Pharmacological Screening of Crude Extracts from Medicinal Plants (I)

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The effects of crude extracts from medicinal plants on biological activity were investigated. The crude ethanol extract of *H. paucistipula* inhibited the growth of the Gram positive bacterium *Bacillus subtilis* ATCC 19659, (2 mm inhibition zone at 150 μg/disc) and the dermatophyte *Trichophyton mentagrophytes* ATCC 28185, (7 mm inhibition zone at 150 μg/disc), and toxic to P388 murine leukaemia cells ATCC CCL 46 P388D1, (IC_{50} 2.48 μg/ml at 75 μg/disc). This crude ethanol extract of *H. paucistipula* is the strongest antimicrobial and cytotoxic activities against P388 murine leukaemia cells ATCC CCL 46 P388D1.

Key words : *Brachyglottis monroi, Trichocolea hatcheri, Hepatostolonophora paucistipula, Bacillus subtilis, Trichophyton mentagrophytes*, P388, Cytotoxic activity

Introduction

Liverworts are colsely related to mosses, and the two groups together form a large and important division of the plant kingdom, technically known as the Bryophytes. Most liverworts contain mono-, sesqui- and diterpenoids and/or aromatic compounds. Liverworts of the genus *Trichocolea* (family Trichocoleaceae) are a treasury of isoprenyl phenyl ethers. *Trichocolea hatcheri* Hodgs, which grows throughout New Zealnd, is distinguished from *T. mollissima* by its smaller size, dark green color, and prostrate habit. Microscopically, *T. hatcheri* is characterized by tapered leaf cilia, which lack swollen septae, and weak or absent cuticular ornamentationn. However, the taxonomy of *Trichocolea* in New Zealand is not settled[1].

Brachyglottis monroi (Hook. f) B. Nordenstam (Asteraceae compositae), previously *Senecio monroi*, is a shrub endemic to New Zealand[2,3]. *B. monroi* has been widely used in Maori traditional medicine for treatment of sores and wounds[4].

Hepatostolonophora paucistipula (Rodw.) J.J. Engel (family Geocalycaceae) is a rich source of sesquiterpenes in the New Zealand liverworts[5]. The species are morphologically very small, therefore, their classification is rather difficult. It is known that the species generally contain a large amount of diterpenoids such as ent-kaurane- and labdane-type[5].

In this study, the antiviral, antimicrobial activities and cytotoxicity of the crude extracts from medicinal plants were examined.

Materials and Methods

1. General experimental procedures

All solvents were distilled before use and were removed by rotary evaporation at temperatures up to 35℃. Octadecyl functionalized silica gel (C 18, Aldrich) was used for reversed-phase flash chromatography, and Davisil, 35-70 μm, 150 Å was used for Si gel flash chromatography. Preparative silica gel TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F_{254}, visualized with an UV lamp then by dipping in a vanillin solution (1% vanillin, 1% H_2SO_4 in EtOH) and heating. NMR spectrum of solutions at 25℃ were recorded at 300 MHz 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 materials

Trichocolea hatcheri (*T. hatcheri*) was collected from a steep earth bank in the Morrisons Creek area, Dunedin, New Zealand, in Fabruary 1996 [University of Otago Herbarium

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(OTA) specimen no. 048094].

Brachyglottis monroi (*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.

Hepatostolonophora paucistipula (*H. paucistipula*) was collected from Port Adventure, Stewart Island, in January 1994. This was identified by D. Glenny, Landcare Research, and a voucher specimen, OTA 046764, has been kept in the Otago University herbarium.

3. Preparation of the extract

Air-dried medicinal plants was macrate in redistilled ethanol in a Waring Blender, and then filtered. the residual marc was reextracted in the same way with more ethanol and chloroform. The combined filtrates were evaporated under reduced pressure to give a dark green gum which was stored at 4℃ until tested.

4. Screening for antiviral activity

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 were observed 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+ representing 25% through to whole well sized zones. The notation used is inhibition/antiviral activity. The type of antiviral 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.

5. Screening for antibacterial and antiyeast activities

Activity against the following bacterial strains and yeast was tested: multiresistant *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/mL. Test plates are prepared from Mueller Hinton agar containing 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, no growth.

6. Screening for antifungal activity

Activity against the following fungal strain was tested: *Trichophyton mentagraphytes* ATCC 28185. Fungal spore suspensions of the test oraganisms were applied to dextrose agar plates. Aliquots of the extract solutions were applied to filter paper discs, at 15 μg extract/disc, and dired at 37℃ for two hours. These discs were applied to the agar plates, two per plate, and incubated at 28℃.

7. Screening for cytotoxic activity

This is a measure of 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/mL, and 15 μL 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/mL. After incubation for three days, the plates were read using an Elisa palte reader at 540 ㎚ 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)[7].

Results and Discussion

1. Biological screening of the crude extract of *T. hatcheri*

Trichocolea hatcheri Hodgs (family Trichocoleaceae) grows throughout New Zealnd. Foliage plant collected from a steep earth bank in the Morrisons Creek area in Dunedin.

An extract of *T. hatcheri* was prepared by grinding dried plant material and extracting separately with ethanol then chloroform. The two extracts were combined, as their ^{1}H-NMR spectra were similar. A crude extract was cytotoxic to P388 murine leukaemia cells ATCC CCL 46 P388D1, (IC_{50} > 12,500 μg/mL) and BSC monkey kidney cells (50% of well at 150 μg/mL). Table 1 shows the mediocre antiviral activity against *Herpes simplex* Type I *virus* ATCC VR 733 and *Polio* Type I *virus* Pfizer vaccine strain, (50% activity, @ 5 mg/mL at 150 μg/disc). The crude extract inhibited the growth of the Gram-positive bacteria and fungus of the extract prepared from New Zealand medicinal plant. As indicated in Table 1, this crude extract inhibited the growth of the Gram-positive bacterium *Bacillus subtilis* ATCC 19659, (1 mm inhibition zone at 150 μg/disc) and the dermatophyte *Trichophyton*

mentagrophytes ATCC 28185, (6 mm inhibition zone at 150 μg/disc). No activity was observed against the fungus *Candida albicans* ATCC 14053 at 150 μg/disc. This extract showed weaker antimicrobial activity than chloramphenicol and nystatin (Table 1). However, this crude extract is stronger antimicrobial activity than the crude extract of *B. monroi* and this extract is inactive against P388 murine leukaemia cells ATCC CCL 46 P388D1.

Table 1. Biological assays of the crude extract from *T. hatcheri*

Extract	Cytotoxicity		
	BSC[a]	*Herpes simplex virus*[b]	*Polio virus*[b]
	+ +	+ +	+ +
	Antimicrobial activity[c]		
	B. subtilis	*C. albicans*	*T. mentagrophytes*
Extract	SM 1	-	HM 6
Chloramphenicol	SM 12	0	0
Nystatin	0	SM 11	HM 8
		P388	
Mitomycin C		59.7[d]	
Extract		〉 12,500[e]	

[a]% of well showing cytotoxic effects. @ 5 mg/mL, 150 μg/disc: + +: 50% activity.
[b]Cytotoxicity in antiviral assays. @ 5 mg/mL, 150 μg/disc: Zone of cytotoxic activity: + +: 50% activity.
[c]Width of zone of inhibition in mm: 150 μg/disc: -: no reduction in growth, 0: not determined. Chloramphenicol: 30 mcg/disc, Nystatin: 100 unit/disc. SM: Sharp margin, HM: hazy margin, numbers refer to zone of inhibition (mm)
[d]Toxicity of sample to P388 murine leukaemia cells ATCC CCL 46 P388D1 in ng/mL at 0.075 μg/disc. P388 : Concentration of the sample required to inhibit cell growth to 50% of a solvent control.
[e]Toxicity of sample to P388 murine leukaemia cells ATCC CCL 46 P388D1 in ng/mL at 75 μg/disc.

2. Biological screening of the crude extract of *B. monroi*

Brachyglottis monroi (Hook. f) B. Nordenstam (Asteraceae compositae) is a shrub endemic to New Zealand[2,3]. Foliage plant collected from the Dunedin Botanical Gardens. An extract of *B. monroi* was prepared by grinding dried plant material and extracting separately with ethanol then chloroform. The two extracts were combined, as their ^{1}H-NMR spectra were similar. A crude extract was cytotoxic to P388 murine leukaemia cells ATCC CCL 46 P388D1, (IC_{50} 23.96 μg/mL) and BSC monkey kidney cells (25% of well at 75 μg/mL). However, this crude extract is stronger cytotoxic activity than the extract of *B. monroi*. Table 2 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). The crude extract inhibited the growth of the Gram-positive bacteria and fungus of the extract prepared from New Zealand medicinal plant, which have been used by Maori for treatment of sores and wounds[4]. The activities are expressed by the diameter of the developed inhibition zones and compared with those of the widely antibious chloramphenicol and nystatin. As indicated in Table 2, this crude extract inhibited the growth of the Gram-positive bacterium *Bacillus subtilis* ATCC 19659, (1 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* ATCC 14053, at 150 μg/disc. This extract showed weaker antimicrobial activity than chloramphenicol and nystatin (Table 2).

Table 2. Biological assays of the crude extract from *B. monroi*

Extract	Cytotoxicity		
	BSC[a]	*Herpes simplex virus*[b]	*Polio virus*[b]
	+	+	0
	Antimicrobial activity[c]		
	B. subtilis	*C. albicans*	*T. mentagrophytes*
Extract	SM 1	-	SM 2
Chloramphenicol	SM 12	0	0
Nystatin	0	SM 12	SM 8
		P388	
Mitomycin C		65.9[d]	
Extract		23,956[e]	

[a]% of well showing cytotoxic effects. @ 5 mg/mL, 150 μg/disc: +: 25% activity.
[b]Cytotoxicity in antiviral assays. @ 5 mg/mL, 150 μg/disc: Zone of cytotoxic activity: +: 25% activity and 0: not determined.
[c]Width of zone of inhibition in mm: 150 μg/disc: -: no reduction in growth, detected, 0: not determined. Chloramphenicol: 30 mcg/disc, Nystatin: 100 unit/disc. SM: Sharp margin, numbers refer to zone of inhibition (mm)
[d]Toxicity of sample to P388 murine leukaemia cells ATCC CCL 46 P388D1 in ng/mL at 0.075 μg/disc. P388 : Concentration of the sample required to inhibit cell growth to 50% of a solvent control.
[e]Toxicity of sample to P388 murine leukaemia cells ATCC CCL 46 P388D1 in ng/mL at 150 μg/disc.

3. Biological screening of the ethanol extract of *H. paucistipula*

H. paucistipula is a rich source of sesquiterpenes in the New Zealand liverworts[5]. Liverwort, collected from Port Adventure, Stewart Island, gave the crude ethanol extract cytotoxic to P388 murine leukaemia cells ATCC CCL 46 P388D1, (IC_{50} 2.48 μg/mL). Table III shows no antiviral activity against *Herpes simplex* Type I *virus* ATCC VR 733 and *Polio* Type I *virus* Pfizer vaccine strain (5 mg/mL at 150 μg/disc). The crude ethanol extract inhibited the growth of the Gram-positive bacteria and fungus of the extract prepared from New Zealand liverwort. The activities are expressed by the diameter of the developed inhibition zones and compared with those of the widely antibious chloramphenicol and nystatin. As indicated in Table III, this crude extract inhibited the growth of the Gram-positive bacterium *Bacillus subtilis* ATCC 19659, (2 mm inhibition zone at 150 μg/disc). Antifungal activity was shown aganist the dermatophyte fungus *Trichophyton mentagrophytes* ATCC 28185, (7 mm inhibition zone at 150 μg/disc). Antiyeast activity was observed against the fungus *Candida albicans* ATCC 14053, (3 mm inhibition zone at 150 μg/disc). This extract showed

weaker antimicrobial activity than chloramphenicol and nystatin (Table 3)[8].

Table 3. Biological assays of the crude ethanol extract from *H. paucistipula*

Extract	Cytotoxicity		
	BSC[a]	*Herpes simplex virus*[b]	*Polio virus*[b]
	0	0	0
	Antimicrobial activity[c]		
	B. subtilis	*C. albicans*	*T. mentagrophytes*
Extract	SM 2	SM 3	SM 7
Chloramphenicol	SM 13	0	0
Nystatin	0	SM 10	SM 6
		P388	
Mitomycin C		61[d]	
Extract		2,482[e]	

[a]% of well showing cytotoxic effects. @ 5 mg/mL, 150 μg/disc; 0: not detected.
[b]Cytotoxicity in antiviral assays. @ 5 mg/mL, 150 μg/disc; Zone of cytotoxic activity: 0: not detected.
[c]Width of zone of inhibition in mm; 150 μg/disc; 0: not detected.
Chloramphenicol: 30 mcg/disc, Nystatin: 100 unit/disc. SM: Sharp margin, numbers refer to zone of inhibition (mm)
[d]Toxicity of sample to P388 murine leukaemia cells ATCC CCL 46 P388D1 in ng/mL at 0.075 μg/disc. P388 : Concentration of the sample required to inhibit cell growth to 50% of a solvent control.
[e]Toxicity of sample to P388 murine leukaemia cells ATCC CCL 46 P388D1 in ng/mL at 75 μg/disc.

Conclusion

In summary, the crude ethanol extract of *H. paucistipula* inhibited the growth of the Gram positive bacterium *Bacillus subtilis* ATCC 19659, (2 mm inhibition zone at 150 μg/disc) and the dermatophyte *Trichophyton mentagrophytes* ATCC 28185, (7 mm inhibition zone at 150 μg/disc), and toxic to P388 murine leukaemia cells ATCC CCL 46 P388D1 (IC_{50} 2.48 μg/mL at 75 μg/disc). This crude ethanol extract of *H. paucistipula* is the strongest antimicrobial and cytotoxic activities against P388 murine leukaemia cells ATCC CCL 46 P388D1. The separation of the main bioactive components from the extracts of plants need to be studied further and the results will be discussed elsewhere.

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