Antifungal Activity of Bioactive Fractions on the Dermatophytic Fungus

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Cytotoxic and antifungal activities were strongly shown by Fr. 4 (218 mg, P388 IC_{50} 652 ng/mL at 75 μg/disc, IC50 834 ng/mL at 30 μg/disc and HM 8). The subfraction 4 - 3 that showed the most activity was cytotoxic to murine leukaemia cells ATCC CCL 46 P388D1, (44.0 mg, P388 IC50 302 ng/mL at 30 μg/disc).

Key words : Cytotoxic and antifungal activities, *Trichophyton mentagrophytes*, murine leukaemia cells

Introduction

Liverworts are generally located in damp forested areas, growing on trees, rotting logs and rocks or on the ground. Frequently, several species of liverwort will be found growing intertwined, and some species grow to resemble a carpet covering large areas of the forest floor. In New Zealand, liverworts can be found throughout the country, generally in rainforest areas, growing on the ground, or on rotting logs in damp and humid regions[1].

Hepatostolonophora paucistipula (Rodw.) J.J. Engel (family Geocalycaceae) is a rich source of sesquiterpenes in the New Zealand liverworts[2]. There are no literature reports on the biological chemistry of this genus. In this study, the antiviral and antimicrobial activities and cytotoxicity of the crude ethanol extract from *H. paucistipula* were examined and have investigated their cytotoxic and antifungl 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-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.

2. Plant material

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 *H. paucistipula* (76.3 g) was ground and macrated in redistilled ethanol (1,000 mL) in a Waring Blender, and then filtered. The residual marc was reextracted in the same way with more ethanol (3 x 300 mL). The combined filtrates were evaporated under reduced pressure to give a crude extract (1.585 g) which was stored at 4 ℃ until tested.

4. Preparation of bioactive fractions

The crude ethanol extract (1.585 g) was subjected to flash column chromatographyon C 18 (10 g) with a H_2O : CH_3CN : $CHCl_3$ gradient. The bioactive subfractions were chromatographed on silica gel with an ethyl acetate : hexane. These fractions and subfractions were stored at 4 ℃ until tested.

5. Screening for antiviral activity

The extract was applied (30 μL of a 5 mg/mL solution)

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to a small filter-paper disc, dried, and assayed for antiviral activity using Schroeder et al. methods[3]. 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.

6. 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.

7. 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 ℃ for two hours. These discs were applied to the agar plates, two per plate, and incubated at 28 ℃.

8. 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 30 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 mg/mL. After incubation for three days, the plates were read using an Elisa palte 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).

Results and Discussion

1. Biological screenings of the crude ethanol extract

H. paucistipula is a rich source of sesquiterpenes in the New Zealand liverworts[2]. Liverwort, collected from Port Adventure, Stewart Island, gave the crude ethanol extract cytotoxic to murine leukaemia ATCC CCL 46 P388D1, (IC_{50} 2.48 μg/mL). Table I shows no antiviral activity against *Herpes simplex* Type I *virus* ATCC VR 733 and *Polio Type* I virus (Pfizer vaccine strain) (5 ng/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 I, 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 dermatophytic 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 (Tables I and Table II)[4].

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

	Cytotoxicity		
Extract	BSC[a]	*Herpes simplex virus*[b]	*Polio virus*[b]
	-	-	-
	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 cytotoxity		
Mitomycin C		61[d]	
Extract		2,482[d]	

a% of well showing cytotoxic effects. @ 5 mg/mL. 150 μg/disc: -: no activity. bCytotoxicity in antiviral assays. @ 5 mg/mL. 150 μg/disc: Zone of cytotoxic activity: -: no activity. cWidth 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, numbers refer to zone of inhibition (mm) dToxicity 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. eToxicity of sample to P388 murine leukaemia cells ATCC CCL 46 P388D1 in ng/mL at 150 μg/disc.

Fig. 1 shows the potent cytotoxic activity of the crude ethanol extract against P388 murine leukaemia cell lines. the cytotoxic activity of the crude extract was in a dose-dependent inhibition of cell proliferation. This extract showed a dose-

dependent increase of cell antiproliferation after treatment with of the crude ethanol extract of H. paucistipula.5-6) This crude extract-mediated cytotoxicity was rapidly increased in the MTT method when its concentrations or absorbances were raised from 1.989 to 2.892. However, the other absorbances were most strong cytotoxicity.

Table II. List of microorganisms used for antimicrobial susceptibility test.

Gram-positive bacterium	
Bacillus subtilis	ATCC 19659
Fungi	
Candida albicans	ATCC 14053
Trichophyton mentagrophytes	ATCC 28185

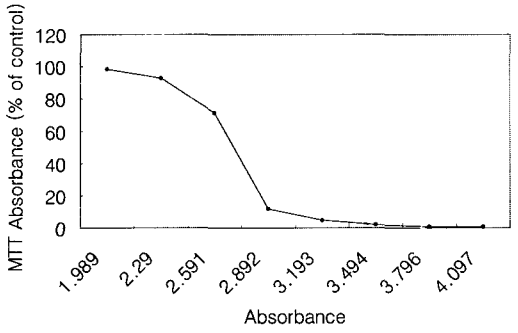

Fig. 1. In vitro cytotoxic effect of the crude ethanol extract of *H. paucistipula* by the MTT method. This extract was serially diluted in RPMI-1640 with 10 % FBS and mixed with equal volume of murine leukaemia cell lines ATCC CCL 46 P388D1 (75 μg/disc). The colometric method was performed as described in materials and methods. Data are mean values of results obtained from three sets of experiments.

2. Cytotoxic and antifungal activities of bioactive fractions

The crude ethanol extract (0.836 g) was fractioned into Fr. 1 - Fr. 9 using C-18 silica gel column chromatography as described previously[7-8]. Chromatography on C-18 (10.0 g) with a H_2O, MeCN, $CHCl_3$, n-hexane gradient gave nine fractions. The column fractions were combined based on visually similar TLC results. These combined fractions were assayed against murine leukaemia cells ATCC CCL 46 P388D1 and *T. mentagrophytes* and the activity was found to be spread over five fractions that were eluted with 1 : 1 H_2O/CH_3CN, 1 : 3 H_2O/CH_3CN, 1 : 9 H_2O/CH_3CN. CH_3CN, C_6H_{12}, $CHCl_3$ and CH_3CN. Also, the fraction 6 was cytotoxic to murine leukaemia cells ATCC CCL 46 P388D1. Reverse-phase flash column chromatography of a crude extract gave most of the mass in the polar fractions, eluted with H_2O : CH_3CN (1 : 3). Among them, the fractions Fr. 3 - Fr. 6, Fr. 8 and Fr. 9 are cytotoxic to murine leukaemia cells ATCC CCL 46 P388D1. The order of cytotoxic activity was shown Fr. 4 > Fr. 3 > Fr. 9 > Fr. 5 > Fr. 6 > Fr. 8 > Fr. 1 = Fr. 2 = Fr. 7 (Table III). The fraction 4 that was the most active against murine leukaemia cells ATCC CCL 46 P388D1 and the dermatophytic fungus *T. mentagrophytes* eluted with 1 : 3 H_20 : CH_3CN (218 mg, P388 IC50 652 ng/mL at 75 μg/disc, IC_{50} 834 ng/mL at 30 μg/disc and HM 8)[4]. All fractions were antifugal activity. However, fractions 1, 2, 6, and 7 were inactive against the dermatophytic fungus *T. mentagrophytes*. (Tables II and III)

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

Fraction No.	Eluent	Vol. (mL)	Mass (mg)	IC_{50} (ng/mL)[b]	*T. mentagrophytes*[c]
1	H_2O	34	79	) 62,500	-
2	3:1 H_2O/CH_3CN	34	33	) 62,500	-
3	1:1 H_2O/CH_3CN	34	62	5,936	HM 5
4	1:3 H_2O/CH_3CN	34	218	652	HM 8
5	CH_3CN	34	6	16,418	HM 3
6	1:1 $CHCl_3/CH_3CN$	34	05	34,955	-
7	$CHCl_3$	34	28	) 62,500	-
8	C_6H_{12}, $CHCl_3$	68	12	41,615	HM 1
9	CH_3CN	34	10	10,929	HM 2

aEach fraction was examined in eight concentrations in triplicated experiments. bIC50 represents the concentration of a fraction required for 50% inhibition of cell growth. Mitomycin C was used as control and exhibited an IC50 61.0 ng/mL. Toxicity of sample to murine leukaemia cells ATCC CCL 46 P388D1 in ng/mL at 75 μg/disc. cWidth of zone of inhibition in mm; 150 μg/disc; -: not detected, Nystatin; HM 6 at 100 unit/disc. HM: Hazy margin, numbers refer to zone of inhibition (mm).

Fig. 2 showed the potent cytotoxic activity of the crude ethanol extract and fractions of *H. paucistipula* against murine leukaemia cells ATCC CCL 46 P388D1. In general, the cytotoxic activity of these fractions was in a dose-dependent inhibition of cell proliferation. All of these fraction showed a dose-dependent increase of cell antiproliferation after treatment with of the crude ethanol fractions of *H. paucistipula*. However, the subfractions 1, 2, and 7 were inactive against murine leukaemia cells ATCC CCL 46 P388D1. The susceptibility of P388 cancer cell lines to fraction 4 was quite sensitive and the most cytotoxic activity. This fraction-mediated cytotoxicity was rapidly increased in the MTT method when their concentrations or absorbances were raised from 1.989 to 2.592. However, the other absorbances were most strong cytotoxicity. Also the subfractions 3, 5, 6 and 9 were gradually increased when their absorbances were raised from 1.989 to 3.796. The other subfractions (1, 2 and 7) which raised from 1.989 to 4.097 absorbances were not sensitive to murine leukaemia cells ATCC CCL 46 P388D1.

Reverse-phase flash column chromatography concentrated the antifungal and cytotoxic activities in fraction 4 eluted with H_2O - CH_3CN 1 : 3. Chromatography on silica gel (1.0 g) with an ethyl acetate - cyclohexane gradient gave six fractions. The second silica gel column chromatography of fraction 4 - 3 gave most of the mass in the less polar fraction, eluted with 5 %

and 10 % ethyl acetate - hexane. Among them, fr. 4 - 3 that eluted with 5 % and 10 % ethyl acetate - hexane is the most cytotoxic activity to murine leukaemia cells ATCC CCL 46 P388D1 (44.0 mg, P388 IC_{50} 302 ng/mL at 30 μg/disc, IC_{50} 577 ng/mL at 7.5 μg/disc). A comparison of IC_{50} (ng/mL) values of these subfractions in cancer cells showed that their susceptibility to these subfractions decreased in the following order; Subfr. 4 - 3 > Subfr. 4 - 2 > Subfr. 4 - 4 > Subfr. 4 - 5 > Subfr. 4 - 1 > Subfr. 4 - 6 (Table IV)[4].

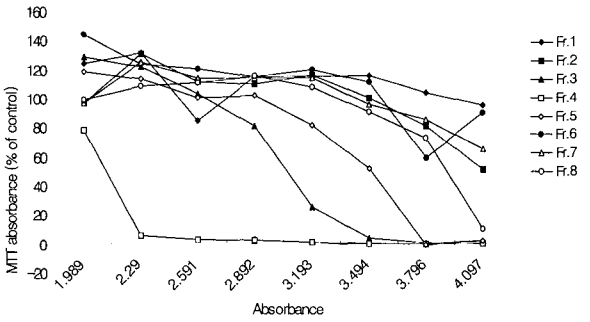

Fig. 2. In vitro cytotoxic effect of the crude ethanol fractions of *H. paucistipula* by the MTT method. This fractions were serially diluted in RPMI-1640 with 10 % FBS and mixed with equal volume of murine leukaemia cells ATCC CCL 46 P388D1 (75 μg/disc). The colometric method was performed as described in materials and methods. Data are mean values of results obtained from three sets of experiments.

Table IV. In vitro cytotoxic activity of bioactive subfractions on murine leukaemia cells ATCC CCL 46 P388D1 by the MTT method[a].

Subfr. No.	Eluent	Vol. (mL)	Mass (mg)	IC_{50} (ng/mL)[c]
4 - 1	Hex, 2 %, 5 % EtOAC/Hex	20	0.4	13,193
4 - 2	5 % EtOAC/Hex	12	6.5	1,492
4 - 3	5 %, 10 % EtOAC/Hex	28	44.0	302
4 - 4	15 %, 20 % EtOAC/Hex	32	8.5	7,595
4 - 5	20 %, 30 % EtOAC/Hex	28	28.5	9,041
4 - 6	30 %, 40 %, 50 %, 75 % EtOAC/Hex, EtOAC	76	11.5	>25,000

[a]Each subfraction was examined in six concentrations in triplicated experiments. [b]IC50 represents the concentration of a subfraction required for 50 % inhibition of cell growth. [c]Mitomycin C was used as control and exhibited an IC50 57.3 ng/mL. Toxicity of sample to murine leukaemia cells ATCC CCL 46 P388D1 in ng/mL at 30 μg/disc. EtOAC: ethyl acetate, Hex: n-hexane.

Fig. 3 showed the potent cytotoxic activity of the crude ethanol subfractions of *H. paucistipula* against murine leukaemia cells ATCC CCL 46 P388D1. In general, the cytotoxic activity of these subfractions was in a dose-dependent inhibition of cell proliferation. All of these subfraction showed a dose-dependent increase of cell antiproliferation after treatment with of the crude ethanol subfractions of *H. paucistipula*. However, the subfraction 4 - 6 was inactive against murine leukaemia cells ATCC CCL 46 P388D1. The susceptibility of P388 cancer cell lines to subfraction 4 - 3 was quite sensitive and the most cytotoxic activity. This subfraction-mediated cytotoxicity was rapidly increased in the MTT method when their concentrations or absorbances were raised from 1.989 to 2.592. However, the other absorbances were most strong cytotoxicity. Also the subfraction 4 - 2 was gradually increased when their absorbances were raised from 1.989 to 3.193. However, the other absorbances were similar to subfraction 4 - 3. The other subfractions (4 - 1, 4 - 4 and 4 - 5) which raised from 1.989 to 4.097 absorbances were gradually sensitive to murine leukaemia cells ATCC CCL 46 P388D1.

Fig. 3. In vitro cytotoxic effect of the crude ethanol subfractions of *H. paucistipula* by the MTT method. This subfractions were serially diluted in RPMI-1640 with 10 % FBS and mixed with equal volume of murine leukaemia cells ATCC CCL 46 P388D1 (30 μg/disc). The colometric method was performed as described in materials and methods. Data are mean values of results obtained from three sets of experiments.

In conclusion, cytotoxic and antifungal activities were strongly shown by Fr. 4 (218 mg, P388 IC_{50} 652 ng/mL at 75 μg/disc, IC_{50} 834 ng/mL at 30 μg/disc and HM 8). The subfraction 4 - 3 that showed the most activity was cytotoxic to murine leukaemia cells ATCC CCL 46 P388D1 (44.0 mg, P388 IC_{50} 302 ng/mL at 30 μg/disc). The separation of the main components from the bioactive subfraction 4 - 3 of *H. paucistipula* extract need to be studied further and the results will be discussed elsewhere.

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