Natural Product Sciences
13(2) : 110-113 (2007)

Cytotoxic Constituents from *Boesenbergia pandurata* (Roxb.) Schltr.

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Abstract – Five flavonoid derivatives, pinostrobin (1), pinocembrin (2), alpinetin (3), cardamonin (4) and boesenbergin A (5) were isolated from the rhizomes of *Boesenbergia pandurata*. All compounds were elucidated based on its spectroscopic data and by the comparison with the previous works. 2D NMR technique was used for the structure elucidation of boesenbergin A to complement the data reported previously. The extracts and pure compounds were screened for cytotoxic activity against HL-60 cancer cell lines (human promyelocytic leukemia). Cytotoxic screening showed most of the extracts and pure compounds isolated from the rhizomes of *Boesenbergia pandurata* were active against HL-60 cancer cell line. The chloroform extract and boesenbergin A showed the most potent cytotoxic activity.
Keywords – *Boesenbergia rotunda*, *Kaempferia pandurata*, Zingiberaceae, Flavonoids, Cytotoxic

Introduction

Zingiberaceae is one of the largest plant family with approximately 50 genera and over 1,000 species widely distributed throughout the tropics. It is estimated that there are 150 species of ginger belonging to 23 genera found in Peninsular Malaysia (Holttum, 1950). Various species of Zingiberaceae are used as spices, medicines, flavouring agents and as the source of certain dyes (Burkill, 1966). Several studies have revealed that the members of the Zingiberaceae family consist of a wide variety of active phytochemicals and possess antioxidative, anti-inflammatory, anticancer and anti-tumour promoter activity (Ling *et al.*, 2005). *Boesenbergia pandurata* (Roxb.) Schltr. is a perennial herb belonging to the Zingiberaceae family. The plant is locally known as 'temu kunci' and is the most abundant *Boesenbergia* species in Malaysia. The fresh rhizomes have a characteristic aroma and a slightly pungent taste. It is commonly used in Southeast Asia as food ingredient and in folk medicine treatment of several diseases (Burkill, 1966).

The plant shows various biological activities including antimutagenic, antitumour, antibacterial, antifungal, analgesic, antipyretic, antispasmodic, anti-inflammatory and insecticidal activities (Cheenpracha *et al.*, 2005). The rhizomes of this species contain active constituents against HIV-1 protease (Tewtrakul *et al.*, 2003). Previous investigations have revealed the isolation of some flavonoid derivatives and chalcone from the rhizomes of *Boesenbergia pandurata* (Jaipetch *et al.*, 1982, Mongkolsuk *et al.*, 1964). In this paper, we wish to report the isolation and characterization of flavonoid derivatives from *Boesenbergia pandurata*, including detailed NMR study on boesenbergin A. The cytotoxic activity of the plant extracts and the pure compounds against HL-60 cancer cell was also investigated.

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Experimental

General – Melting points (uncorrected) were determined on Kohfler melting points apparatus. Infrared spectra were recorded on Perkin Elmer FTIR model 1725X spectrophotometer; using potassium bromide (KBr) disc. MS was measured on equipped Shimadzu model QP5050A at 70 eV. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a JEOL FTNMR 400 and 100 MHz transformed spectrometer, respectively with tetramethylsilane (TMS) as internal standard. Column chromatography was carried out using silica gel (Merck 7749) and Merck silica gel 60 PF_{254} was used for TLC analysis.

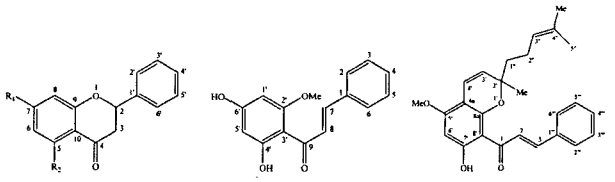
Plant Material – The rhizomes of *Boesenbergia pandurata* were collected from Puchong, Selangor, Malaysia. Voucher specimens are deposited in the Chemistry Department, University Putra Malaysia.

Extraction and Isolation – Finely ground air-dried rhizomes of *Boesenbergia pandurata* (1.0 kg) was extracted three times with hexane and chloroform sequentially for seventy two hours for each solvent at room temperature. The solvents were removed under reduced pressure to give 36.2 g orange coloured semi-solid of hexane extract and 42.6 g brown coloured semi-solid of chloroform extract. Around 34 g of each hexane and chloroform extract was purified by silica gel column chromatography using various ratios of hexane, hexane/ethyl acetate, ethyl acetate, ethyl acetate/methanol and methanol to give 60 fractions for hexane extract and 70 fractions for chloroform extract. Purification using silica gel column chromatography and recrystallization afforded five pure compounds. The hexane extract gave pinostrobin **(1)** (2.60 g) and boesenbergin A **(5)** (27 mg), while chloroform extract afforded pinocembrin **(2)** (0.17 g), alpinetin **(3)** (39 mg) and cardamonin **(4)** (8 mg).

Pinostrobin (1) colorless crystals, $C_{16}H_{14}O_4$, m.p 96 - 98 °C (Jaipetch *et al.*, 1982, m.p. 100 - 101 °C). IR ν_{max} (cm^{-1}, KBr disc): 3444 (OH), 1646 (C = O), 1382, 1340, 1302, 1210, 1158, 768, 742. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 12.00 (1H, s, 5-OH), 7.41 (5H, m, H-2', H-3', H-5', H-6'), 6.05 (2H, d, J = 2.72 Hz, H-6, H-8), 5.39 (1H, dd, J = 12.84, 2.76 Hz, H-2), 3.79 (3H, s, 7-OMe), 3.06 (1H, dd, J = 15.14, 12.84 Hz, H-3), 2.79 (1H, dd, J = 14.68, 2.76 Hz, H-3). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 195.7, 167.8, 164.0, 162.7, 138.3, 128.8, 126.0, 103.0, 95.0, 94.1, 79.0, 55.6, 43.2. MS (*m/z*, rel. int.): *m/z* 270 (M^+, 100), 269 (67), 252 (8), 193 (99), 166 (68), 138 (41), 114 (14), 110 (18), 95 (34), 77 (20), 69 (19), 51 (19).

Pinocembrin (2) yellow needle-shaped crystals, $C_{15}H_{12}O_4$, m.p 191 - 193 °C (Jaipetch *et al.*, 1982, m.p. 200 - 201 °C). IR ν_{max} (cm^{-1}, KBr disc): 3436 (OH), 3092, 1632 (C = O), 1584, 1488, 1358, 1168. ^{1}H-NMR (400 MHz, MeOD): δ 12.00 (1H, s, 5-OH, 7-OH), 7.34 (5H, m, H-3', H-4', H-5', H-6'), 5.87 (2H, d, J = 1.84 Hz, H-6, H-8), 5.33 (1H, dd, J = 12.84, 3.68 Hz, H-2), 2.98 (1H, dd, J = 17.40, 12.88 Hz, H-3), 2.67 (1H, dd, J = 16.96, 3.68 Hz, H-3). ^{13}C-NMR (100 MHz, MeOD): δ 197.3, 168.3, 165.4, 164.6, 140.3, 129.7, 129.6, 127.3, 103.3, 97.2, 96.2, 80.4, 44.1. MS (*m/z*, rel. int.): *m/z* 256 (M^+, 100), 238 (14), 179 (92), 152 (77), 124 (52), 103 (18), 96 (20), 78 (29), 69 (31), 51 (16).

Alpinetin (3) colorless needle-shaped crystals, $C_{16}H_{14}O_4$, m.p 222-224 °C (Mongkulsok *et al.*, 1964, m.p. 223-227 °C). IR ν_{max} (cm^{-1}, KBr disc): 3520 (OH), 3362, 3090, 1612 (C = O), 1578, 1436, 1362, 1108, 1072, 702. ^{1}H-NMR (400 MHz, MeOD): δ 7.32 (5H, m, H-2', H-3', H-4', H-5', H-6'), 6.01 (1H, *d*, J = 1.84 Hz), 5.96 (1H, d, J = 2.76 Hz, H-6), 5.33 (1H, dd, J = 10.08, 2.80 Hz, H-2), 3.74 (3H, s, 5-OMe), 2.90 (1H, dd, J = 12.84, 4.60 Hz, H-3), 2.63 (1H, dd, J = 12.84, 3.68 Hz, H-3). ^{13}C-NMR (100 MHz, MeOD): δ 191.8, 167.2, 166.6, 164.3, 140.6, 129.7, 129.5, 127.3, 105.7, 97.2, 94.3, 80.2, 56.2, 46.4. MS (*m/z*, rel. int.): *m/z* 270 (M^+, 51), 269 (14), 193 (22), 166 (100), 138 (51), 104 (15), 95 (16).



(1) R_1 = OMe, R_2 = OH
(2) R_1 = OH, R_2 = OH
(3) R_1 = OH, R_2 = OMe

(4) (5)

Cardamonin (4) yellow needle-shaped crystals, $C_{16}H_{14}O_4$, m.p 186 - 188 °C (Jaipetch *et al.*, 1982, m.p. 199 - 200 °C). IR ν_{max} (cm^{-1}, KBr disc): 3154 (OH), 1628 (C = O), 1542, 1486, 1286, 1320, 1224, 1188, 1114, 926. ^{1}H-NMR (400 MHz, MeOD): δ 7.84 (1H, d, J = 15.60 Hz, H-7), 7.59 (1H, d, J = 15.60 Hz, H-8), 7.43 (5H, m, H-2, H-3, H-4, H-5, H-6), 5.93 (1H, d, J = 2.76 Hz, H-1'), 5.85 (1H, d, J = 2.72 Hz, H-5'), 4.54 (1H, s, 4'-OH, 6'-OH), 3.84 (3H, s, 2'-OMe). ^{13}C-NMR (100 MHz, MeOD): δ 193.9, 168.8, 167.0, 164.8, 142.9, 137.0, 131.2, 130.1, 129.3, 128.9, 106.6, 97.1, 92.6, 56.3. MS (*m/z*, rel. int.): *m/z* 270 (M^+, 52), 269 (57), 253 (9), 193 (100), 131 (7), 103 (22), 77 (31).

Boesenbergin A (5) orange needle-shaped crystals, $C_{26}H_{28}O_4$, m.p 87 - 89 °C (Jaipetch *et al.*, 1982, m.p. 89 - 91 °C). IR ν_{max} (cm^{-1}, KBr disc): 3442 (OH), 2962, 1636 (C = O), 1590, 1546, 1446, 1344, 1152 (C-O). MS (*m/z*, rel. int.): *m/z* 404 (M^+, 20), 389 (4), 321 (100), 217 (99), 77 (10), 55 (6).

Biological Assay – The extracts and pure compounds isolated from the rhizomes of *Boesenbergia pandurata* were screened for cytotoxic activity against HL-60 cancer cell lines (human promyelocytic leukemia). The assay was carried out according to the methods described previously (Mackeen, 1997). The medium was used to dilute the cells to a concentration of 5×10^3 cells/mL. From this cell suspension, 100 ml of various concentrations of the extracts was pipetted into a 96-well micro titer plate and incubated in 37 °C, 5% CO_2 for 72 hours. The various concentration be used is 30, 15, 7.5, 3.75, 1.875, 0.9375 and 0.46875 µg/mL. The assays of each concentration of extracts were performed in triplicates and the control wells of untreated population were also included. After 3 days, the fraction of surviving cells were determined

relative to the untreated cell population by the colorimeter MTT (3-[4,5-dimethylthiozol 1-2yl]-2,5-diphenyltetrazolium bromide) method where the viability of cells was measured by 20 μL of blue formazan crystals of MTT solution (5 mg/mL in PBS, freshly prepared before assay) was added to each well at 37 °C for 4 hours incubation. 100 μl of the medium was removed from each well. The plate was left at room temperature for 30 minutes before reading the absorbance. The absorbance was read with the ELISA reader test wavelength of 570 nm and references wavelength of 630 nm. The IC_{50} value was defined as the concentration of the test compound resulting in a 50% reduction of absorbance (Mackeen, 1997).

Results and Discussion

Extraction and separation on the hexane and chloroform extracts of *Boesenbergia pandurata* have led to the isolation and characterization of five flavonoid derivatives. Pinostrobin **(1)** and boesenbergin A **(5)** were isolated from hexane extract, while chloroform extract afforded pinocembrin **(2)**, alpinetin **(3)** and cardamonin **(4)**. The compounds were identified by comparison of their spectral data with those reported in the literature. As for boesenbergin A, the high field NMR study including HMQC, HMBC and COSY correlation techniques was undertaken to complement the published NMR data, with respect to the assignment of the peaks.

Boesenbergin A **(5)** was obtained as orange needle-shaped crystals. The EI-MS of compound **5** indicated the presence of molecular ion peak at *m/z* 404 corresponding to molecular formula $C_{26}H_{28}O_4$, with the base peak of at *m/z* 321. The IR spectrum displayed a strong absorption band at 1636 cm^{-1} which was characterized for a conjugated ketone with intramolecular hydrogen bonding and a C-O stretching band at 1152 cm^{-1}. The IR spectra suggested compound 5 to be a chalcone derivative. In the ^{1}H-NMR spectrum of compound 5, a sharp singlet at δ 14.23 was observed due to a strong hydrogen-bonded hydroxyl group. An AB quartet (J = 15.60 Hz) at δ 7.78 and δ 8.18 were assigned to the (*E*)-vinylic protons of a chalcone moiety which are assigned to H-2 and H-3. Two multiplets at δ 7.37 and δ 7.60 indicate the protons on a monosubstituted benzene ring (H-2‴, H-3‴, H-4‴, H-5‴, H-6‴). The peak at δ 5.42 and δ 6.62 (J = 10.12 Hz) were due to the presence of a chromene ring (H-3', H-4') with an AB pattern. A singlet at δ 6.05 was assigned to H-6' of the second aromatic ring. A triplet at δ 5.08 with J = 6.40 Hz indicated the presence of an olefinic proton. These signals suggested the presence of an isoprene unit which is not linked to an aromatic ring, as indicated by the high-field resonance of the methylene protons. The peak of a singlet at δ 3.86 indicates the presence of methoxyl protons (5'-OMe). Two methylene (H-1", H-2") and three methyl groups (4"-Me, H-5", 2'-Me) were present at δ 1.72-2.23 as multiplets, and at δ 1.63 as singlet and at δ 1.46 as doublet (J = 7.32 Hz), respectively.

The ^{13}C-NMR and DEPT spectrum of compound **5** indicated the presence of 26 carbons as 12 aliphatic carbons (3Me, $2CH_2$, 1C, 1C = CH–, 2-CH = CH), 12 aromatic carbons (6CH, 4C, 2C-O), one carbonyl and one methoxyl carbon. From the previous report (Mahidol *et al.*, 1984), 4 signals in ^{13}C-NMR had been assigned incorrectly. The signals were indicated as C-2 at δ 123.4, C-3' at δ 117.2, C-4' at δ 130.0 and C-4‴ at δ 127.5. In this study, 2D NMR (HMQC, HMBC, DEPT and COSY) was carried out for compound **5** to complement the

Table 1. NMR spectral data for boesenbergin A **(5)**

position	δ_H	δ_C	HMBC correlations	COSY correlations
1	–	192.7	–	–
2	8.18, d	127.5	C-1, C-1"	H-3
3	7.78, d	142.2	C-1‴, C-1, C-2	H-2
1'	–	–	–	–
2'	–	80.6	–	–
2'-Me	1.63, s	26.6	C-2', C-3', C-1", C-4"	H-5"
3'	5.42, d	123.4	C-2', C-4a'	H-4'
4'	6.62, d	117.2	C-2', C-4a', C-7', C-8a'	H-3'
4a'	–	102.8	–	–
5'	–	167.4	–	–
5'-OMe	3.86, s	55.8	C-8a'	–
6'	6.05, s	92.5	C-4a', C-5', C-8', C-8a'	–
7'	–	155.9	–	–
7'-OH	14.23, s	–	C-5', C-6', C-8'. C-8a'	–
8'	–	106.1	–	–
8a'	–	161.2	–	–
1"	1.72-2.23, m	41.4	C-2', C-3', C-2", C-4"-Me	H-2"
2"	1.72-2.23, m	23.0	C-3", C-4"	H-1"
3"	5.08, t	123.6	C-2", C-4"-Me, C-5"	–
4"	–	132.1	–	–
4"-Me	1.46, d	25.6	–	–
5"	1.46, d	17.5	C-3", C-4", C-4"-Me	2'-Me
1‴	–	135.6	–	–
2‴	7.59, m	128.3	C-1, C-3, C-3‴, C-5‴	H-3‴, H-4‴, H-5‴
3‴	7.39, m	128.9	C-1‴, C-2‴, C-6‴	H-2‴, H-6‴
4‴	7.39, m	130.0	C-1‴, C-2‴, C-6‴	H-2‴, H-6‴
5‴	7.39, m	128.9	C-1‴, C-2‴, C-6‴	H-2‴, H-6‴
6‴	7.59, m	128.3	C-1, C-3, C-3‴, C-5‴	H-3‴, H-4‴, H-5‴

Table 2. IC_{50} values of extracts and pure compounds of *Boesenbergia pandurata* towards HL-60 cell line (human promyelocytic leukemia)

extracts/pure compounds	IC_{50} (μg/mL)
hexane	8.5
chloroform	5.8
methanol	> 30.0
boesenbergin A	5.8
alpinetin	11.0
cardamonin	23.2
pinostrobin	> 30.0
pinocembrin	> 30.0

< 10 = strong activity, 10 – 20 = moderate activity, 20 – 30 = low activity

previous report. The correlations observed in HMQC and HMBC spectrum confirmed the signals of C-2 was present at δ 127.5, C-3' present at δ 123.4, C-4' present at δ 117.2 and C-4''' at δ 130.0 . The correlations observed in COSY and HMBC spectra linked all the connectivities in the structure (Table 1).

As for the cytotoxic activity, all extracts and pure compounds isolated from the rhizomes of *Boesenbergia pandurata* were screened against HL-60 cancer cell line (Human promyelocytic leukemia). Most of the extracts and pure compounds of *Boesenbergia pandurata* were active against HL-60 cancer cell line (Table 2). The chloroform and hexane extracts showed strong activity with the IC_{50} values of 5.8 μg/ml and 8.5 μg/ml, respectively. For the pure compounds, boesenbergin A **(5)** showed the most potent cytotoxic activity with IC_{50} value of 5.8 μg/mL. The strong activity of the hexane extract most probably was due to the presence of high cytotoxic boesenbergin A **(5)**. However, the cytotoxic activity of pinocembrin **(2)**, alpinetin **(3)** and cardamonin **(4)** were found to be less than the activity of chloroform extract. It is suggested that the cytotoxic compounds have been in the chloroform extract, but its have not been isolated successfully. The cytotoxic activity of *Boesenbergia pandurata* against HL-60 cancer cell line has not been reported previously.

Acknowledgement

We thank The Ministry of Science Technology and Innovation Malaysia for the fund under Intensified Research in Priority Area (IRPA) Program.

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(Accepted April 30, 2007)