

Antifungal Activity of Chloroform Extract from *Riccardia marginata* on the Dermatophytic Fungus *Trichophyton mentagrophytes*

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The effects of chloroform extract from *Riccardia marginata* on antifungal activity were investigated. The crude chloroform extract of *R. marginata* inhibited the growth of the Gram positive bacterium *Bacillus subtilis* ATCC 19659, (4 mm inhibition zone at 150 µg/disc) and the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (6 mm inhibition zone at 150 µg/disc), and inactive to P388 murine leukaemia cells ATCC CCL 46 P388D1, (IC₅₀ >25,000 µg/mL at 150 µg/disc). This crude chloroform extract of *R. marginata* showed strong antifungal activity against the dermatophytic fungus *Trichophyton mentagrophytes*.

Key words : *Riccardia marginata*, *Bacillus subtilis*, *Trichophyton mentagrophytes*, antifungal activity

Introduction

Liverworts are the only class of the Bryophytes that contain complex oil bodies¹⁾, and so are capable of synthesising a vast range of lipophilic aromatic and terpenoid compounds; it is perhaps because of this that liverworts have been more thoroughly investigated than mosses or hornworts for their rich and varied chemistry.^{2,3)} A recent review by Asakawa states more than 700 terpenoids and 220 aromatic compounds (excluding flavonoids) have been isolated from or detected in the Hepaticae⁴⁾. It is possible that the chemicals synthesised by these primitive soft-bodied plants protect them from external attack, whereas higher plants protect themselves physically with thorns, and spikes and thick bark (as well as chemical forms of defence)³⁾. Perhaps the best evidence for this chemical form of defence is that liverworts are rarely found damaged by insects, bacteria, fungi or mammals. *Riccardia marginata* (Colenso) Pearson (family Aneuraceae) is endemic to New Zealand⁵⁾, found growing on old logs in wet forests⁶⁾. No previous chemistry of *R. marginata* has been reported, but the genus *Riccardia* has yielded a range of compounds²⁾, including sesquiterpene-linked phenol derivatives^{7,8)}, indole alkaloids^{9,10)}

and bis-bibenzyls^{11,12,13)}. Brominated bibenzyls have previously been found in the red alga *Polysiphonia urceolata*¹⁴⁾. A crude extract of one of our collections of *R. marginata* showed activity against *Bacillus subtilis*, *Candida albicans* and *Trichophyton mentagrophytes* in our antimicrobial screening¹⁵⁾.

In this study, the antimicrobial activity of the crude ethanol extract from *Riccardia marginata* was 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°C. Preparative silica gel TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F₂₅₄, visualized with a UV lamp then by dipping in a vanillin solution (1% vanillin, 1% H₂SO₄ in EtOH) and heating.

2. Plant materials

Riccardia marginata (*R. marginata*) was collected from New Zealand's sub-Antarctic Auckland Islands in January 1994 (OTA046755), and near the Kiahoka Lakes, Cape Farewell, West Coast, New Zealand (OTA 050850) in March 1999. Collections were identified by *R. Tangney* and voucher specimens are deposited in the University of Otago Herbarium.

3. Preparation of the extract

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Air-dried *R. marginata* was macerate in redistilled chloroform in a Waring Blender, and then filtered. the residual marc was reextracted in the same way with chloroform. The combined filtrates were evaporated under reduced pressure to give a dark green gum which was stored at 4°C until tested.

4. Screening for antiviral activity

The extract was applied (30 µL of a 5 mg/mL solution) to a small filter-paper disc, dried, and assayed for antiviral activity using Schroeder et al.'s methods¹⁶. 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), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Candida albicans* (ATCC 14053) and *Cladosporium resinae* (ATCC 52833). 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 10⁴ colony forming units onto the test, control (solvent), and blank (agar only) plates with a multipoint inoculator. 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 as follows: (-) inhibition, no reduction in growth compared with the control, (+) inhibition, no growth. Solutions of compound 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. HM and SM refer to the observed margin surrounding the zone of inhibition. (H= hazy, S= sharp).

6. Screening for antifungal activity

Activity against the following fungal strain was tested:

Trichophyton mentagrophytes (ATCC 28185) local strain. Fungal spore suspensions of the test organisms were applied to dextrose agar plates. Aliquots of the extract solutions were applied to filter paper discs, at 30 µg extract/disc, and dried at 37°C for two hours. These discs were applied to the agar plates, two per plate, and incubated at 28°C.

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 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 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).

Results and Discussion

Riccardia marginata (Colenso) Pearson (family Aneuraceae) grows throughout New Zealand. Foliage plant collected from sub-Antarctic Auckland Islands and near the Kiahoka Lakes, Cape Farewell, West Coast, New Zealand. A crude extract of *R. marginata* was prepared by grinding dried plant material and extracted with chloroform. A crude extract was not cytotoxic to P388 murine leukaemia cells ATCC CCL 46 P388D1, (IC₅₀ > 25,000 µg/mL) and BSC monkey kidney cells (150 µg/mL). Table 1 does not show the 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 extract inhibited the growth of the Gram-positive bacteria and fungus of the extract prepared from *R. marginata*. As indicated in Table 1, this crude extract inhibited the growth of the Gram-positive bacterium *Bacillus subtilis* ATCC 19659, (4 mm inhibition zone at 150 µg/disc) and the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (6 mm inhibition zone at 150 µg/disc). No activity was observed against the fungus *Candida albicans* (ATCC 14053) and *Cladosporium resinae* (ATCC 52833), against the Gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) at 150 µg/disc. This extract showed weaker antimicrobial activity than chloramphenicol and nystatin (Tables 1 and 2)¹⁷.

In conclusion. the crude ethanol extract of *R. marginata*

inhibited the growth of the Gram positive bacterium *Bacillus subtilis* ATCC 19659, (4 mm inhibition zone at 150 µg/disc) and the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (6 mm inhibition zone at 150 µg/disc), and inactive to P388 murine leukaemia cells ATCC CCL 46 P388D1, (IC₅₀ >25,000 µg/mL at 150 µg/disc). We suppose that this crude chloroform extract of *R. marginata* is the strong antimicrobial activity. The separation of the main bioactive components from the extracts of plants need to be studied further and the results will be discussed elsewhere.

Table 1. Biological activities of the crude extract from *R. marginata*

Assay	Tested material			
	Crude extract	Chloramphenicol	Nystatin Gentamycin	Mitomycin C
Cytotoxicity BSC-1 cells ^a P388 IC ₅₀	25,000 ^b	-	-	59.7 ^c
Antiviral activity ^d <i>Herpes simplex virus</i> <i>Polio virus</i>	-	-	-	-
Antimicrobial activity ^e				
<i>B. subtilis</i>	SM 4	SM 12	0	0
<i>E. coli</i>	-	0	0	SM 9
<i>P. aeruginosa</i>	-	0	0	SM 10
<i>C. albicans</i>	-	0	SM 11	0
<i>C. resinae</i>	-	0	SM 10	0
<i>T. mentagrophytes</i>	SM 6	0	HM 8	0

^a% of well showing cytotoxic effects, with virus growing in cytotoxic zone. @ 5 mg/mL, 150 µg/disc: -: no activity. BSC-1 cells: African green monkey kidney cells. ^bToxicity of sample to P388 murine leukaemia cells (ATCC CCL 46 P388D1) in ng/mL at 150 µg/disc. ^cToxicity 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. ^dAntiviral assays. @ 5 mg/mL, 150 µg/disc: Zone of cytotoxic activity: -: no activity. ^eWidth 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)

Table 2. List of microorganisms used for antimicrobial susceptibility test.

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

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