Anticholinesterase and Anti-inflammatory Constituents from Beilschmiedia pulverulenta Kosterm

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Abstract – Phytochemical investigation from the stem bark of Beilschmiedia pulverulenta resulted in the isolation of five lignans, (+)-yangambin (1), (+)-sesartemin (2), (+)-excelsin (3), (+)-sesamin (4), and (+)-syringaresinol (5), together with lupeol (6), lupenone (7), β-sitosterol (8), and β-sitostenone (9). Their structures were established by the analysis of their spectroscopic (1D and 2D NMR) and spectrometric (MS) data, as well as by comparison with those reported in the literature. The isolated lignans were tested for their anticholinesterase (AChE: acetylcholine esterase and BChE: butyryl cholineesterase) and anti-inflammatory (COX-2: cyclooxygenase-2 and LOX: lipoxygenase) activities. All the isolated lignans (1 – 5) exhibited significant inhibition activities in AChE/BChE and COX-2/LOX assays with IC50 values ranging from 168.8 – 504.2 µM and 21.0 – 59.4 µM, respectively.

Keywords – Anticholinesterase, Anti-inflammatory, Lignan, Beilschmiedia pulverulenta

Introduction

Alzheimer disease (AD) is an irreversible neurodegenerative disorder which effects memory loss, learning disabilities, cognitive impairments, and diverse range of neuropsychiatric symptoms.1 Acetylcholinesterase (AChE) has proven to be the most viable therapeutic target for symptomatic improvement in AD. Besides AChE which is found primarily in the blood and neural synapses is butyrylcholinesterase (BChE), an enzyme found in the liver. Many of drugs available for treatment of AD target both AChE and BChE but some are more selective than others.2 The role of anti-inflammatory agents in the prevention or treatment of AD has been the focus of current research. According to Borovikova et al.,3 there is an established link between the cholinergic system and inflammation, with acetylcholine playing a role in cytokine release. In addition, Tabet4 reported on evidence that acetylcholinesterase inhibitors have an anti-inflammatory role by indirectly increasing the production of antioxidants in the brain, thereby acting against free radicals, amyloid toxicity and a reduction in release of cytokines from activated microglia in the brain and blood.

The genus Beilschmiedia comprises nearly 250 species and is abundant in Africa and Asia. Previous phytochemical investigations have shown that several classes of natural products including, endiandric acid derivatives, alkaloids, flavonoids, terpenoids, lignans, neolignans and essential oils.5,6,7 A few of these compounds are reported to exhibit antibacterial, antimalarial and anti-tuberculosis activities.8,9 Beilschmiedia pulverulenta is locally known as ‘medang merah’ in Malaysia and distributed in the Peninsular Malaysia, Borneo and Indonesia. It grows on sandy loam soils in mixed dipterocarp forest.10 The chemical compositions and biological activities of the essential oil from B. pulverulenta have been reported by us.11 Herein, we would like to report the chemical constituents from the stem bark of B. pulverulenta together with their anticholinesterase and anti-inflammatory activities. To the best of our knowledge, there is no report on the constituents and bioactivity studies from this species.

Experimental

General experimental procedures – The optical rotations were recorded on a Perkin Elmer 341 Polarimeter

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equipped with a sodium lamp and chloroform as solvent. The mass spectra were recorded on Bruker Mass Spectrometry Services, obtained from National University of Singapore (NUS). The UV spectra were obtained in methanol on a Shimadzu UV 1601PC spectrophotometer. The IR spectra were obtained on a Perkin-Elmer 1600 FTIR spectrophotometer. The 1H-NMR and 13C-NMR spectra were recorded in deuterated chloroform on a Bruker Avance 400 MHz spectrometer, chemical shifts were reported in ppm on δ scale, and the coupling constants were given in Hz. Column chromatography was carried out on silica gel 70 - 230 mesh (Merck). Silica gel 60 F254 precoated aluminium plates (0.2 mm, Merck) were used for TLC analysis. The TLC and PTLC spots were visualized under UV light (254 and 366 nm) followed by spraying with 5% H2SO4 in methanol and 1% vanillin in MeOH, followed by heating at 120 °C for 5 min. All solvents were AR grade.

**Plant materials** – A sample of *B. pulverulenta* was collected from mixed dipterocarp forests located in Kuching and Samarahan Districts of Sarawak in January 2010 and identified by Mohizar Mohamad. The voucher specimens (UiTMKS 4014) were deposited at the Natural Product Research and Development Centre (NPRDC), Universiti Teknologi MARA Sarawak.

**Extraction and isolation** – Cold extraction of the powdered stem bark (300 g) with n-hexane, EtOAc and MeOH yielded the crude stem bark of *n*-hexane (BPBH: 5.02 g), EtOAc (BPBE: 8.78 g) and MeOH (BPBM: 5.02 g), EtOAc (BPBE: 8.78 g) and MeOH (BPBM: 5.02 g) respectively. The EtOAc extract was fractionated by VLC hexane:CHCl3 (10.5 mg: white solid) and eluted with n-hexane:EtOAc:MeOH to give six fractions (BPBE1-6). Fractions BPBE5-6 were subjected to column chromatography (CC) over silica gel (800 g, 12×100 cm) eluted with n-hexane:CHCl3 (90:10, 80:20, 75:25, 70:30, 60:40, 50:50) to afford 150 fractions. Fraction 50 - 80 were combined and followed by preparative TLC (n-hexane:EtOAc, 1:1) to yield (3) (10.5 mg: white solid).

(+) - Yangambin (1) – White solid. [α]D25 +85.5° (c 0.02, CHCl3); MP: 125 - 128 °C; IR (KBr) νmax: 3076, 2952, 1590, 1511, 1237 cm−1; UV (MeOH) λmax nm: 257 and 271; 1H NMR (400 MHz, CDCl3): δ 3.12 (2H, m, H-8/H-8'), 3.86 (6H, s, 2×OCH3), 3.96 (2H, dd, J = 9.2, 3.6 Hz, H-β/H-9β'), 4.34 (2H, dd, J = 9.2, 3.6 Hz, H-9α/H-9α'), 4.76 (2H, J = 4.4 Hz, H-7/H-7').

(+)-Sesartemin (2) – White solid. [α]D25 +42.6° (c 0.02, CHCl3); MP: 110 - 112 °C; IR (KBr) νmax: 3071, 2952, 1590, 1511, 1237 cm−1; UV (MeOH) λmax nm: 257 and 271; 1H NMR (400 MHz, CDCl3): δ 3.09 (2H, m, H-8/H-8'), 3.85 (6H, s, 2×OCH3), 3.93 (3H, s, 5-OCH3), 3.93 (4H, m, H-9α/H-9α'), 4.74 (2H, J = 2.8 Hz, H-7/H-7'), 5.98 (1H, s, OCH3), 6.54 (1H, s, H-2/H-6/H-2'/H-6'); 13C NMR (100 MHz, CDCl3): δ 54.3 (C-3/C-3'), 56.2 (4×OCH3), 60.8 (2×OCH3), 71.9 (C-9/C-9'), 85.9 (C-7/C-7'), 102.9 (C-2/C-2'/C-6/C-6'), 136.7 (C-1/C-1'), 137.6 (C-4/C-4'), 153.4 (C-3/C-3'/C-5/C-5'); EIMS: m/z 446 [M]+, C21H20O10.12

(+) - Excelsin (3) – White solid. [α]D25 +124.9° (c 0.02, CHCl3); MP: 109 - 112 °C; IR (KBr) νmax: 3071, 2953, 1587, 1510, 1237 cm−1; UV (MeOH) λmax nm: 259 and 275; 1H NMR (400 MHz, CDCl3): δ 3.06 (2H, m, H-8/H-8'), 3.88 (2H, m, H-9β/H-9β'), 3.93 (6H, s, 2×OCH3), 4.27 over silica gel (300 g, 5×60 cm) eluted with n-hexane:EtOAc to afford 100 fractions. Fraction 55 - 65 and 70 - 90 recrystallized from cold n-hexane to yield (4) (10.5 mg: colourless needle) and (5) (15.9 mg: colourless rhombohedral crystal), respectively. Fractions BPBE1-2 were subjected to CC over silica gel (500 g, 5×60 cm) eluted with n-hexane:CHCl3 (90:10, 80:20, 75:25, 70:30, 60:40, 50:50) to afford 100 fractions. Fraction 20-35 was recrystallized from cold n-hexane and yielded (8) (18.2 mg: white crystalline needles) and (9) (21.4 mg: white solid). The MeOH extract was fractionated by VLC and eluted with n-hexane:EtOAc:MeOH to give five fractions (BPBE1-5). Fractions BPBE3-5 was subjected to CC over silica gel (200 g, 5×60 cm) eluted with n-hexane:CHCl3 (90:10, 80:20, 75:25, 70:30, 60:40, 50:50) to afford 100 fractions. Fraction 50 - 80 were combined and followed by preparative TLC (n-hexane:EtOAc, 1:1) to yield (8) (18.2 mg: white solid).
Electric eel AChE and horseradish peroxidase were employed for the measurement of the cholinesterase activity. Briefly, 140 μL of sodium phosphate buffer (pH 8.0), 20 μL of DTNB, 20 μL of the test samples and 20 μL of AChE/BChE solution were added by multichannel automatic pipette to a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated by the addition of 10 μL of acetylthiocholine iodide/butyrylthiocholine chloride. Hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocyanates, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer, USA). Percentage of inhibition (I%) of AChE/BChE was determined by comparison of the rate of reaction of each sample relative to a blank sample (ethanol in phosphate buffer pH = 8) using the following formula:

\[ I\% = \left(1 - \frac{E}{S}\right) \times 100 \]

where E is the activity of enzyme without the test sample and S is the activity of enzyme with test sample. Galantamine was used as the standard. The experiments are reported as mean ± SD of triplicates.

**Anti-inflammatory activity**

**Cyclooxygenase-2 (COX-2) assay** – The anti-inflammatory activity of the compounds on COX-2 was determined by measuring prostaglandin E2 (PGE2) using a COX Inhibitor Screening Kit (Catalog No 560131, Cayman Chemicals, Ann Arbor Michigan USA) as well as the reported method.16 Reaction mixtures containing the reaction buffer (950 μL), heme (10 μL), COX-2 (10 μL) and sample/control (20 μL) were incubated at 37 °C in a water bath for 15 min. The reaction was initiated by addition of arachidonic acid (10 μL) at a final concentration of 100 μM. After 2 min incubation, the reactions were stopped by addition of 1 M HCl (50 μL), followed by saturated solution of stannous chloride (100 μL). Then, prostaglandins (PG) were quantified by means of the ELISA method. The contents of the reaction tubes were diluted and transferred to a 96-well plate coated with a mouse antirabbit IgG, followed by addition of the PG screening acetylcholinesterase tracer and the PG screening antiserum. Plates were incubated in an orbital shaker for 18 h, at room temperature. The reaction mixtures were removed, and the wells were washed five times with a buffer containing 0.05% Tween 20. Acetyltiocholine and 5,5’-dithio-bis-2-nitrobenzoic acid known as Ellman’s reagent (200 μL) was then added to each well, and the plate was incubated in an orbital shaker for 60 min, at room temperature, until the control wells yielded an anticholinesterase activity. The absorbance was measured at 412 nm using a spectrophotometer.
optical density lying between 0.3 - 0.8 at 415 nm. A standard curve with PG was generated from the same plate, which was used to quantify the PG levels produced in the presence of the samples. The compound DuP697 (Cayman Chemicals) was used to standardize the assay for COX-2 and indomethacin was employed as positive control. Results were expressed as a percentage relative to a control (100% initial activity, solvent treated samples). All determinations were performed in triplicate. Regression analysis was employed for the calculation of IC_{50} values.

Lipoxygenase (LOX) assay – The reagents were prepared according to the standard protocol (Lipoxygenase inhibitor screening assay kit, Item No. 760700 Cayman Chemicals Co). Stock solutions of compounds were prepared so as to obtain concentrations of 100 - 6.25 µM in the respective wells. The prepared solutions were then introduced onto 96 well plates where the cells were distributed as blanks 1A-2A-1D (triplicate), positive control 1B-2B (duplicate), and 100% initial activity wells 1C-2C-2D (triplicate). The remaining wells were designated for inhibitor (tested sample) solutions in duplicate. The addition of the reagents was done according to the standard protocol, according to which, 100 µL of assay buffer was added to the blank wells and 90 µL of lipoxygenase (5-LOX) enzyme and 10 µL of assay buffer were added to positive control wells. For the 100% initial activity wells, 90 µL of lipoxygenase enzyme and 10 µL of solvent (DMSO) were added. The inhibitor (tested sample) wells were charged with 90 µL of lipoxygenase enzyme and 10 µL of respective stock (tested sample) solution. The reaction was initiated by adding 10 µL of the substrate (AA) to all wells. The plate was then shaken for 5 min on an orbital shaker. Ultimately, 100 µL of chromogen solution (prepared according to standard protocol) was added to each well to stop the enzyme catalysis. The plate was incubated for 30 min and was read at 500 nm. The percentage inhibitions (I%) of the tested sample were calculated using the following equation:

\[ I\% = \left( \frac{A_{\text{initial activity}} - A_{\text{inhibitor}}}{A_{\text{initial activity}}} \right) \times 100 \]

where \( A_{\text{initial activity}} \) is the absorbance of 100% initial activity wells without sample and \( A_{\text{inhibitor}} \) is the absorbance of sample/reference. Analyses were expressed as means ± SD of triplicates.

Statistical analysis – Data obtained from biological activity was expressed as mean values. The 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.

Result and Discussion

In continuation of our research on the medicinal plants from Malaysian flora, we have performed a phytochemical investigation on the stem bark of a Malaysian Lauraceae, \textit{B. pulverulenta}, which has led to the isolation of nine compounds (Fig. 1), characterised as five lignans two triterpenes, and two steroids. These metabolites were identified by analysing their spectroscopic data and comparing them with the literature data, as (+)-yangambin

Fig. 1. Chemical structures of isolated compounds (1 - 9) from \textit{B. pulverulenta}. 

\[ \text{(1)} \ \ R_1 = R_2 = \text{OCH}_3 \\
\text{(2)} \ \ R_1 = \text{OCH}_3 \\
\text{(3)} \ \ R_1 = R_2 = \text{OCH}_3 \\
\text{(4)} \ \ R_1 = R_2 = \text{H} \\
\text{(5)} \ \ R_1 = R_2 = \text{H} \\
\text{(6)} \ \ R_1 = \text{OH} \\
\text{(7)} \ \ R_1 = \text{OH} \\
\text{(8)} \ \ R_1 = \text{OH} \\
\text{(9)} \ \ R_1 = \text{OH} \]
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References


