Pharmacological Effects of Bioactive Fractions from *Brachyglottis monroi*

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The effects of bioactive fractions from *Brachyglottis monroi* on biological activity were investigated. this bioactive subfraction 6-5 is the most cytotoxic to P388 murine leukaemia cell lines. A comparison of IC_{50} values of these subfraction in cancer cell lines showed that their susceptibility to these subfractions descreased in the following order; Fr. 6-5 〉 Fr. 6-3 〉 Fr. 6-6 〉 Fr. 6-1 〉 Fr. 6-2 〉 Fr. 6-4 by the MTT method. Silica gel flash column chromatography concentrated the cytotoxic activity in subfraction 6-5 which eluted with 30% and 40% ethyl acetate : hexane gave a major bioactive (51 mg, P388 IC_{50} 8,286 ng/mL at 7.5 μg/disc).

Key words : *Brachyglottis monroi*, P388 murine leukaemia cell lines, Cytotoxic activity

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]. Bloor et al have studied extracts of *Brachyglottis bidwillii* var. bidwillii (Hook. f) Nordenstam (Asteraceae), a medium-sized shrub found in most higher altitude parts of central New Zealand. Extracts of the leaf and twig material showed inhibitory activity against one of our target bacteria, methicillin resistant Staphylococcus aureus. bioassay-guided fractionation showed the activity to be associated with labdane-type diterpenoids all present in significant quantity[4].

In this study, the antiviral and antimicrobial activities and cytotoxicity of bioactive fractions from *B. monroi* were examined and have investigated their cytotoxic fractions.

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-19 Aldrich) as the adsorbent. Further column fractionation was performed using Davisil silica 60 Å (35-70 μm 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℃ were recorded at 300 ㎒ for ^1H on a varian VXR-300 spectrometer. Chemical shifts are given in ppm on the scale referenced to the solvent peaks of $CDCl_3$, ^1H-NMR referenced to 7.25 ppm.

2. Plant material

Brachyglottis 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. Preparation of bioactive fractions

Air-dried *Brachyglottis 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 x 150 mL) and chloroform (100 mL). The combined filtrates were evaporated under reduced pressure to give a dark green gum (2.60 g) which was subjected to flash

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column chromatographyon C 18 (10 g) with a H_2O : CH_3CN : $CHCl_3$ gradient. These fractions was stored at 4℃ until tested.

4. Disk diffusion assays

Antimicrobial assays against *Bacillus subtilis* ATCC 19659, *Candida albicans* ATCC 14053 and *Trichophyton mentagrophytes* ATCC 28185 were performed by Gill Ellis at the Chemistry Department, University of Canterbury, Christchurch[5]. Solutions of compounds for assay were dried onto 6 mm filter paper disks, which were then placed onto seeded agar Petri dishes and incubated. Activity was observed as a zone of inhibition around the disk, with its width recorded from the edge of the disk in mm. SM refers to the observed margin surrounding the zone of inhibition (S=sharp).

5. P388 assay

This is a measure of the ability of a sample (i.e., plant extract) to inhibit the multiplication of murine leukaemia cells. The sample was dissolved in a suitable solvent, usually ethanol, at 2 mg/mL, and 20 μL of this solution was placed in the first well of a multiwell plate. After addition of the cell solution the concentration range in the test wells was 25,000 down to 195 ng/mL. After incubation for three days the plates were read using an ELISA plate reader at 540 nm wavelength. Automated reading of the plates was possible with the addition of a MTT tetrazolium salt (yellow color). Healthy cells reduce this salt to MTT formazan (purple color). The plate reader was able to determine the concentration at which 50% inhibition of cell growth has occurred. These IC_{50} are presented in ng/mL.

6. Antiviral assays

The extract was applied (15 μL of a 5 mg/mL solution) to a small filter-paper disc, dried, and assayed for antiviral activity using Schroeder et al.'s methods[6]. The results observed were either cell death (cytotoxicity), inhibition of virus replication, no effect (i.e., all of the cells show viral infection), or a combination of all three. The results were noted as the approximate size of the circular zone, radiating from the extract sample, from 1+ to 4+ repressing 25% through to whole well sized zones. The notation used is inhibition/cytotoxicity. The type of cytotoxic effect, indicated by a number after the size of the zone, was also considered important and may give some indication as to the mode of cytotoxic action.

7. Antibacterial and antiyeast activities

Activity against the following bacterial strains and yeast was tested; multiresistant *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 14053. Extracts were dissolved and diluted in an appropriate solvent (usually ethanol : water) to a concentration of 2 mg/mL. Test plates are prepared from Mueller Hinton agar + extract to give a final concentration of 100 μg extract/mL agar. Activity growing cultures of the test strains were diluted in saline so as to deliver 10^4 colony forming units onto the test, control (solvent), and blank (agar only) plates with a multipoint inoculator. Inoculated plates were incubated overnight at 37℃. Growth on the blank and control plates was checked and, if satisfactory, growth on the test plates was scored for each test strain as follows: (-) inhibition, no reduction in growth compared with the control, (±) inhibition, some reduction in growth, (+) inhibition, no growth.

8. 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. Cytotoxic activity of bioactive fractions

Brachyglottis monroi (Hook. f) B. Nordenstam(Asteraceae compositae) is a shrub endemic to New Zealand.1,2 Foliage plant, collected from the Dunedin Botanical Gardens, gave a crude ethanol extract cytotoxic to P388 murine leukaemia (IC_{50} 23.96 μg/mL) and BSC monkey kidney cells (25% of well at 75 μg/mL). This extract shows the weak antiviral activity against *Herpes simplex* type I *virus* ATCC VR 733 and *Polio* type I *virus* Pfizer Vaccine strain (25% activity, @ 5 mg/mL at 75 μg/disc).

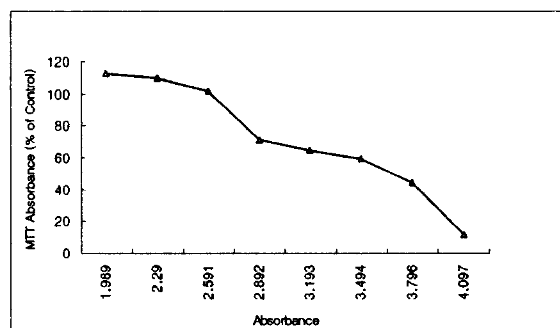

Fig. 1. ***In vitro* cytotoxic effect of the crude extract of *B. monroi* by the MTT method.** This crude extract was serially diluted in RPM1-1640 with 10% FBS and mixed with equal volume of P388 murine leukaemia (75 μg/disc) cells. The colorimetric method was performed as described in materials and methods. Data are mean values of results obtained from three sets of experiments.

As indicated in Table 1, this crude extract inhibited the growth of the Gram-positive bacterium *Bacillus subtilis* ATCC

19659, 91 mm inhibition zone at 150 μg/disc) and the dermatophyte *Trichophyton mentagrophytes* ATCC 28185, (2 mm inhibition zone at 150 μg/disc). No activity was observed against the fungus *Candida albicans* at 150 μg/disc. This extract showed weaker antimicrobial activity than chloramphenicol and nystatin.

Fig. 1 shows the potent cytotoxic activity of the crude extract against P388 murine leukaemia cell lines. The cytotoxic activity of the crude extract was in a dose-dependent inhibition of cell proliferation[6,7]. This crude extract showed a dose-dependent increase of cell antiproliferation. After treatment with the crude ethanol extract of *B. monroi*, this extract-mediated cytotoxicity was gradually increases in the MTT method when its concentrations or absorbances were raised from 1.989 to 4.097.

This method that evolved is to coat the extract, containing compounds ranging from hydrocarbons to labdanoic diterpenoids, on to a reverse-phase support. This can then be loaded, as either an aqueous slurry or a powder, onto a flash chromatography column that has been slurry-packed with the same support. Elution with H_2O, followed by a steep, stepped gradient through MeCN, $CHCl_3$ to EtOH generally gives very satisfactorily partitioning of crude extracts. The recovery of material is usually very good. The strongly cytotoxic extract prepared from *B. monroi* was partitioned[8]. The results of the partitioning are shown in Table 1.

Table 1. ***In vitro cytotoxic* activity of the crude ethanol fractions of B. *monroi* on P388 murine leukaemia cell lines by the MTT method.a**

Fraction No.	Eluent	Volume (mL)	Mass (mg)	IC_{50} (ng/mL)[b]
1	H_2O, 9 : 1 H_2O/MeCN	90	133	>62,500
2	9 : 1 H_2O/MeCN	30	140	>62,500
3	3 : 1 H_2O/MeCN	60	139	>62,500
4	1 : 1 H_2O/MeCN	60	145	17,914
5	1 : 3 H_2O/MeCN	60	285	9,730
6	1 : 9 H_2O/MeCN	60	245	1,967
7	MeCN	60	222	10,312
8	3 : 1 MeCN/$CHCl_3$, $CHCl_3$, 1 : 1 $CHCl_3$/EtOH, EtOH	240	1152	>62,500

[a]Each fraction was examined in eight concentrations in triplicate experiments. [b]IC_{50} represents the concentration of a fraction required for 50% inhibition of cell growth, Mitomycin C was used as control and exhibited an IC_{50} 52.3 ng/mL. Toxicity of sample to P388 murine leukaemia cells in ng/mL at 75 μg/disc.

The crude extract was fractioned into Fr. 1 - Fr. 8 using C-18 silica gel column chromatography as described previously. Chromatography on C-18 (26.0 g) with a H_2O, MeCN, $CHCl_3$, EtOH gradient gave eight fractions. 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[9] with 1 : 1 H_2O/ MeCN, 1 : 3 H_2O/MeCN 1 : 9 H_2O/MeCN and MeCN (Table 1). Among them, the fractions Fr. 4 - Fr. 7 are cytotoxic to P388 murine leukaemia cells. The order of cytotoxic activity was shown Fr. 6 > Fr. 5 > Fr. 7 > Fr. 4 > Fr. 1 = Fr. 2 = Fr. 3 = Fr. 8 (Table 1). Reverse-phase flash column chromatography concentrated the cytotoxic activity in fractions which gave a major active (245 mg, P388 IC_{50} 1,967 ng/mL at 75 μg/disc) eluted with 1 : 9 H_2O : MeCN. This bioactive fraction 6 with the high activity was cytotoxic to P388 murine leukaemia cells (P388 IC_{50} 8,268 ng/mL at 30 μg/disc; P388 IC_{50} > 6,250 ng/mL at 7.5 μg/disc).

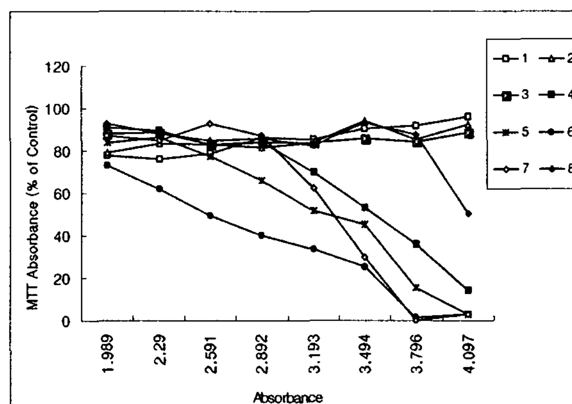

Fig. 2. ***In vitro* cytotoxic effect of the crude ethanol fractions of *B. monroi* by the MTT method.** This crude ethanol fractions were serially diluted in RPM1-1640 with 10% FBS and mixed with equal volume of P388 murine leukaemia (75 μg/disc) cells. The colorimetric method was performed as described in materials and methods. Data are mean values of results obtained from three sets of experiments.

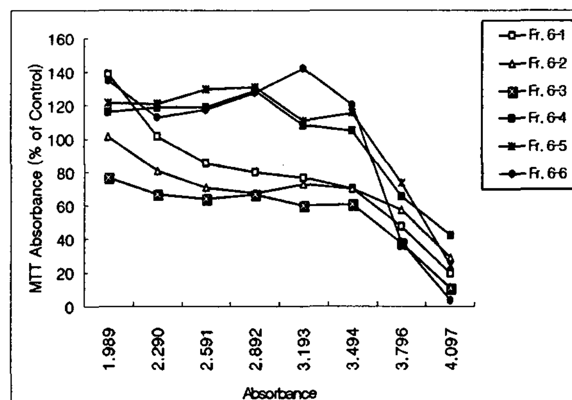

Fig. 3. ***In vitro* cytotoxic effect of the crude ethanol subfractions of *B. monroi* by the MTT method.** This crude ethanol subfractions were serially diluted in RPM1-1640 with 10% FBS and mixed with equal volume of P388 murine lukaemia (30 μg/disc) cells. The colorimetric method was performed as described in materials and methods. Data are mean values of results obtained from three sets of experiments.

Fig. 2 showed the potent cytotoxic activity of the crude ethanol fractions of *B. monroi* against P388 murine leukaemia cells. In general, the cytotoxic activity of these fractions was in a dose-dependent inhibition of cell proliferation and the

susceptibility of P388 cancer cell lines to fraction 6 was quite sensitive. Fractions (4, 5, 6 and 7) - mediated cytotoxicity were increased in the MTT method when their absorbances or concentrations were raised from 1.989 to 4.097. However, the other fractions had no effect.

Fig. 3 showed the potent cytotoxic activity of the crude ethanol subfractions of *B. monroi* against P388 murine leukaemia cells (30 μg/disc). In general, the cytotoxic activity of these subfractions showed a dose-dependent inhibition of cell proliferation. Subfractions (6-1, 6-2 and 6-3) showed a dose-dependent increase of cell antiproliferation after treatment with subfractions of *B. monroi*. However, subfractions (6-4, 6-5 and 6-6) had no effect from A= 1.989 to A= 3.494. After treatment with subfractions the inhibitory activity showed a dose-dependent inhibition of P388 murine leukaemia cells. As shown in Fig. 4, treatment of P388 murine leukaemia cells with subfractions (6-1, 6-2 and 6-3) showed dose- dependent inhibitions of cell proliferation at 7.5 μg/disc, whereas subfractions (6-4, 6-5 and 6-6) had no effect[10].

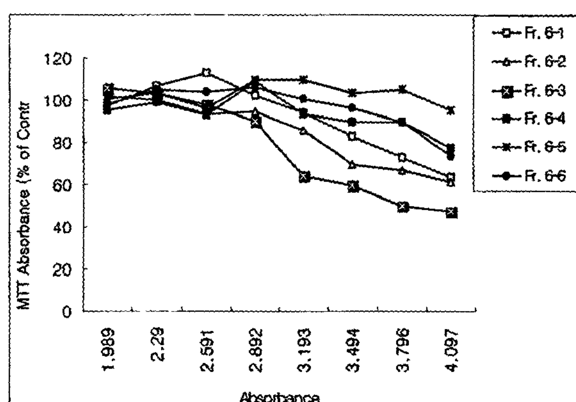

Fig. 4. *In vitro* cytotoxic effect of the crude ethanol subfractions of B. minori by the MTT method. This crude ethanol subfractions were serially diluted in RPM1-1640 with 10% FBS and mixed with equal volume of P388 murine leukaemia (7.5 μg/disc) cells. The colorimetric method was performed as described in materials and methods. Data are mean values of results obtained from three sets of experiments.

Table 2. *In vitro* cytotoxic activity of the crude ethanol subfractions of *B. monroi* on P388 murine leukaemia cell lines by the MTT method.[a]

Subfraction No.	Eluent	Volume (mL)	Mass (mg)	IC_{50} (ng/mL)[b]
6-1	2%, 4% EA/HX	30	23	11,745
6-2	4% EA/HX	25	30	14,819
6-3	4%, 10% EA/HX	50	56	8,531
6-4	20%, 30% EA/HX	30	22	19,249
6-5	30%, 40% EA/HX	20	51	8,286
6-6	60%, 90% EA/HX, 100% EA, 10% EtOH/EA	75	11,408	11,408

[a]Each fraction was examined in six concentrations in triplicate experiments. [b]IC_{50} represents the concentration of a subfraction required for 50% inhibition of cell growth, Mitomycin C was used as control and exhibited an IC_{50} 52.3 ng/mL. Toxicity of sample to P388 murine leukaemia cells in ng/mL at 30 μg/disc.

Silica gel flash column chromatography with ethyl acetate, n-hexane, ethanol gradient gave six subfractions. The second silica gel column chromatography of subfraction 6-3 gave most of the mass in the less polar fraction, eluted with 4% and 10% ethyl acetate-hexane. Among them, subfraction 6-5 that eluted with 30% and 40% ethyl acetate-hexane is the most cytotoxic activity to P388 murine leukaemia cells (51 mg, P388 IC_{50} 8.286 ng/mL at 30 μg/disc). These subfractions are cytotoxic to P388 murine leukaemia cell lines. A comparison of IC_{50} values of these subfraction in cancer cell lines showed that their susceptibility to these subfractions descreased in the following order; Fr. 6-5 〉 Fr. 6-3 〉 Fr. 6-6 〉 Fr. 6-1 〉 Fr. 6-2 〉 Fr. 6-4 by the MTT method (Table 2). Slilca gel flash column chromatography concentrated the cytotoxic activity in subfraction eluted with 30% and 40% ethyl acetate : hexane which gave a major bioactive (51 mg, P388 IC_{50} 8,286 ng/mL at 30 μg/disc). This bioactive subfraction 6-5 was cytotoxic to P388 murine leukaemia cell lines (IC_{50} 8,286 ng/mL at 7.5 μg/disc). The bioactive subfraction 6-5 was shown by TLC and ^{1}H-NMR spectrum which appeared signals due to an olefinic group (δ 5.38 ppm), methylene protons and five methyl groups (δ 0.72, 0.73, 0.80, 1.07 and 1.63 ppm). By ^{1}H-NMR spectrum analysis, the main components of Fr. 6-5 were identified as labdane-type diterpenes.

In conclusion, this bioactive subfractions are cytotoxic to P388 murine leukaemia cell lines. A comparison of IC_{50} values of these subfraction in cancer cell lines showed that their susceptibility to these subfractions descreased in the following order; Fr. 6-5 〉 Fr. 6-3 〉 Fr. 6-6 〉 Fr. 6-1 〉 Fr. 6-2 〉 Fr. 6-4 by the MTT method. Slilca gel flash column chromatography concentrated the cytotoxic activity in subfraction 6-5 which eluted with 30% and 40% ethyl acetate : hexane gave a major bioactive (51 mg, P388 IC_{50} 8,286 ng/mL at 7.5 μg/disc).

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