 12.1 µg/mL in DPPH radical scavenging, and FRAP value of 1904.2 µM Fe(II)/mg extract [14].

In continuation of our search for bioactive compounds from Malaysian *Beilschmiedia* species, we have investigated the phytochemicals present in the stem bark of *B. insignis.* To the best of our knowledge, this is the first report on the phytochemical study of this species and their acetylcholinesterase inhibitory activity.

EXPERIMENTAL

Plant material. The stem bark of *Beilschmiedia insignis* was collected from Gambang Pahang (3°42'59.99" N, 103°05'60.00" E) (September 2019) and identified by Shamsul Khamis. The voucher specimen (SK39/19) was deposited at UKMB Herbarium.

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_2) gel F_{254} plates (0.2 mm thickness) to detect and monitor the presence of compounds in the samples. The TLC and PTLC spots were visualized under UV light (254 and 366 nm) followed by spraying with Dragendorff's reagent for an alkaloid detection. Melting points were measured using melting point apparatus equipped with a microscope, Leica Gallen III and were uncorrected. The ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Bruker Avance 400 Spectrometer. Chemical shifts were reported in ppm and $CDCl_3$ as the solvent. The residual solvent was used as an internal standard. The IR spectra were recorded on Perkin Elmer ATR and 1600 spectrophotometer series as KBr disc. The mass spectra of alkaloids were obtained from LCMS-IT-TOF, Shimadzu, whereas for triterpenoids were obtained from gas chromatography–mass spectrometry (GC-MS).

Extraction and isolation. The dried stem bark (300 g) of *B. insignis* was ground and extracted exhaustively for 12 hours by Soxhlet extraction with hexane, followed by dichloromethane (DCM). The extraction of alkaloids was carried out in the usual manner, which has been described in detail [15] and gave 10 g of crude alkaloid. The crude alkaloid was submitted to exhaustive column chromatography over silica gel using DCM gradually enriched with methanol (MeOH) to

yield 16 fractions. Fractions were combined on the basis of TLC behavior. Fractions 5-8 (1.5 g), afforded two alkaloids identified as (+)-isocorydine (**1**) (12 mg) (PTLC; DCM:MeOH 92:8; R_f 0.25) and (+)-norisocorydine (**2**) (13 mg) (PTLC; DCM:MeOH95:5; R_f 0.40). Fraction 10-12 (1.5 g) produced (+)-laurotetanine (**3**) (15 mg) (PTLC; DCM:MeOH 95:5; R_f 0.50) and (+)-*N*-methyllaurotetanine (**4**) (15 mg) (PTLC; DCM:MeOH 94:6; R_f 0.52). Fraction 13-16 (1.0 g) produced (+)-boldine (**5**) (12 mg) (PTLC; EtOAc:MeOH 96:4; R_f 0.45). The hexane extract was separated on a silica gel column using a gradient Hex:DCM to give β-sitosterol (**6**) (30 mg), β-sitostenone (**7**) (35 mg), lupeol (**8**) (28 mg), and lupenone (**9**) (20 mg).

Acetylcholinesterase inhibitory activity. AChE inhibitory activities were measured by slightly modifying the spectrophotometric method [15]. Acetylcholinesterase from *Electrophorus electricus* (electric eel) 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 the enzyme with test sample. Galantamine (1 mg/mL) was used as a positive control. 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 ($p > 0.05$).

Statistical analysis. Data obtained from the biological 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

Chemical constituents on *Beilschmiedia* species have resulted in the isolation and identification of various phytochemicals. In view of the attributed medicinal properties, studies were undertaken on the stem bark of *B. insignis* which resulted in the isolation and structure elucidation of alkaloids and steroids. Five alkaloids have been successfully isolated which are (+)-isocorydine (**1**), (+)-norisocorydine (**2**), (+)-laurotetanine (**3**), (+)-*N*-methyl laurotetanine (**4**), and (+)-boldine (**5**), together with β-sitosterol (**6**), β-sitostenone (**7**), lupeol (**8**), and lupenone (**9**).The chemical structures are shown in Figure 1. All secondary metabolites were identified by analyzing their spectroscopic data and comparing them with the literature data. To the best of our knowledge, all compounds were isolated for the first time from this species. These alkaloids have been previously isolated from several *Beilschmiedia* genus. Compound (**1**) has been isolated previously from *B. podagrica* [16], while compounds (**3**) and (**5**) from *B. alloiophylla* [10] and *B. kunstleri* [11]. Interestingly, compounds (**2**) and (**4**) were isolated for the first time from *Beilschmiedia* species. Furthermore, all the alkaloid compounds were also reported from plants of various families such as Amaryllidaceae [17], Piperaceae [18], Asteraceae [19], and Rutaceae [20]. The presence of

those valuable alkaloids in *Beilschmiedia* species enriches their chemical diversity and provides evidence for chemotaxonomic studies of *Beilschmiedia* species and the family Lauraceae as well [6].
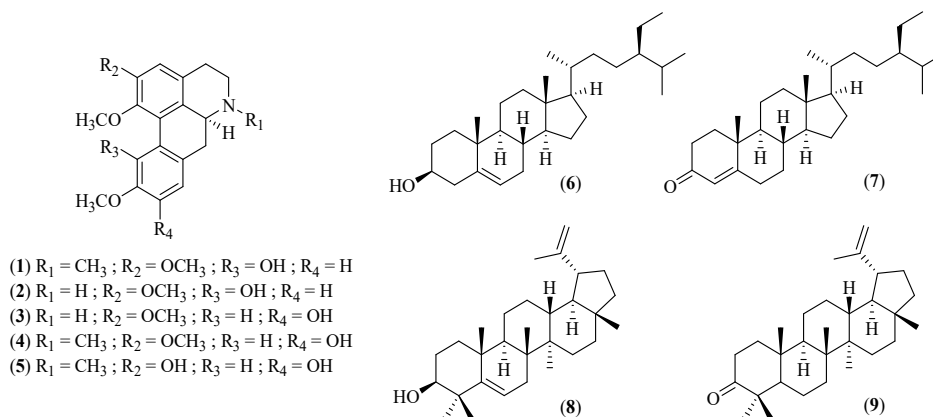

(**1**) R$_1$ = CH$_3$; R$_2$ = OCH$_3$; R$_3$ = OH ; R$_4$ = H
(**2**) R$_1$ = H ; R$_2$ = OCH$_3$; R$_3$ = OH ; R$_4$ = H
(**3**) R$_1$ = H ; R$_2$ = OCH$_3$; R$_3$ = H ; R$_4$ = OH
(**4**) R$_1$ = CH$_3$; R$_2$ = OCH$_3$; R$_3$ = H ; R$_4$ = OH
(**5**) R$_1$ = CH$_3$; R$_2$ = OH ; R$_3$ = H ; R$_4$ = OH

Figure 1. Chemical constituents isolated from *B. insignis*.

(+)-Isocorydine (1). Brownish amorphous powder; m.p. 183-185 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ$_H$ 2.45-2.50 (2H, m, H-4), 2.50 (3H, s, *N*-CH$_3$), 2.65-2.70 (2H, dd, *J* = 16.6, 3.2 Hz, H-7), 2.95-3.04 (2H, m, H-5), 3.12-3.20 (1H, m, H-6a), 3.68 (3H, s, 1-OCH$_3$), 3.88 (3H, s, 2-OCH$_3$), 3.90 (3H, s, 10-OCH$_3$), 6.68 (1H, s, H-3), 6.80 (1H, d, *J* = 8.0 Hz, H-8), 6.85 (1H, d, *J* = 8.0 Hz, H-9); ^{13}C NMR (100 MHz, CDCl$_3$): δ$_C$ 29.2 (C-4), 35.8 (C-7), 43.5 (*N*-CH$_3$), 52.6 (C-5), 55.8 (2-OCH$_3$), 56.2 (10-OCH$_3$), 62.0 (1-OCH$_3$), 62.8 (C-6a), 111.0 (C-3), 111.2 (C-9), 119.0 (C-8), 120.2 (C-11a), 125.8 (C-1a), 129.2 (C-1b), 130.0 (C-3a), 130.2 (C-7a), 142.0 (C-1), 144.0 (C-11), 149.5 (C-10), 151.2 (C-2); MS *m/z* 342 [M$^+$, C$_{20}$H$_{24}$NO$_4$] [21].

(+)-Norisocorydine (2). Brownish amorphous powder; m.p. 203-205 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ$_H$ 3.63 (1H, d, *J* = 3.6 Hz, H-6a), 3.65 (1-OCH$_3$), 3.85 (10-OCH$_3$), 3.90 (2-OCH$_3$), 6.87 (1h, d, *J* = 8.4 Hz, H-8), 6.90 (1H, s, H-3), 6.95 (1H, d, *J* = 8.4 Hz, H-9); ^{13}C NMR (100 MHz, CDCl$_3$): δ$_C$ 29.5 (C-4), 38.5 (C-7), 42.5 (C-5), 54.2 (C-6a), 56.0 (2-OCH$_3$), 56.2 (10-OCH$_3$), 62.5 (1-OCH$_3$), 111.0 (C-9), 111.5 (C-3), 119.0 (C-8), 120.2 (C-11a), 125.5 (C-1a), 129.8 (C-1b), 130.5 (C-7a), 130.8 (C-3a), 142.0 (C-1), 144.5 (C-11), 149.5 (C-10), 151.4 (C-2); MS *m/z* 328 [M$^+$, C$_{19}$H$_{22}$NO$_4$] [22].

(+)-Laurotetanine (3). Brownish amorphous solid; m.p. 124-125 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ$_H$ 2.71 (2H, m, H-7), 3.02 (2H, m, H-4), 3.38 (2H, m, H-5), 3.65 (3H, m, 1-OCH$_3$), 3.82 (1H, m, H-6a), 3.88 (3H, m, 2-OCH$_3$), 3.87 (3H, m, 10-OCH$_3$), 6.59 (1H, s, H-3), 6.82 (1H, s, H-8), 8.05 (1H, s, H-11); ^{13}C NMR (100 MHz, CDCl$_3$): δ$_C$ 29.0 (C-4), 36.5 (C-7), 43.0 (C-5), 53.5 (C-6a), 55.8 (10-OCH$_3$), 56.0 (2-OCH$_3$), 60.2 (1-OCH$_3$), 110.5 (C-3), 111.0 (C-11), 113.8 (C-8), 124.0 (C-11a), 126.5 (C-1a), 127.5 (C-1b), 128.5 (C-3a), 129.2 (C-9), 129.5 (C-7a), 144.2 (C-1), 145.5 (C-10), 152.0 (C-2); MS *m/z* 328 [M$^+$, C$_{19}$H$_{21}$NO$_4$] [11].

(+)-N-Methyl laurotetanine (4). Brownish amorphous solid; m.p. 125-128 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ$_H$ 2.48 (2H, d, *J* = 3.9 Hz, H-5), 2.52 (2H, d, *J* = 3.6 Hz, H-7), 2.55 (3H, s, *N*-CH$_3$), 2.65 (2H, dd, *J* = 16.0, 4.4 Hz, H-4), 2.98 (1H, m, H-6a), 3.65 (3H, m, 1-OCH$_3$), 3.87 (3H, m, 2-OCH$_3$), 3.90 (3H, m, 10-OCH$_3$), 6.58 (1H, s, H-3), 6.85 (1H, s, H-8), 8.08 (1H, s, H-11); [13]C

NMR (100 MHz, CDCl$_3$): δ_C 29.0 (C-4), 34.5 (C-7), 53.0 (C-5), 55.8 (10-OCH$_3$), 56.0 (2-OCH$_3$), 60.2 (1-OCH$_3$), 62.5 (C-6a), 110.2 (C-3), 111.0 (C-11), 113.5 (C-8), 124.0 (C-11a), 127.0 (C-1a), 127.2 (C-3a), 128.5 (C-1b), 130.2 (C-7a), 144.0 (C-1), 144.5 (C-9), 145.5 (C-10), 151.5 (C-2); MS *m/z* 342 [M$^+$, C$_{20}$H$_{22}$NO$_4$] [18].

(+)-Boldine (5). Light brown powder; m.p. 161-163 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ_H 2.55 (3H, s, *N*-CH$_3$), 2.40-2.75 (3H, m, H-7b, H-4), 2.92-3.10 (4H, m, H-6a, H-5, H-7a), 3.60 (3H, s, 2-OCH$_3$), 3.90 (3H, s, 10-OCH$_3$), 6.65 (1H, s, H-3), 6.80 (1 H, s, H-8), 7.92 (1H, s, H-11); ^{13}C NMR (100 MHz, CDCl$_3$): δ_C 28.5 (C-4), 35.0 (C-7), 43.2 (N-CH3), 53.0 (C-5), 56.0 (10- OCH$_3$), 60.5 (1-OCH$_3$), 62.5 (C-6a), 110.0 (C-11), 113.0 (C-3), 114.0 (C-8), 123.5 (C-11c), 126.0 (C-11b), 126.5 (C-11a), 130.0 (C-3a), 130.0 (C-7a), 142.0 (C-1), 145.0 (C-9), 145.2 (C-10), 148.0 (C-2); MS *m/z* 328 [M$^+$, C$_{19}$H$_{21}$NO$_4$] [11].

β-Sitosterol (6). White crystalline needles; m.p. 133-134 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ_H 0.70 (3H, s, H-18), 0.84 (3H, d, *J* = 6.6 Hz, H-27), 0.86 (3H, d, *J* = 6.6 Hz, H-26), 0.88 (3H, d, *J* = 3.9 Hz, H-29), 0.95 (3H, d, *J* = 6.3 Hz, H-21), 1.03 (3H, s, H-19), 1.27−2.31 (29H, m, overlapping CH and CH$_2$), 3.54 (1H, m, H-3), 5.37 (1H, d, J = 4.8 Hz, H-6); GC-MS *m/z* 414 [M$^+$, C$_{29}$H$_{50}$O] [15].

β-Sitostenone (7). White solids; m.p. 77-79 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ_H0.73 (3H, s, H-18), 0.82 (3H, d, *J* = 6.4 Hz, H-27), 0.84 (3H, d, *J* = 6.4 Hz, H-26), 0.86 (3H, t, *J* = 7.6 Hz, H-29), 0.93 (3H, d, *J* = 6.8 Hz, H-21), 1.18 (3H, s, H-19), 1.25−2.44 (29H, m, overlapping CH and CH$_2$), 5.74 (1H, s, H-4); GC-MS *m/z* 412 [M$^+$, C$_{29}$H$_{48}$O] [15].

Lupeol (8). White needles; m.p. 214-216 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ_H 0.71 (1H, d, J = 9.2 Hz, H-5),0.77 (3H, s, H-28); 0.80 (3H, s, H-25), 0.94 (3H, s, H-27), 0.96 (3H, s, H-23),0.98(3H, s, H-24), 1.00 (3H, s, H-26), 1.67 (3H, s, H-30), 1.95 (2H, m, H-21), 2.36 (1H,dt, J = 11.2 and 5.6 Hz, H-19), 3.19 (1H, dd, J = 11.2 and 5.4 Hz, H-3), 4.58 (1H, s,H-29), 4.70 (1H, s, H-29); GC-MS *m/z* 426 [M+, C$_{30}$H$_{50}$O] [15].

Lupenone (9). Colourless needle; m.p. 169-171 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ_H 0.81 (3H, s, H-28), 0.95 (3H, s, H-25), 0.97(3H, s, H-27), 1.04 (3H, s, H-24), 1.09 (3H, s, H-23), 1.22 (3H, s, H-26), 1.68 (3H, s,H-30), 1.89−1.94 (2H, m, H-21), 2.40−2.51 (1H, m, H-19), 4.59 (1H, s, H-29a), 4.71 (1H, s, H-29b); GC-MS *m/z* 424 [M$^+$, C$_{30}$H$_{48}$O] [15].

Some of the most relevant therapies for the treatment of the disease are based on the acetylcholinesterase (AChE) inhibitor activity. AChE contains three key motifs: an active site, a peripheral anionic site and a long narrow hydrophobic gorge connecting the active and peripheral anionic site. The gorge is lined with hydrophobic residues to facilitate the passage of different molecules and wherein appears to take place hydrolysis of acetylcholine [23].

In recent years, alkaloids have received great attention due to their well-known anticholinergic activity, which generally has in common the presence of nitrogen atoms in a cyclic ring. This fact has motivated the screening of isolated alkaloids as possible AChE inhibitors. The galanthamine used as a standard in this study, was the first alkaloid isolated from different species of Amaryllidaceae being the most recently AChE inhibitor approved in Europe and the United States for the symptomatic treatment of AD [23]. In the current study, the isolated alkaloids of *B. insignis* were subjected to the acetylcholinesterase inhibitory activity and the results are shown in Table 1.

The aporphine type alkaloid is an important class of natural AChE inhibitors and there are several sub-type aporphine alkaloids that have been obtained as AChE inhibitors [24]. However, of the five aporphine alkaloids from *B. insignis*, compound (**5**) showed the highest AChE inhibitory activity. By comparing the structure-activity relationship of compounds **1-5**, we found

that the hydroxyl at C-2 position may be important to activity. The relationship was also confirmed by another two aporphine alkaloids reported before [25], which had similar structures to compound **5** but no hydroxyl at C-2 position and showed weak activity against AChE.

Table 1. Acetylcholinesterase inhibitory activity of isolated alkaloids from *B. insignis*[a].

Samples	AChE inhibition (I%)
Isocorydine (**1**)	50.2±0.25
Norisocorydine (**2**)	52.8±0.22
Laurotetanine (**3**)	44.9±0.30
N-Methyl laurotetanine (**4**)	46.5±0.15
Boldine (**5**)	74.5±0.20
Galantamine[b]	85.5±0.21

[a]Data represent mean ± standard deviation of three replicate experiments; ($p < 0.05$); [b]positive control.

CONCLUSION

In the present study, the phytochemical investigation from the stem bark of *B. insignis* furnished five alkaloids. This study is the first report of the occurrence of alkaloids from this species. Thus, high variants of alkaloids compounds from this species may be used as chemotaxonomic markers for this *Beilschmiedia* species. In addition, the AChE activity also provides a preliminary indication that isolated alkaloids may have a big potential for neuroprotective activities against both hypoxic injury and oxidative damage inneuronal cells. To validate the above-mentioned activity, clinical trials should be carried out to ensure the safe use of the compounds as therapeutic agents.

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