Cytotoxic Activity of 13(*E*)-labd-13-ene-8α,15-diol on Human Epithelioid Carcinoma Cells

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The cytotoxic activity of 13(*E*)-labd-13-ene-8α,15-diol (1), isolated from the ethanol extract of *Brachyglottis monroi*, was evaluated against human oral epithelioid carcinoma cells(KB).

Key words : *Brachyglottis monroi*

Introduction

Brachyglottis monroi (Hook. f) B. Nordenstam (Asteraceae compositae), previously *Senecio monroi*, is a shrub endemic to New Zealand[1,2]. *B. monroi* has been widely used in Maori traditional medicine for treatment of sores and wounds[3]. Kulanthaivel et al have investigated n-butyl caffeate and n-butyl traversate derivatives, and flavonoids from the leaf gum of *Traversia baccharoides* Hook. f. (Asteraceae: Senecionae). This plant is a low shrub of montane to sub-alpine habitat, indigenous to the South Island of New Zealand. medium-sized shrub found in most higher altitude parts of central New Zealand. Among the characteristic features of the plant is a distinct stickiness to the touch, a yellow stain with a delicately resinous aroma remaining upon the skin[4].

We now describe the cytotoxicity of 13(*E*)-labd-13-ene-8 α,15-diol isolated from *B. monroi* using the MTT method.

Materials and Methods

1. General experimental procedures.

All solvents were distilled before use. Removal of solvents from chromatography fractions were removed by rotary evaporation at temperature up to 40℃. Initial fractionation of crude plant extract using reverse phase column chromatography was performed with octadecyl-functionalized silica gel (C-18, Aldrich) as the adsorbent. Further column fractionation was performed using Davisil silica 60 Å (35-70 μ m silica gel, Allth) as adsorbent. TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F_{254} visualized first with a UV lamp, then by dipping in a vanillin solution (1% vanillin, 1% H_2SO_4 in EtOH) followed by heating. NMR spectra of $CDCl_3$ solutions at 25℃ of extracts and fractions were recorded at 200 ㎒ for ^{1}H-NMR on a Varian Germini 200 spectrometer, or at 300 ㎒ for ^{1}H-NMR and 75 ㎒ for ^{13}C-NMR on a Varian Inova 300, or a Varian VXR-30 spectrometer. Chemical shifts are given in ppm on the δ scale and are referenced to TMS at 0.00 ppm (^{1}H) or to solvent peaks at either: $CDCl_3$ (^{1}H at 7.25 ppm, ^{13}C at 77.0 ppm).

Two-dimensional (2-D) NMR experiments were recorded on a Varian VXR-300 fitted with a Nalorac inverse-detection probe, or a Varian Inova 300 fitted with a Varian "Autoswitchable" 5 mm Z-axis PFG Direct 300 ㎒ probe (in both cases). Proton and carbon shifts were referenced as described for 1-D NMR experiments. Proton-carbon coupling correlations were observed from gCOSY experiments; one bond ^{1}H - ^{13}C correlation (^{13}C resonances with directly attached protons) were assigned using results from HMQC experiments; the ^{13}C resonance's of quaternary carbons and two and three bond ^{1}H - ^{13}C correlations were assigned using HMBC and DEPT experiments[5,6].

Solids were ground with KBr to a fine powder and pressed into a KBr disk. Spectra were recorded using a Perkin-Elmer 1600 Fourier Transform spectrophotometer. Melting point was determined in duplicate using a Mettler

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Toledo electronic melting point apparatus to an accuracy of ± 0.2°. High resolution mass spectra was recorded by Bruce Clark (Chemistry Department, University of Canterbury, Christchurch, NZ), on a Kratos MS80 (electron impact, 70 eV) mass spectrometer. Microanalyses were performed by Marianne Dick and Bob McAllister (Campbell Microanalytical Laboratory, Chemistry Department, University of Otago). All other chemicals were of reagent grade.

2. Plant material.

B. monroi was collected from the Dunedin Botanical Garden, Dunedin, New Zealand, in June 1998. This was identified by Dr. Glenny, Landcare Research, and a voucher specimen, OTA 980309-63, has been kept in the Otago University herbarium.

3. Extraction and isolation.

Air-dried *B. monroi* (26.88 g) was macrate in redistilled ethanol (200 mL) in a Waring Blender and then filtered. The residual marc was reextracted in the same way with more ethanol (2 × 150 mL) and chloroform (100 mL). The combined filtrates were evaporated under reduced pressure to give a dark green gum (2.60 g). A sample was subjected to flash chromatography on C_{18} (10 g) with a H_2O : MeCN : $CHCl_3$ gradient. The most cytotoxic fraction was eluted with 1 : 9 H_2O : MeCN (245 mg, IC_{50} 8.286 μg/mL). A sub-sample (245 mg) was subjected to flash chromatography on sillica gel (2.5 g) with a hexane : ethyl acetate gradient.

A fraction eluted with 1 : 9 hexane : ethyl acetate was subjected to give 13(*E*)-labd-13-ene-8α,15-diol (1, 51 mg). m.p. 138.1-139.9 ℃ (lit, 131-134℃),[7] $[\alpha]_{18}$-2.256 (C, 3.0 $CHCl_3$) High-resolution mass spectrum m/z, 308.2724 ($C_{20}H_{36}O_2$ required 308.4986), TLC, MeCN : H_2O 3 : 1, R_f 0.37, black spot on H_2O : H_2SO_4 : MeOH 895 : 5 : 10 dip; $UV_{\lambda max}$ (MeOH) log ε 266 (3.01) nm; $IR_{\lambda max}$ (film), 3341, 2931, 2861, 1455, 1384, 1214, 1014, 756 cm^{-1}; EI-MS (70 eV), 308.2724 (0.1, M^+, $C_{20}H_{36}O_2$ required 308.4986), 290 (M^+-H_2O), 275 (10), 257 (8), 245 (12), 204 (9), 192 (91), 177 (100), 163 (12), 149 (22), 137 (34), 123 (45), 109 (45), 95 (54), 81 (66), 69 (66), 55 (47), 43 (51); ^{1}H-NMR ($CDCl_3$) δ5.38 (1H, qt, J=1.2, 6.9 Hz), 4.08 (2H, dd, J=2.4, 7.1 Hz), 2.03 (2H, t, J=8.1. Hz), 1.80 (2H, dt, J=2.0, 12.3 Hz), 1.63 (3H, t, J=0.6 Hz), 1.60 (2H, t, J=1.0 Hz), 1.56 (1H, t, J=2.7 Hz), 1.38 (2H, m), 1.33 (1H, dd, J=1.4, 3.9 Hz), 1.29 (1H, dd, J=1.4, 3.9 Hz), 1.07 (3H, t, J=0.6 Hz), 0.99 (2H, t, J=3.9 Hz), 0.99 (1H, t, J=3.9 Hz), 0.90 (1H, t, J=2.7 Hz), 0.86 (1H, dd, J=1.4, 9.6 Hz), 0.80 (3H, s), 0.73 (3H, s), 0.72 (3H, s); ^{13}C-NMR ($CDCl_3$) δ42.04 (C-1), 18.51 (C-2), 39.80 (C-3), 33.31 (C-4), 56.17 (C-5), 20.64 (C-6), 44.60 (C-7), 74.22 (C-8), 61.25 (C-9), 36.30 (C-10), 23.64 (C-11), 42.96 (C-12), 141.01 (C-13), 123.23 (C-14), 59.35 (C-15), 16.53 (C-16), 23.99 (C-17), 33.46 (C-18), 21.58 (C-19), 15.55 (C-20).

4. Cell culture.

KB cells was grown at 37℃ in RPMI medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL). The cells were grown in a humidified atmosphere of 95% air/5% CO_2. Cells were dissociated with 0.25% trypsin and were counted using a Hemacytomer just before transferring them for the experiment.

5. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay.

The assay is dependent on the cellular reduction of water-soluble MTT (Sigma Chemical Co. St. Louis, M.O.) by mitochondrial dehydrogenase of vial cells to a blue water-insoluble formazan crystal product, which can be measured spectrophotometrically[8,9]. KB cell lines were cultured in RPMI-1640 medium (Gibco Laboratories) containing 10% FBS. Exponentially growing tumor cells (5 x 10^4) were cultured for 24 hrs at 37℃ in a humidified 5% CO_2 incubator in the presence or absence of 13(*E*)-labd-13-ene-8α,15-diol (1).

6. Morphology.

Morphological changes of KB cells cultured in a medium with 13(*E*)-labd-13-ene-8α,15-diol(1) were observed by microphotography.

7. Statistical analysis.

All values, expressed as mean ± S. D., were statistically analyzed through analysis of Students's t test. The p values less than 0.05 was considered as significant.

Results and Discussion

1. Isolation and identification of 13(*E*)-labd-13-ene-8α,15-diol (1)

A sub-sample of the extract was subjected to reverse-phase (C-18) silica gel column chromatography. The column fractions were combined based on visually similar TLC results. These combined fractions were assayed against P388 murine leukaemia cells and the activity was found to be spread over four fractions that were eluted with 1 : 1 H_2O/MeCN, 1 : 3 H_2O/MeCN, 1 : 9 H_2O/MeCN and MeCN. Because of this, the fraction 6 chromatographed on a silica gel column using hexane-ethyl acetate and ethyl acetate-ethanol gradients. The fraction 6-5 with the highest activity was shown by TLC and ^{1}H-NMR spectrum to consist of one main UV-active compound (1). The unique absorption bands due to

olefinic band (1,455 cm^{-1} and 1,384 cm^{-1}) were shown in the IR spectrum along with hydroxyl group (3,331 cm^{-1}). Si-gel column chromatography spread cytotoxic activity across ethyl acetate-hexane 30 : 70 and 40 : 60 fractions containing 13(*E*)-labd-13-ene-8α,15-diol (1). The MS supported a molecular of $C_{20}H_{36}O_2$. The ^{1}H-NMR spectrum of 1 showed the presence of olefinic group with signal at δ5.38 (^{1}H, qt J= 1.2, 6.9 Hz) and the protons at C-15 in an allylic alcohol as two-proton double doublets at δ4.08 (J= 2.4, 7.1 Hz) together with five methyl groups at δ0.72, 0.73, 0.80, 1.07 and 1.63. The ^{13}C-NMR spectrum showed 20 peaks and a DEPT experiment confirmed these to arise from eight CH_2 groups, five CH_3 groups, three CH groups and four quaternary carbons, one of the which was oxygenated, probably with an alcohol group. The ^{13}C-NMR assignments of compound (1) were confirmed by extensive 2D-NMR experiments involving the determination of its COSY, NOE, HMQC and HMBC spectra. The HMBC spectrum showed the important following correlation; i) δ_c= 23.64 (C-11)/δ_H= 0.72 (H-19) and 0.73 (H-20); ii) δ_c= 42.96 (C-12)/δ_H= 1.63 (H-16); iii) δ_c= 141.01 (C-13)/δ_H= 2.03 (H-12) and 1.63 (H-16; iv) δ_c= 123.23 (C-3)/δ_H= 2.03 (H-12 and 1.63 (H-16). The assignment of the other groups of 1 was established by its HMBC spectrum. The NOE difference experiments produced a surprising result. Irradiation of the H-12 protons gave to enhancement of the H-14 olefinic proton and the H-9 tertiary proton signals which links with bicyclic ring. Irradiation of the H-14 olefinic proton signal gave to enhancement of both the H-12 and H-15 protons. Structure 1 would be expected to give enhancements of both the H-14 and H-16. An NOE interaction between H-9 and H-12 protons of the 3-methylpentenol group was also consistent with structure 1 (Fig. 1).

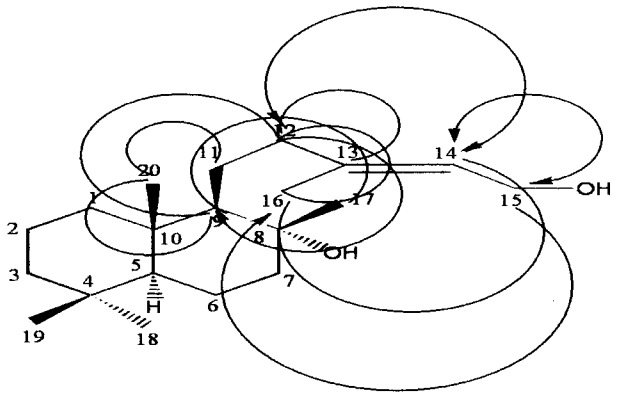

Fig. 1. Important NMR correlations establishing the structure of 13 (E)-labd-13-ene-8α,15-diol (1). ↔, Selected NOE interaction; Selected HMBC correlations.

Structure 1 was derived from a combination of NMR experiments and confirmed by comparison with published NMR data[10]. Both (1) and its enantiomer have been reported from a variety of natural sources; the absolute stereochemistry shown was supported by a small negative optical rotation[11]. Compound (1) was toxic to KB cells (IC_{50} 20.43 μg/mL).

2. Cytotoxic effect of 13(*E*)-labd-13-ene-8α,15-diol (1) on KB cells.

In our investigation, the cytotoxicity of 13(*E*)-labd-13-ene-8α,15-diol (1) on KB cells was studied by the MTT method. In general, the cytotoxic activity of 13(*E*)-labd-13-ene-8α,15-diol (1) was in a concentration-dependent manner over the concentration range 8 μg/mL to 32 μg/mL It can be observed that increasing 13(*E*)-labd-13-ene-8α,15-diol (1)-concentration resulted in decreased cell numbers in KB cells[12-14].

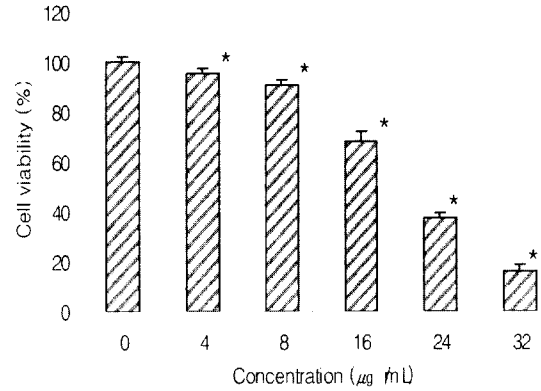

Fig. 2. *In vitro* cytotoxicity of 13(*E*)-labd-13-ene-8α,15-diol (1) by the MTT method. This compound was serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of tumor cell lines (5 x 10^4). The colorimetric assay was performed as described in the materials and methods section. Data are mean of results obtained from three sets of experiments. Significantly different from the control value* $p<0.05$ (Student's t-test).

As shown in Fig. 3A, control KB cells had abundant cytoplasm and cytoplasmic process. However, KB cells treated with 32 μg/mL of 13(*E*)-labd-13-ene-8α,15-diol (1) were seen to have detached from the culture dish as well as cell rounding, cytoplasmic blebing and irregularities in shape were also observed (Fig. 3B)

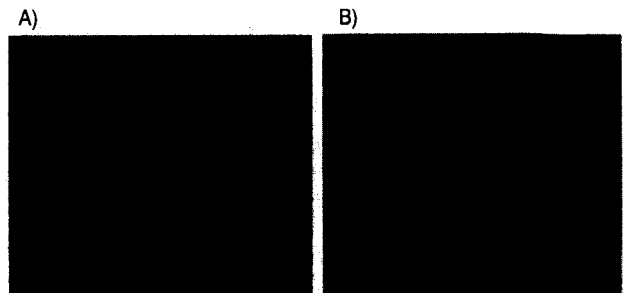

Fig. 3. Morphological change of KB cells after incubation in unmodified medium (control, A) and modified medium containing 32 μg/mL of 13(*E*)-labd-13-ene-8α,15-diol (1, B) for 1 day x 200.

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