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Bioactive compounds from the bark of beilschmiedia palembanica (MIQ.) kosterm

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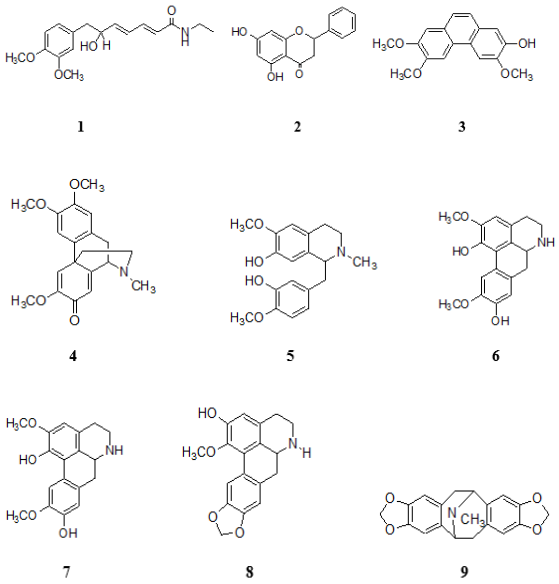
Abstract
A dienamide, named (-) kunstleramide (1), were isolated from the bark of *Beilschmiedia Palembanica (Miq.) Kosterm* together with one flavanone: pinocembrin (2), one phenanthrenol: perakensol (3) and 12 known alkaloids: sebiferine (4), reticuline (5), norisoboldine (6), norboldine (7), norisodomesticine (8), crychine (9), cassythicine (10), argentinine (11), isoboldine (12), o-methylnorflavinantine (13), nornantenine (14), and milonine (15). Their structures were established from spectroscopic techniques, most notably 1D- and 2D-NMR, UV, IR, OR, circular dichroism (CD) spectra and LCMS-IT-TOF. (-)-Kunstleramide (1) exhibited very poor dose-dependent inhibition of DPPH activity, with an IC_{50} value of 179.5 ± 4.4 µg/mL, but showed a moderate cytotoxic effect on MTT assays of A375, A549, HT-29, PC-3 and WRL-68 with EC_{50} values of 64.65, 44.74, 55.94, 73.87 and 70.95 µg/mL, respectively.

Keywords: beilschmiedia palembanica, lauraceae, antioxidant, alkaloid, dienamide, cytotoxicity

1. Introduction

In continuation of our research on the medicinal plants from Malaysian flora [1, 3], we have performed a phytochemical investigation on the bark of a Malaysian Lauraceae, *Beilschmiedia Palembanica (Miq.) Kosterm*, which has led to the isolation of a dienamide, named (-)-kunstleramide [1], were isolated from the bark of *Beilschmiedia Palembanica (Miq.) Kosterm* together with one flavanone: pinocembrin [2], one phenanthrenol: perakensol [3] and 12 known alkaloids: sebiferine [4], reticuline [5], norisoboldine [6], norboldine [7], norisodomesticine [8], crychine [9], cassythicine [10], argentinine [11], isoboldine [12], o-methylnorflavinantine [13], nornantenine [14], and milonine [15] were also isolated (Figure 1). This paper describes the structural elucidation, the DPPH activity with an IC_{50} value and cytotoxic effect of (−)-kunstleramide [1].



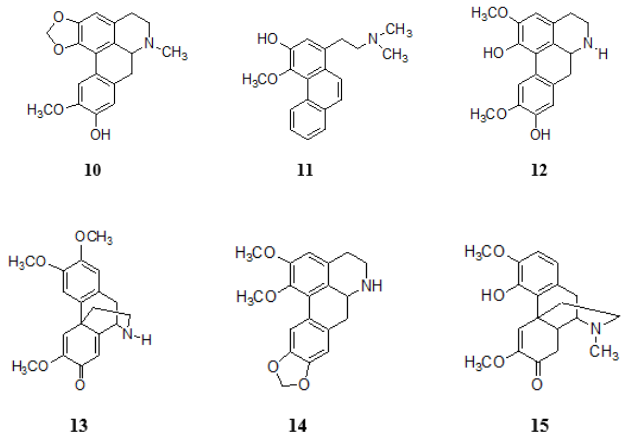


Fig 1: Chemical structures of compounds 1–15

2. Material and Method

2.1. General

The Fourier Transform Infrared (FT-IR) spectra were obtained with $CHCl_3$ (NaCl window technique) on a Perkin Elmer 2000 instrument. The ultraviolet spectra (UV) were obtained in MeOH on a Shimadzu UV-310 ultraviolet-visible spectrophotometer. The OR (optical rotation) was recorded on a JASCO (Japan) P1020 Polarimeter equipped with a tungsten lamp (MeOH as solvent) and CD (circular dichroism) data was recorded on a JASCO (Japan) J-815 spectrometer equipped with a tungsten lamp (MeOH as solvent). Mass spectra were obtained using LCMS-IT-TOF, Shimadzu spectrometer Series Mass Selective Detector, Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS, with ZORBAX Eclipse XDB-C18 Rapid Resolution HT 4.6 mm i.d. × 50 mm × 1.8 µm column. Solvent used was methanol (CH_3OH). The Auto mass Multi Thermofinnigan was used for HR ESI analysis and EIMS spectra were obtained on Shimadzu LCMS-IT-TOF Mass Spectrometer, QP2000A

spectrometer 70 eV. NMR spectra were recorded in deuterated chloroform ($CDCl_3$) and deuterated methanol CD_3OD) on JEOL LA400 FT-NMR and JEOL ECA 400 FT-NMR as a JEOL JNM-FX400 (400 MHz for ^1H and 100 MHz for ^{13}C; unless stated otherwise) and signal of spectra calibrated using TMS. Chemical shifts were reported in ppm on δ scale, and the coupling constants were measured in Hertz (Hz). Silica gel 60, 70–230 mesh ASTM (Merck 7734) was used for various column chromatography methods such as CLC and FLC.TLC aluminum sheets and PTLC (20 × 20 cm Silica gel 60 F_{254}) were used in the thin layer chromatography analysis. The TLC and PTLC spots were visualized under UV light (254 and 366 nm) followed by spraying with Dragendorff's reagent for detection of alkaloids. Dragendorff's reagent and Mayer's reagent were used for alkaloid screening. All solvents used were of AR grade except those used for bulk extraction.

2.2. Plant Materials

The bark of Beilschmiedia palembanica (Miq.) Kosterm (KL 5373) was collected from Hutan Simpan Sungai Temau, Kuala Lipis, Pahang by L.E. Teo & Din. at the Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia and at the Herbarium of the Forest Research Institute, Kepong, Malaysia.

2.3. Extraction and Isolation of Chemical Constituents

The air-dried bark (4.00kg) of *Beilschmiedia palembanica* (Miq.) Kosterm was extracted comprehensively and defatted with hexane (12.0L) for 72 hours then hexane extract was filtered. The residual plant material was dried and left to soak for 6.30h after moistening and rinsed with 10% NH_4OH. It was then macerated with CH_2Cl_2 (13.0L) for 7 days. After filtration, the supernatant was concentrated to 500 mL at room temperature (30°C) followed by acidic extraction with 5% HCl until a negative Mayer's test result was obtained. This procedure was repeated three times and the hydrochloric acid portion was kept and washed with CH_2Cl_2. The aqueous solution was made alkaline to pH=11 with NH_4OH and re-extracted with CH_2Cl_2. This was followed by washing with distilled H_2O, dried over anhydrous sodium sulphate, and evaporation to give an alkaloid fraction (3.07g). The extraction of alkaloids was repeated by using MeOH solvent and after acid base extraction obtained another (11.07g) of crude alkaloid. The CH_2Cl_2 extracts were dried with Na_2SO_4 and evaporated to dryness to give crude alkaloid. Finally the yields of the crude alkaloid extracts from this plant.

The crude alkaloid (3.07g of CH_2Cl_2) was submitted to exhaustive column chromatography over silica gel (column dimension =3 cm, length =100 cm, silica gel 60, 70-230 mesh ASTM; Merck 7734) using CH_2Cl_2 gradually enriched and increasing the polarity with methanol (1% until 90% MeOH) to give 5 fractions. Fractions 2, afforded five alkaloids as identified as (−)-kunstleramide [1] (2.88%), norboldine [7] (1.08%), norisodomesticine [8] (1.38%), argentinine [11] (1.63%), cassythicine [10] (1.79%) and nornantenine [14] (1.64%) (PTLC Merck KGaA silica gel 60 F254; CH_2Cl_2-MeOH; 90:10). Fraction3 also produced one phenanthrenol identified as perakensol [3] (1.49%), and five alkaloids as recognized as sebiferine [4] (0.54%), reticuline [5] (0.91%), crychine [9] (1.28%), *o*-methylnorflavinantine [13] (0.82%) and isoboldine [12] (2.38%) (PTLC Merck KGaA silica gel 60 F254; CH_2Cl_2-MeOH; 97:3). Fraction 4 afforded one flavanone as pinocembrin [2] (0.80%) (PTLC Merck KGaA silica gel 60 F_{254}; CH_2Cl_2-MeOH; 95:5). Fraction 5 produced two alkaloids as norisoboldine [6] (0.66%) and milonine [15] (2.15%) using PTLC (Merck KGaA silica gel 60 F_{254}; CH_2Cl_2-MeOH; 85:15).

2.4. Antioxidant Assay

The DPPH assay was performed according to the method reported by Orhan *et al*. [4] and Brem *et al*. [5], with modifications. Briefly, 0.02% stable DPPH free radical (50 µL) in methanol (100 mL) was added to standard/sample/control (20 µL) and methanol (130 µL, total assay volume 200 µL) in a 96-well plate. Ascorbic Acid (vitamin C) was used as the standard and blank solvent methanol as the negative control. The absorbance was read at 517 nm using SUNRISE Microplate Absorbance Reader after 30 min of incubation at room temperature. The percentage of DPPH free radical inhibition activity was determined according to the formula:

$$\text{Percentage of DPPH inhibition (\%)} = \frac{[\text{A (Blank)} - \text{A (Standard/Sample)}]}{\text{A (Blank)}} \times 100\%$$

Where a (Blank) refers to the absorbance of the blank solvent and DPPH at 517 nm while A (Standard/Sample) refers to the absorbance of Ascorbic Acid and the samples at 517 nm. This formula was also used to determine the concentration of each sample required to quench 50% of the DPPH free radical activity (IC_{50} value) [6].

2.5. Statistical Analyses

Each experiment was performed at least twice. Results are expressed as the means value ± standard deviation (SD). Log IC_{50} calculations were performed using the built-in algorithms for dose-response curves with variable slope using GraphPad Prism software (version 4.0; Graph Pad Software Inc., San Diego, CA, USA). A fixed maximum value of the dose-response curve was set to the maximum obtained value for each drug.

2.6. Cytotoxic Activity Studies

2.6.1. Cell Culture

All the cells used in this study were obtained from American Type Cell Collection (ATCC) and maintained in a 37 °C incubator with 5% CO_2 saturation. A375 human melanoma, HT-29 human colon adenocarcinoma cells and WRL-68 normal hepatic cells were maintained in Dulbecco's modified Eagle's medium (DMEM), whereas A549 non-small cell lung cancer cells and PC-3 prostate adenocarcinoma cells were maintained in RPMI medium. Both medium were supplemented with 10% fetus calf serum (FCS), 100 units/mL penicillin, and 0.1 mg/mL streptomycin.

2.6.2. Cellular Viability

Different cell types mentioned above were used to evaluate the inhibitory effect of (-) kunstleramide [1] on cell growth using the MTT assay. The MTT assay was modified as described by Cheah *et al*. and Mosmann [7, 8]. Briefly, cells were seeded at a density of 1×10^5 cells/mL in a 96-well plate and incubated for 24 h at 37 °C, 5% CO_2. The next day, cells were treated with the compounds respectively and incubated for another 24 h. After 24

h, MTT solution at 2 mg/mL was added and incubatefor 1 h. Absorbance at 570 nm was measured and recorded using a Plate Chameleon V microplate reader (Hidex, Turku, Finland). Results were expressed as a percentage of control giving percentage cell viability after 24 h exposure to test agent. The potency of cell growth inhibition for each test agent was expressed as an EC_{50} value, defined as the concentration that caused a 50% loss of cell growth. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells [9].

3. Results and Discussion

3.1. Antioxidant Activity

The antioxidant activity of (−)-kunstleramide (1) was tested using a DPPH assay. Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals [10]. Free radicals from oxidative stress are involved in many disorders like neurodegenerative diseases and cancer [11]. The new dienamide, (−)-kunstleramide (1) exhibited very poor DPPH activity, with an IC_{50} value of 179.5 ± 4.4 µg/mL compared to ascorbic acid (Figure 2). Factors such as growth conditions, stability of the specific antioxidant components, including variations in the process of extraction can influence the variations in the antioxidant activity [12].

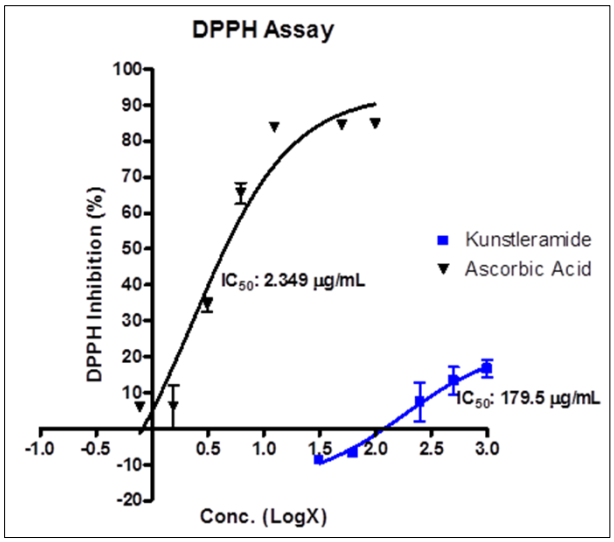


Fig 2: IC_{50} values of (−)-kunstleramide (**1**). AA: ascorbic acid as control. Results are means ± SD of two replicates

3.2. Cytotoxic Activity

To evaluate the cytotoxic activity, the new compound (−)-kunstleramide (1) was tested with a series of different doses on A549, PC-3, A375, HT-29 and WRL-68, respectively (Figure 3). After 24 h, cell viability was determined by the MTT assay. Test agents induced cell cytotoxicity in a concentration dependent manner. These dose titration curves allowed determining EC_{50} for the test agents towards different cell lines (Table 1).

From Figure 3, (−)-kunstleramide (1) showed cytotoxic effect on Several of the cancer cell lines with different EC_{50} values as compared to the standard, Doxorubicin (Figure 4). This compound showed moderately cytotoxic effect. (−)-Kunstleramide (1) demonstrated dose-depended cytotoxic effects EC_{50} values of 64.65, 44.74, 55.94, 73.87 and 70.95 µg/mL; in A375, A549, HT-29, PC-3 and WRL-68, respectively. These results indicate that cell lines differ in their sensitivity to the same test agent, which may be determined by multiple cell type-specific signalling cascades and transcription factor activities.

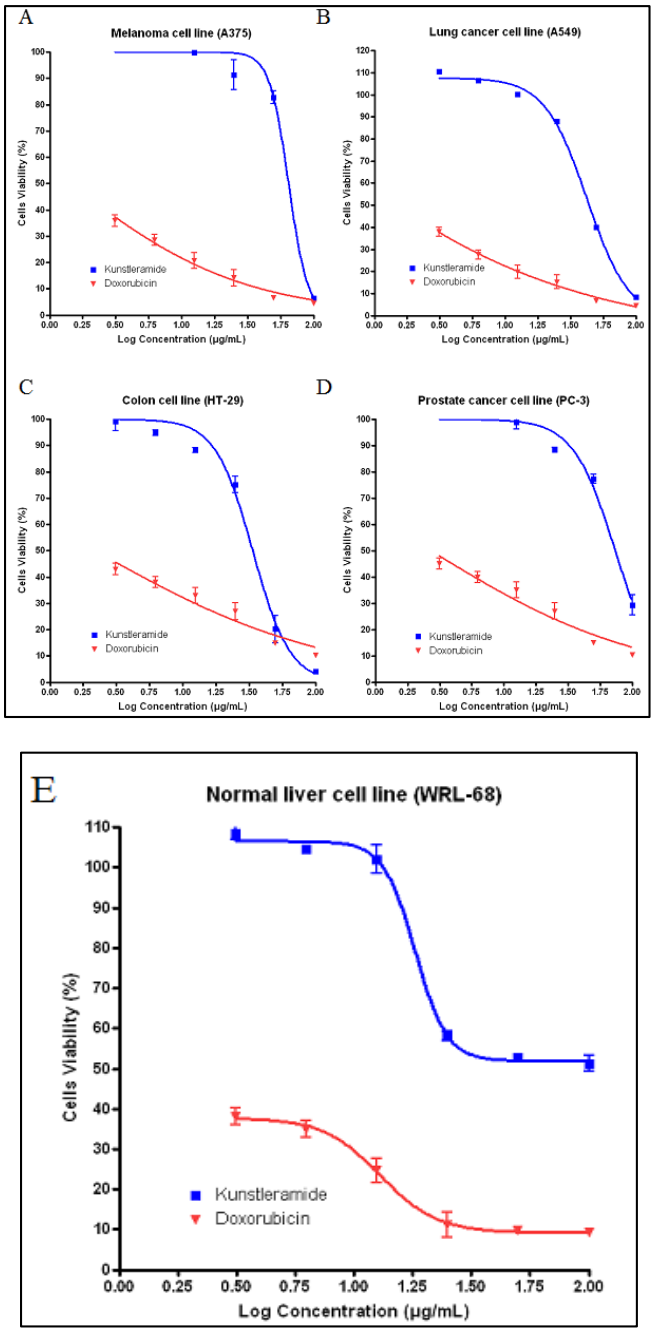


Fig 3: Dose-response curves (using GraphPad Prism) tested with (−)-kunstleramide (1) and doxorubicin (positive control) in the MTT assays towards (A) A375,(B)A549, (C) HT-29, (D) PC-3 and (E) WRL-68.

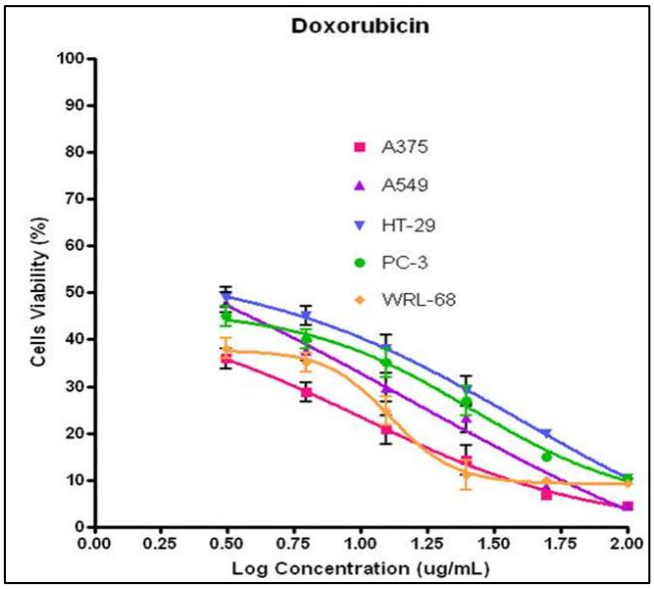


Fig 4: Dose-response curves (using GraphPad Prism) tested with doxorubicin (positive control) in the MTT assays towards A375, A549, HT-29, PC-3 and WRL-68.

Table 1: Effect of compounds (−)-kunstleramide (1) and doxorubicin (positive control) on different cells type expressed as EC_{50} values in 24 h MTT assay. [EC_{50} ± S.D (µg/mL)].

Cell line	(-)-kunstleramide (1)	Doxorubicin
A375	64.65	1.364
A549	44.74	1.550
HT-29	55.94	1.957
PC-3	73.87	2.125
WRL-68	70.95	1.731

4. Conclusions

(-)-kunstleramide (1) as a dienamide, were isolated from the bark of *Beilschmiedia Palembanica (Miq.) Kosterm* together with one flavanone named pinocembrin (2), one phenanthrenol named perakensol (3) and 12 known alkaloids: sebiferine (4), reticuline (5), norisoboldine (6), norboldine (7), norisodomesticine (8), crychine (9), cassythicine (10), argentinine (11), isoboldine (12), o-methylnorflavinantine (13), nornantenine (14), and milonine (15). (−)-Kunstleramide (1) exhibited very poor DPPH, activity with an IC_{50} value of 179.5 ± 4.4 µg/mL compared with the DPPH inhibitor ascorbic acid. Kunstleramide (1) showed moderate cytotoxic effect with EC_{50} values. This study revealed that this plant showed promising cytotoxic activity but poor DPPH activity. Further investigation should be carried out to evaluate the cytotoxicity of compound 1 at lower concentrations.

Acknowledgements

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