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Antifungal Effect of *Brachyglottis repanda* Ethanol Extract

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The crude ethanol extract of *B. repanda* showed the cytotoxic activity against *Polio virus* (25% activity at 150 μg/disk) and the minor cytotoxic activity against BSC cells (African green monkey kidney). However, the crude ethanol extract of *B. repanda* was non-toxic to murine leukaemia cells CCL 46 P388D1 (IC_{50}, > 62,500 ng/m*l*). Cytotoxic and antifungal activities were strongly shown by Fr. 64-3 which was eluted with 90% CH_3CN/H_2O, 100% CH_3CN, and 50% CH_3CN/H_2O (SM 2 at 150 μg/disk). The fraction 64-3 also showed the most cytotoxic activity against murine leukaemia cells (128 mg, IC_{50} 10,051 ng/m*l* at 75 μg/disk). These results suggest that this fraction has a potent antifungal activity against the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185.

***Key words*:** *Brachyglottis repanda*, Cytotoxic activity, Murine leukaemia cell lines, *Trichophyton mentagrophytes*, Antifungal activity, Antiviral activity

INTRODUCTION

Brachyglottis genus contains several shrubs, previously botanically classified in the *Senecio* genus, and is closely related to the *Olearia* genus. Many are especially useful garden plants for dry, sunny area and a number of excellent *Brachyglottis* cultivars are now common in cultivation. *Brachyglottis monroi* (Hook. f) B. *Nordenstam* (*Asteraceae* compostae), previously *Senecio monroi*, is a shrub endemic to New Zealand (Connor and Edger, 1987; Allan, 1960). *B. monroi* and *B. repanda* have been widely used in Maori traditional medicine for treatment of sores and wounds (Riley, 1994). Bloor *et al*. (1993) have studied extracts of *B. bidwillii*, a medium-sized shrub found in high 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 that the activity was associated with labdane-type diterpenoids which were present in significant quantity. *B. repanda* 'Purpurea' is a purple leaf form of *B. repanda*. It needs to be planted in a dry are and plenty of air circulation is needed to counter moulds (http://www.google.co.nz). Mortimer *et al.* (1967) reported that a shrub endemic to New Zealand, *B. repanda*, J. E. et G. Forst. (family Compositae, Maori name "Rangiora"), is suspected to poison farm animals, particularly horses. Feeding tests on sheep gave evidence of toxicity, but details are lacking. The presence of alkaloid (0.02%) in leaves was reported and from the close botanical alliance of *Brachyglottis* to *Senecio*, pyrrolizidine bases might be expected.

Choi *et al.* (2010) reported the antiviral and anticancer activities of 13(*E*)-labd- 13-ene-8a,15-diol (**1**) isolated from *B. monroi*, which was examined against human rhinovirus 2 (HRV 2) and 3 (HRV 3), and human cancer cells (A549 and Hep2). 13(*E*)-Labd-13-ene-8a,15-diol (**1**) showed strong anti-HRV 2 and HRV 3 activity with a 50% inhibitory concentration (IC_{50}) of 2.68 and 0.87 mg/m*l*, respectively, and a 50% cytotoxicity concentration (CC_{50}) of 59.45 mg/m*l*. Furthermore, A549 and Hep2 cells incubated with 32 mg/m*l* of 13(*E*)-labd-13-ene-8a,15-diol (**1**) for 48 hrs exhibited antilung and antilaryngeal cancer activities, with a viability of less than 50% (Choi *et al.*, 2010). Lim *et al.* reported that the cytotoxic and antimicrobial activities of 13(*E*)-labd-13-

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ene-8a,15-diol (**1**) against tumor cell lines, bacteria, and fungi. 13(*E*)-Labd-13-ene-8a,15-diol (**1**) was active against various tumor cell lines. The potencies (IC_{50}s 8.3~21.3 μg/m*l*) are similar to those reported (IC_{50}s 11.4~> 50 μg/m*l*) for 13(*E*)-labd-13-ene-8a,15-diol (**1**) isolated from another plant, *Cistus creticus* (*Cistaceae*). 13(*E*)-Labd-13-ene-8a,15-diol (**1**) was the most effective growth inhibitor of P388 murine leukaemia cell lines, producing approximately 8.3 μg/m*l* of IC_{50} in the MTT method. 13(*E*)-Labd- 13-ene-8a,15-diol (**1**) also inhibited the growth of the Gram-positive bacteria (*Staphylococcus aureus, Bacillus cereus* and *Listeria monocytogenes*) and Gram-negative bacteria (*Bacillus subtilis* (*Vibrio parahaemolyticus, Escherichia coli* and *Salmonella enteritidis*) with minimum inhibitory concentration (MIC) ranging from 0.092 to 0.598 mg/m*l*. However, gram-negative bacteria were more sensitive to 13(*E*)-labd-13-ene-8a,15-diol (**1**, MIC 0.092 mg/m*l*).

In this study, the antiviral and antimicrobial activities and cytotoxicity of crude ethanol extract from *B. repanda* were examined and their cytotoxic and antifungal fractions were investigated.

MATERIALS AND METHODS

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°C. 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 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°C, were recorded at 300 MHz for ^{1}H-NMR on a Varian VXR-300 spectrometer. Chemical shifts are given in parts per million on the δ scale referenced to the solvent peak $CHCl_3$ at 7.25 ppm and are referenced to TMS at 0.00 ppm.

Plant material. *Brachyglottis repanda* (*B. repanda*) was collected from Botanical garden, Dunedin, in December 1999. This was identified by D. Glenny, Landcare Research, Dunedin, New Zealand, and a voucher specimen, OTA 990119, has been kept in the Otago University herbarium, Dunedin, New Zealand.

Preparation of the extract. Air-dried *B. repanda* (82.6 g) was ground and macerated in redistilled ethanol (800 m*l*) in a Waring Blender, then filtered. The residual marc was re-extracted wisely with ethanol (800 m*l*) and chloroform (400 m*l*). The combined filtrates were evaporated under reduced pressure to give a crude extract (4.26 g, 5.2%), which was stored at 4°C until tested.

Screening for antiviral activity. The extract was applied (15 μ*l* of a 5 mg/m*l* solution) to a small filter-paper disc, dried, and assayed for antiviral activity using Schroeder *et al*. (1981) methods.

Screening for antibacterial and antiyeast activities. Activity against the following bacterial strains and yeast (Canterbury, Christchurch, NZ) were tested: multi-resistant *Bacillus subtilis* ATCC 19659, and *Candida albicans* ATCC 14053. Extracts were dissolved and diluted in an appropriate solvent (usually ethanol : water) to a concentration of 5 mg/m*l*. Test plates are prepared from Mueller Hinton agar containing extract to give a final concentration of 100 μg extract/m*l* agar. Activity growing cultures of the test strains were diluted in saline to deliver 10^4 colony forming units onto the test, control (solvent), and blank (agar only) plates with a multipoint inoculators. Inoculated plates were incubated overnight at 37°C. Growth on the blank and control plates was checked and, if satisfactory, growth on the test plates was scored for each test strain.

Screening for antifungal activity. Fungal spore suspensions of *Trichophyton mentagraphytes* ATCC 28185 were applied to dextrose agar plates. Aliquots of the extract solutions were applied to filter paper discs, at 30 μg extract/disc, and dired at 37°C for two hours. These discs were applied to the agar plates, two per plate, and incubated at 28°C.

Screening for cytotoxic activity. This is to measure the ability of a sample to inhibit the multiplication of murine leukaemia cells. The sample was dissolved in a suitable solvent, usually ethanol, at 5 mg/m*l*, and 15 μ*l* of this solution was placed in the first well of a multiwell plate. Seven two-fold dilutions were made across the plate. After addition of the cell solution, the concentration range in the test wells was 25,000 down to 195 ng/m*l*. 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) (Mosmann, 1983; Keepers *et al*., 1991).

RESULTS AND DISCUSSION

B. repanda, which contains a source of alkaloids, is a small tree native to New Zealand. The crude ethanol extract was not cytotoxic to murine leukaemia cells (IC_{50} > 62,500 ng/m*l*). However, this crude extract showed slight BSC cytotoxic activity. This plant showed much weaker cytotoxic activity than that of *H. paucistipula* extract (IC_{50} 2,480 ng/m*l*) (Baek *et al*., 2003). The ethanol extract of *B. monroi* (IC_{50} 23,960 ng/m*l*), which collected from the Dunedin

Table 1. Biological assays of the crude ethanol extract from *B. repanda*

Extract	BSC[a]	*Herpes simplex virus*[b]	*Polio virus*[b]
	+–	–	+
		P388	
Mitomycin C		75[c]	
Extract		> 62,500[d]	

[a]% of well showing cytotoxic effects. @ 5 mg/m*l*, 150 μg/disk. +–: minor effects located under the disk.
[b]Cytotoxicity in antiviral assays. @ 5 mg/m*l*, 150 μg/disk; Zone of cytotoxic activity: +: 25% activity.
[c]Toxicity of sample to murine leukaemia cells in ng/m*l* at 0.075 μg/disk. P388; Concentration of the sample required to inhibit cell growth to 50% of a solvent control.
[d]Toxicity of sample to murine leukaemia cells in ng/m*l* at 150 μg/disk. –: not detected.

Table 2. List of microorganisms used for antimicrobial susceptibility test

Gram-positive bacterium	
Bacillus subtilis	ATCC 19659
Gram-negative bacteria	
Escherichia coli	ATCC 25922
Pseudomonas aeruginosa	ATCC 27853
Fungi	
Cladosporium resinae	ATCC 52833
Candida albicans	ATCC 14053
Trichophyton mentagrophytes	ATCC 28185

Botanical Gardens, showed stronger cytotoxic activity than that of *B. repanda* (Kwag *et al*., 2004). As indicated in Table 1, the extract showed 25% antiviral activity against *Polio* Type I *virus* (Pfizer vaccine strain) (5 mg/m*l* at 150 μg/disk). However, the ethanol extract of *B. monroi* (25% activity, 5 mg/m*l* at 75 μg/disk) indicated stronger

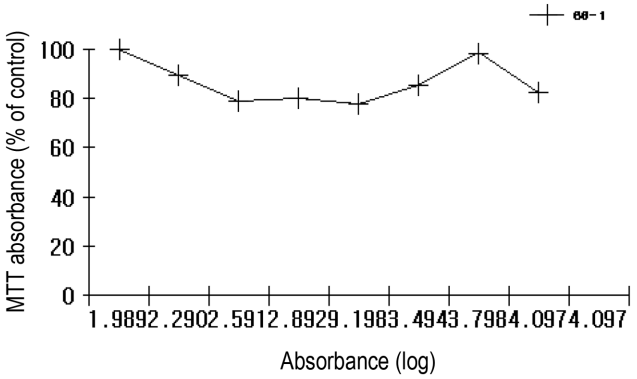

Fig. 1. *In vitro* cytotoxic effect of the crude extract of *B. repanda* by the MTT method. This crude extract was serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of P388 murine leukaemia cells (5 mg/m*l*, 150 μg/disk). Data are mean values of results obtained from three sets of experiments.

antiviral activity against *Herpes simplex* Type I *virus* ATCC VR 733 than that of *B. repanda* (Kwag *et al*., 2004). This ethanol extract did not show any antimicrobial activity against microorganisms (Table 2) (Yook *et al*., 2007; Kim *et al*., 2009).

Our data showed the extremely weak cytotoxic activity of the crude ethanol extract against P388 murine leukaemia cell lines (Fig. 1). This weak cytotoxic activity of the crude extract was concentration-independent, when its concentration or absorbances were raised from 1.989 to 4.097 (Schroeder *et al*., 1981; Baek *et al*., 2000).

This method was used to coat the extract, containing compounds ranging from hydrocarbon to alkaloids, onto a reverse-phase support. This can then be loaded, as either 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 CH_3CN, $CHCl_3$, to EtOH generally gives very sat-

Table 3. *In vitro* cytotoxic and antifungal activities of the crude ethanol fractions of *B. repanda* on *T. mentagrophytes* and murine leukaemia cells ATCC CCL 46 P388D1 by the MTT method[a]

Fraction No.	Eluent	Vol. (m*l*)	Mass (mg)	IC_{50} (ng/m*l*)[b]	*T. mentagrophytes*[c]
64-1	H_2O, 1:1 CH_3CN/H_2O	66	1,707	> 62,500	–
64-2	1:1, 3:1, 9:1 CH_3CN/H_2O	96	709	> 62,500	–
64-3	9:1 CH_3CN/H_2O, CH_3CN 1:1$CH_3CN/CHCl_3$	90	128	10,051	SM 2
64-4	1:1$CH_3CN/CHCl_3$, $CHCl_3$ Hexane, EtOH	168	402	> 62,500	–
64-5	EtOH, 1:1 $EtOH/H_2O$ Hexane	72	391	> 62,500	–
64-6	Hexane	30	152	57,139	–

Each fraction was examined in eight concentrations of triplicated 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 IC_{50} of 75.0 ng/m*l*. Toxicity of sample to murine leukaemia cells in ng/m*l* at 75 μg/disk.
[c]Width of zone of inhibition in mm; 150 μg/disk; –: not detected, Nystatin; HM 6 at 100 unit/disk. SM; Sharp margin, numbers refer to zone of inhibition (mm).

isfactorily partitioning of crude extracts (Shin *et al*., 2001). The recovery of material was usually very good. The results of the partitioning are shown in Table 3. Reversed- phase flash column chromatography on C18 silica gel (35.0 g) with H_2O, CH_3CN/ H_2O, $CH_3CN/CHCl_3$ and *n*-hexane gradient yielded six fractions. The column fractions were combined based on visually similar TLC results. These combined fractions were assayed against murine leukaemia cells and *T. mentagrophytes.* The activity was found to be spread over two fractions 64-3 and 64-6 that were eluted with 90% CH_3CN/H_2O, 100% CH_3CN, 50% CH_3CN/H_2O and 100% hexane. C18 silica gel column chromatography of the most polar fraction 64-1, which was eluted with 100% H_2O and 50% CH_3CN/H_2O, yielded the highest quantity. Among them, fraction 64-3, which was eluted with 90% CH_3CN/H_2O, 100% CH_3CN, and 50% CH_3CN/H_2O, showed the most cytotoxic activity against murine leukaemia cells (IC_{50}, 10.051 μg/m*l*). This fraction also indicated the most antifungal activity against the dermatophytic fungus *T. mentagrophytes* (SM 2). However, the other fractions were inactive against the dermatophytic fungus *T. mentagrophytes*. (Tables 2 and 3). A comparison of IC_{50} (ng/m*l*) values of these fractions in cancer cells showed that their susceptibility to these fractions decreased in the following order; Fr. 64-3 > Fr. 64-6 > Fr. 64-1 = Fr. 64-2 = Fr. 64-4 = Fr. 64-5 (Table 3) (Baek *et al*., 2003). The fraction 64-3 showed equal antifungal activity than that of the ethanol extract of *B. monroi* (SM 2, 5 mg/m*l* at 150 μg/disk) against the dermatophytic fungus *T. mentagrophytes*. However, the ethanol extract of *B. monroi* (SM 1, 5 mg/m*l* at 150 μg/disk) showed stronger antimicrobial activity than that of the fraction 64-3 against *B. sutilis* (Kwag *et al*., 2004).

As shown in Table 1, a potent cytotoxic activity of the crude ethanol extract from *B. repanda* against P388 murine leukaemia cell lines (P 388 IC_{50} > 62,500 ng/m*l*) was not observed. However, fractions 64-3 and 64-6 showed the cytotoxic activity against P388 murine leukaemia cell lines. The polar fraction 64-3 (P 388 IC_{50} 10,051 ng/m*l*) indicated more cytotoxic activity than that of non-polar fraction 64-6. As indicated in Table 3, the least polar fraction 64-6 showed cytotoxic activity against P388 murine leukaemia cell lines (P 388 IC_{50} 57,139 ng/m*l*). In ^{1}H-NMR spectrum, the fraction 64-3 indicated lactone-type compounds based on the chemical shift at 1.60~2.50 and 4.50~6.30 ppm. The cytotoxic activity of *B. repanda* ethanol extract and fractions inhibited cell proliferation in a concentration-dependent manner (Keepers *et al*., 1991; Kwag *et al*., 2004; Yook *et al.*, 2007). Among these fractions, the fraction 64-3 showed the most cytotoxic activity due to the presence of lactones which was shown in ^{1}H-NMR spectrum (Williams and Fleming, 1995; Becconsall, 1993).

In general, the cytotoxic activity of these fractions was in a concentration-dependent manner (Fig. 2). All of these fractions showed a concentration-dependent increase of cell

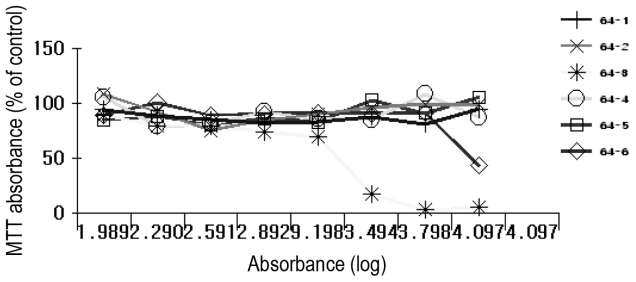

Fig. 2. *In vitro* cytotoxic effect of the crude extract ethanol fraction of *B. repanda* by the MTT method. This crude ethanol fractions were serially diluted in RPM1-1640 with 10% FBS mixed with equal volume of P 388 murine luekaemia (75 μg/disk) cells. The colorimetric method was performed as described in materials and methods. Data are mean values of results obtained from three sets of experiments.

anti-proliferation after treatment with the crude ethanol fractions of *B. repanda.* However, the fractions 64-1, 64-2, 64-4 and 64-5 were inactive against murine leukaemia cells (IC_{50} > 62,500 ng/m*l*). The susceptibility of P388 cancer cell lines to fraction 64-3 was highly sensitive and showed the most cytotoxic activity. This fraction-mediated cytotoxicity was rapidly increased in the MTT method when their concentrations or absorbances were raised from 2.892 to 3.798. The fraction 64-6 was sensitive to murine leukaemia cells in a concentration-dependent manner. However, the other fractions were inactive (Fig. 2 and Table 3) (Yook *et al*., 2007; Kim *et al*., 2009). The separation of the main components from the bioactive fraction 64-3 of *B. repanda* extract needs be studied further and the results will be discussed elsewhere.

In conclusion, the crude ethanol extract of *B. repanda* showed the cytotoxic activity against *Polio virus* (25% activity at 150 μg/disk). It also had minor cytotoxic effect against BSC cells (African green monkey kidney) but was non-toxic to P388 murine leukaemia cells (IC_{50}, > 62,500 ng/m*l*). Cytotoxic and antifungal activities were strongly shown by Fr. 64-3 which were eluted with 90% CH_3CN/H_2O, 100% CH_3CN, and 50% CH_3CN/H_2O (SM 2 at 150 μg/disk). The fraction 64-3 showed the most cytotoxic activity against murine leukaemia cells (128 mg, IC_{50} 10,051 ng/m*l* at 75 μg/disk). This fraction indicated the most antifungal activity against the dermatophytic fungus *T. mentagrophytes*.

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