Research Article

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Biological activities of undescribed North American lichen species

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Abstract

BACKGROUND: Lichens provide a large array of compounds with the potential for pharmaceutical development. In the present study, extracts from three previously undescribed North American lichen species were examined for antioxidant, antibacterial and anticancer activities.

RESULTS: The results from this study demonstrated the following: (i) *Acarospora socialis* ethanol extract exhibited significant DPPH antioxidant scavenging activities, which were concentration dependent; (ii) acetone and ethyl acetate extracts of *Xanthoparmelia mexicana* inhibited Gram-positive bacteria but had no effect on Gram-negative bacteria; *X. mexicana* acetone extract yielded a minimum inhibitory concentration (MIC) of 20.9 μg mL^{-1} against *Staphylococcus aureus*, and 41.9 μg mL^{-1} against *Enterococcus faecalis*; (iii) acetone extract of *Lobothallia alphoplaca* inhibited growth of cultured breast cancer MCF-7 cells with an effective concentration (EC_{50}) of 87 μg mL^{-1} ; the MCF-7 cell cycle appears arrested in the G2 phase, whereas the DNA synthesis cell cycle (S) may be inhibited.

CONCLUSION: New lichen species that possess strong biological activities have been identified. These lichens comprise secondary metabolites that possess antioxidant, antibacterial and anticancer properties.

Keywords: lichens; North America; biological activities; *Acarospora socialis*; *Xanthoparmelia mexicana*; *Lobothallia alphoplaca*

INTRODUCTION

Lichens and their diverse metabolic products provide a large array of compounds with the potential for pharmaceutical development.[1] General characteristics of lichens and their historical ethnobotanical uses have been described previously.[2] However, the search for novel antibiotic compounds and for anticancer compounds is particularly relevant in view of the spread of antibiotic-resistant bacteria and the growing need for better and improved cancer drugs, respectively.

The antioxidant, antibacterial and antiproliferative activities of lichens have been the focus of several reports. Generally, antioxidant activity may be relevant as a mechanism for reversing cellular damage induced by metabolic dysfunction or by external factors. Radical scavenging capacity by several lichen extracts was reported recently,[3,4] as well as in older work using extracts from cultured lichen material.[5] Reports by Turkez and co-workers[6] suggest a protective role of lichen extracts against specific types of oxidative cell damage.

The search for lichen-derived antibiotic compounds started in the post-WWII era[7] and has continued to the present. Shrestha and St Clair[8] tabulated some of the more recent work, and additional reports have appeared since.[9–11] Unfortunately, of the hundreds of natural products investigated, only a few have reached clinical trials and none of these originated from lichens.[12] We are not aware of any current clinical work with lichen-derived compounds. Commercial natural health products tend to be used topically, and internally consumed lichen extracts have proven toxic.[13]

Nevertheless, the toxicity of lichen extractives may yet turn out to be beneficial in relation to their potential use as anticancer drugs. Usnic acid, a common lichenic acid, is reported to have some effect against lung carcinoma in mice.[14] Much of the work on cultured neoplastic cells performed between 1979 and 2012 has been summarized.[8,11] More recent investigations report antiproliferative activity against a variety of cultured cell lines.[8,10,11,15] To our knowledge, only two investigations included non-neoplastic cells as controls and showed a degree of selectivity (SI) of the lichen compounds against the transformed cell as compared to normal cells.[16,17] Studies on living model animals are rarely performed or

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reported, but of note is the report of inhibition of tumor angiogenesis by olivetoric acid.[18]

The objective of this study was to investigate the *in vitro* biological effects of lichens, with the expectation that further work will be possible to characterize the chemical nature of the active constituents and elucidate in some detail their mechanisms of action. Three lichen species occurring in the western USA, which have not been studied to date, were selected for investigation: *Acarospora socialis*, *Xanthoparmelia mexicana* and *Lobothallia alphoplaca*. The biological activity of these lichens was assessed by determining their antioxidant, antibacterial and antiproliferative activities.

EXPERIMENTAL

Lichens

Two of the lichens – *A. socialis* and *L. alphoplaca*– were obtained from Joshua Tree National Park in California, whereas *X. mexicana* was collected on Catalina Island, 22 miles southwest of Los Angeles, California. The images of the lichen species are depicted in Fig. 1. These species were collected and identified by lichenologist Kerry Knudsen, and voucher specimens were deposited in the herbarium of the University of California at Riverside, under accession numbers 15106.1, 15119.1 and 15436, respectively. The collected material was kept until needed in sealable plastic bags at room temperature.

Chemicals and instruments

Extraction solvents, bacterial media, dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and CellTiter-Blue® were purchased from Thermo Fisher Scientific, Waltham, MA, USA. Materials for cell viability staining including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide were obtained from Sigma-Aldrich (St Louis, MO, USA). The instruments utilized in this study included a Biotek PowerWaveXS microplate spectrophotometer (Biotek Instruments Inc., Winooski VT, USA), a BD ACCURI C6 flow cytometer (BD Biosciences, San Jose, CA, USA) and a BMG FLUOstar Optima Microplate Reader (BMG Labtech, Offenburg, Germany).

Preparation of lichen extracts

Lichens were pulverized in a mortar and pestle; the lichen powder was stirred with 100 mg mL^{-1} solvent (99.5% ethanol, acetone or ethyl acetate) at 120 rpm and 37 °C for 24 h in an Innova© R42 incubator shaker (New Brunswick, CT, USA), and thereafter filtered through Whatman No. 1 filter paper. The extraction yield was calculated by subtracting the mass of the filtered solids from the original mass of ground lichen.

Antioxidant activity

The procedures of Sharma and Bhat[19] and Ranković *et al.*[20] were followed to determine the free radical scavenging activity of lichen extracts. A 1 mmol L^{-1} solution of DPPH was prepared (in the same solvent as extract); 1 mL of the DPPH solution was mixed with 25 µL solvent or extract and absorbency at 517 nm (A_{517}) was read in a Shimadzu UV-2450 spectrophotometer (Shimadzu Scientific, MD, USA) at 30 and 60 min. Results from three independent and parallel experiments were expressed as percent absorbance compared to solvent only. The

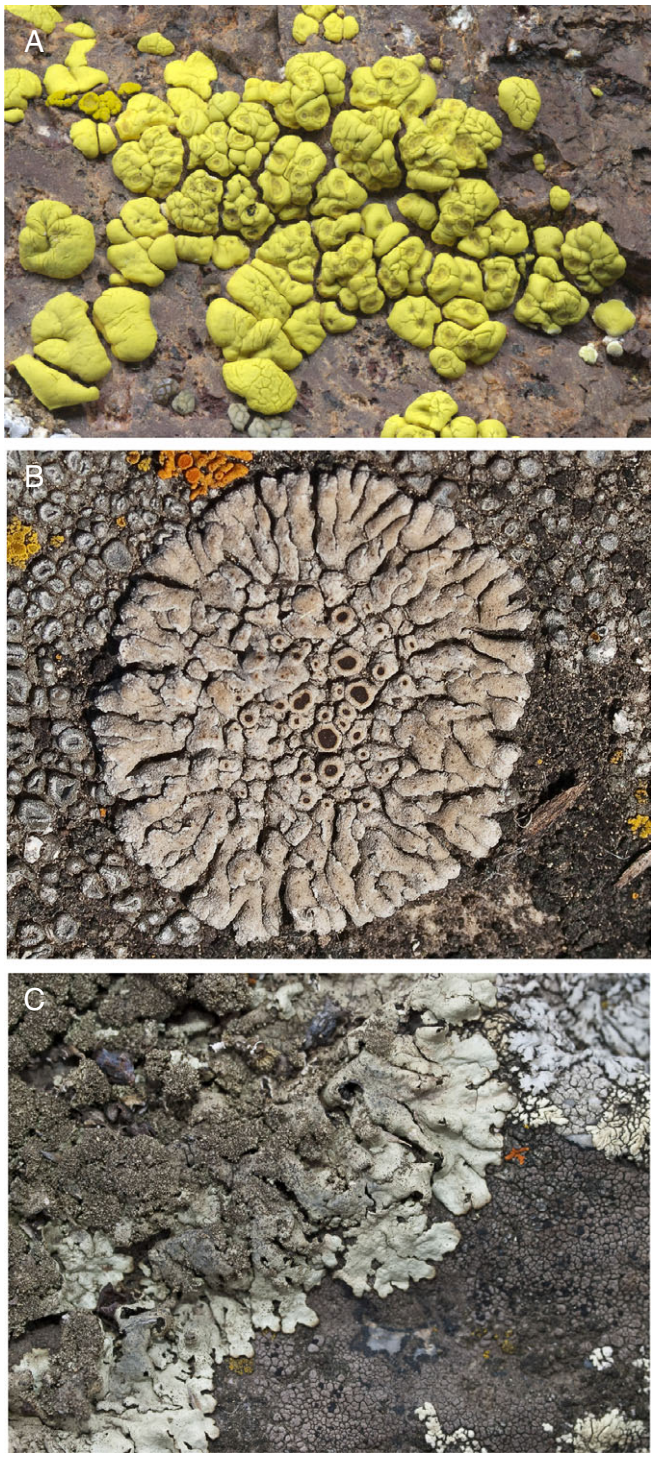

Figure 1. Lichens investigated: (A) *Acarospora socialis*; (B) *Lobothallia alphoplaca*, (C) *Xanthoparmelia mexicana* (all images credited to S. Sharnoff).

DPPH radical concentration was calculated using the following equation:

$$\text{Antioxidant (DPPH – scavenging) activity (\%)} = \left[\left(A_0 - A_1\right) / A_0\right] \times 100$$

where A_0 is the absorbance of the negative control and A_1 is the absorbance of the reaction mixture or standard.

Anticancer activity

Cell culture

Human breast cancer MCF-7 cells (ATCC HTB-22) were purchased from the American Type Cell Culture Collection (ATCC, Manassas, VA, USA) and maintained in supplemented Dulbecco's Modified Eagle Medium (DMEM). The DMEM was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-amphotericin B. The cell line was incubated at 37 °C in a humidified 5% CO_2 atmosphere.

Anticancer activity assay

MCF-7 cells were seeded in a 96-well tissue culture microplate at a density of 10 000 cells per well, then incubated at 37 °C under 5% CO_2 for 24 h. Next, the lichen extract was concentrated five times to a final metabolite concentration of 17.25 mg mL^{-1} in order to reduce the amount of acetone used in the treatment. Upon the MCF-7 cells reaching confluency, the medium was removed from the wells and 200 µL lichen extract diluted in non-supplemented DMEM was added in triplicate. The concentration of lichen metabolites in the treatments ranged from 5.39 to 517.5 µg mL^{-1}. Note that the lichen metabolite concentration must be expressed as a µg mL^{-1} value, as the active compounds have not yet been isolated and identified. For clarity, the concentration of the positive control, *cis*-diammineplatinum(II) dichloride (cisplatin), is given in both molar concentration and mass per volume concentration. Appropriate negatives and controls were also added. The positive control, *cis*-diammineplatium(II) dichloride (cisplatin), was prepared as a 198 µg mL^{-1} (0.66 mmol L^{-1}) stock solution in sterile 0.9% NaCl solution. It was serially diluted from 0.6 µg mL^{-1} (2 µmol L^{-1}) to 24 µg mL^{-1} (80 µmol L^{-1}) for treatment conditions. The negative control was an untreated control group of MCF-7 cells that were grown in non-supplemented DMEM. A 12 mmol L^{-1} solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in phosphate-buffered saline was prepared, filter sterilized and stored in the dark at 4 °C. The plate was incubated at 37 °C and 5% CO_2 atmosphere for 44 h, followed by the addition of 20 µL MTT solution to each well. Next, the plates were incubated at 37 °C and 5% CO_2 for 4 h. The MTT solution was then removed from each well and replaced with 50 µL dimethyl sulfoxide (DMSO). The plates were covered in foil and mixed on a Belly Dancer shaker for 10 min. The absorbance was then measured at 490 nm and 650 nm with a BioTek Powerwave XS spectrophotometer. To determine cell survival, the absorbance of the samples, controls and negatives was measured and compared to baseline and negative control. All experiments were run in triplicate.

Cell culture treatment and analysis

Cell cycle analysis was performed in triplicate by treating MCF-7 cells with 5 µL extract per 200 µL media, incubating for 48 h, trypsinizing and collecting them, then fixing the cells with 70% ethanol. The fixed cells were then stained with propidium iodide (50 µg mL^{-1}) for 30 min at room temperature. The cell cycle analysis was performed on a BD ACCURI C6 flow cytometer and was used to determine the frequency of cells that had their cell cycles in the G1, S and G2 phases.

Antibacterial activity

Microorganisms and media

Bacteria used as test organisms in this study included both Gram-positive (*Streptococcus pyogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*). All bacteria were acquired from ATCC and stored at −80 °C as a solution comprising 50% glycerol and 50% cell suspension in Luria–Bertani (LB) broth.

When taken out of cryostorage, one loop of bacteria was streak plated on Mueller Hinton II (MHII) agar and incubated at 37 °C until individual colonies were sighted. Next, one colony was taken from the plate and used to inoculate 10 mL MHII broth. The broth culture was incubated at 37 °C until it reached a turbidity level equal to that of a 0.5 McFarland Turbidity standard (~1.5 × 10^8 CFU mL^{-1}).

Table 1. Zone of inhibition (mm) by *X. mexicana* extracts

Solvent	Method[a]	*S. aureus* (G+)	*S. pyogenes* (G+)	*E. faecalis* (G+)
Acetone	Dry	11 ± 0.5	18 ± 1.0	11 ± 0.5
Acetone	Wet	15 ± 1.0	20 ± 1.5	13 ± 1.0
Ethyl acetate	Dry	12 ± 0.5	19 ± 2.0	11 ± 1.0
Ethyl acetate	Wet	15 ± 1.0	21 ± 1.5	14 ± 1.5

[a] Wet: disk placed on agar before extract treatment; Dry: disk soaked in lichen extract, dried, then placed on agar.

Zone of inhibition

Initial tests as reported in Table 1 were carried out by agar disk diffusion tests, also commonly referred to as the Kirby–Bauer susceptibility procedure.[21] The plates used were LB agar plates inoculated with bacterial isolates via cotton swab, which covered the entire agar surface with bacteria. The disks were sterile 6 mm sizes punches of Whatman No. 1 filter paper, which was then loaded with lichen sample. Dry disks were prepared by pipetting 5 µL of 100 mg mL^{-1} extract (500 µg lichen metabolites dissolved in 5 µL solvent) onto each disk; the disks were then dried until all the solvent evaporated and placed on LB agar plates. The wet disks were prepared by placing sterile disks on the agar plates; then 5 µL of 100 mg mL^{-1} extract (500 µg lichen metabolites dissolved in 5 µL solvent) was pipetted onto the disk. The plates were then immediately incubated at 37 °C for 24 h. After incubation, the zone of inhibition was determined by measuring the diameter of the growth-inhibited area with a ruler. Appropriate solvent control disks were evaluated as well. All experiments were performed in triplicate. The sensitivity of microorganisms to the acetone extracts of the investigated lichen species was tested by determining the minimum inhibitory concentration (MIC).

Minimum inhibitory concentration

The MIC was determined in triplicate as follows: an 18 h broth culture (37 °C and 150 rpm) was diluted to 10 × 10^6 CFU mL^{-1} and 100 µL added to the microplate, together with lichen extract dilutions (from 0.86 to 860 µg mL^{-1}) and appropriate solvent and negative controls. Baseline absorbances at 600 nm were measured using a BioTek PowerwaveXS spectrophotometer, followed by measuring the same plates incubated at 37 °C for 24 h. At 24 h, 40 µL CellTiter-Blue® was added to each well of the microplate. The plates were incubated for 4 h at 37 °C, then fluorescence was measured at 544 nm excitation/590 nm emission. To ensure accuracy of the spectrophotometer absorbance and fluorescence readings, 10 µL of each treatment condition was streaked onto LB agar plates, then incubated at 37 °C for 24 h. After 24 h, the growth on each plate was noted.

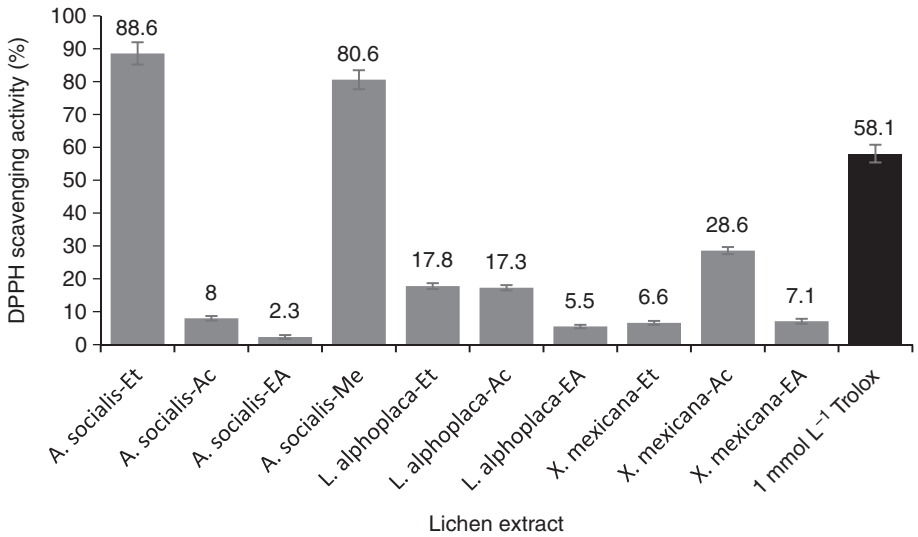

Figure 2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging ability of lichen extracts. Et, ethanol; Ac, acetone; EA, ethyl acetate; Met, methanol; Trolox, positive control.

Statistical analyses

Statistical analyses were performed with Excel, GraphPad Prism 6 and FlowJo software packages. To determine the statistical significance of antioxidant activity, Student's *t*-test was used. All values were expressed as mean ± standard deviation (SD) of three parallel measurements. To determine the statistical significance of cell death, a two-sample *t*-test assuming unequal variance was used.

RESULTS AND DISCUSSION

Antioxidant activity

The antioxidant properties of both *A. socialis* ethanolic and methanolic extracts were more effective than any other extracts examined in this study (Fig. 2). Furthermore, the *A. socialis* ethanolic and methanolic extracts showed higher DPPH free radical scavenging activities (88.6% and 80.6%, respectively) than the 1 mmol L^{-1} Trolox positive control (58.1%). There was a statistically significant difference between these extracts and the Trolox control ($P < 0.05$). The *X. mexicana* acetone extract exhibited half the DPPH free radical scavenging activity of 1 mmol L^{-1} Trolox treatment, while the *L. alphoplaca* extracts did not show any significant antioxidant effects (Fig. 2).

Many compounds with marked antioxidant capabilities have been isolated from lichen species. The activities of these compounds have proven to be comparable to those of common antioxidants. Some examples of such compounds are sekikaic acid (depside), lobaric acid (depsidone) and lecanoric acid (depside).[22] These compounds exhibit superoxide radical scavenging abilities very similar to that of a common antioxidant food additive, propyl gallate.[22] Another study found that 8′-methylmenegazziaic acid and antranorin – both lichen-derived compounds – have antioxidant activities that are comparable in activity to a Trolox® standard.[23] A lichen-derived bisnaphthoquinone, cuculloquinone, was reported to produce 80% DPPH inactivation, which was twofold more effective than the butylated hydroxytoluene (BHT) standard it was compared to.[24] As evident from Fig. 2, the *Acarospora* ethanolic extracts exhibited high antioxidant potential. The other two lichen extracts tested – *L. alphoplaca* and *X. mexicana* – did not exhibit statistically significant antioxidant abilities (data not shown). The results from our study and those of others[25] have shown that naturally occurring lichen metabolites produce *in vitro* antioxidant effects similar to or exceeding those of common synthetic antioxidant compounds. The impetus of natural antioxidant research is to find novel and effective antioxidant compounds that do not exhibit carcinogenic effects, as some of the synthetic antioxidants do.[25]

Antibacterial activity

The antibacterial activity of the lichen extracts against Gram-negative and Gram-positive bacteria was evaluated using the disk diffusion method. None of the lichen extracts was active against Gram-negative bacteria (data not shown). However, the acetone and ethyl acetate extracts of *X. mexicana* were the only two extracts that showed antibiotic activity against Gram-positive bacteria (Table 1). The *L. alphoplaca* and *A. socialis* extracts did not have significant antibacterial activity (data not shown). Moreover, the solvent control disks did not show any significant zones of inhibition (data not shown). Hence there was a statistically significant difference between the extracts and the solvent control ($P < 0.05$), suggesting that the antibacterial activity was attributed to the presence of compound(s) present in the extract. Further investigation into the *X. mexicana* acetone extract using the microdilution assay yielded an MIC of 20.9 μg mL^{-1} against *S. aureus*, and 41.9 μg mL^{-1} against *E. faecalis* (data not shown). MIC tests performed with acetone controls showed no statistically significant effect on bacterial viability ($P < 0.05$). The differences regarding the activity of *X. mexicana* extract against Gram-negative and Gram-positive bacteria cannot be delineated from the results of this study, but it is conceivable to suggest that they may be attributed to differences in the permeability of the cell walls of bacteria to the more hydrophilic extracts. The cell wall of Gram-positive bacteria consists of a thick layer of peptidoglycan and lipoteichoic acids, whereas Gram-negative bacteria possess a lipid-rich outer membrane and a thin peptidoglycan layer.[26]

The search for novel antibiotic compounds is critical,[27] given the incidence of antibiotic resistance, particularly among hospital-acquired infections.[28] Screening for natural compounds in unusual sources such as lichens may provide new leads. *Xanthoparmelia mexicana* showed promise for further investigation

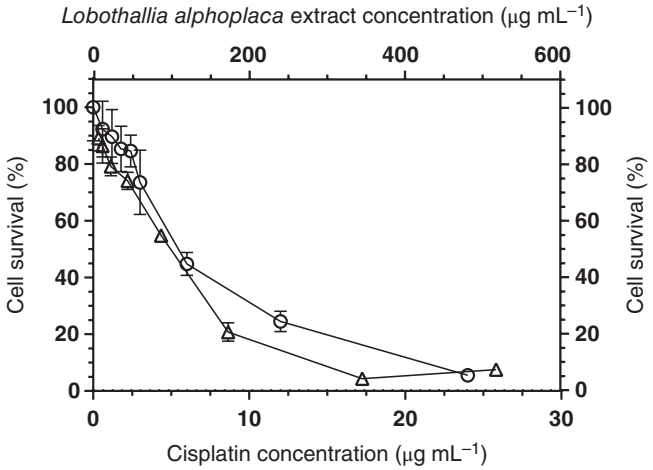

Figure 3. MCF-7 cell sensitivity to cisplatin (open circles) and *L. alphoplaca* acetone extract (open triangles).

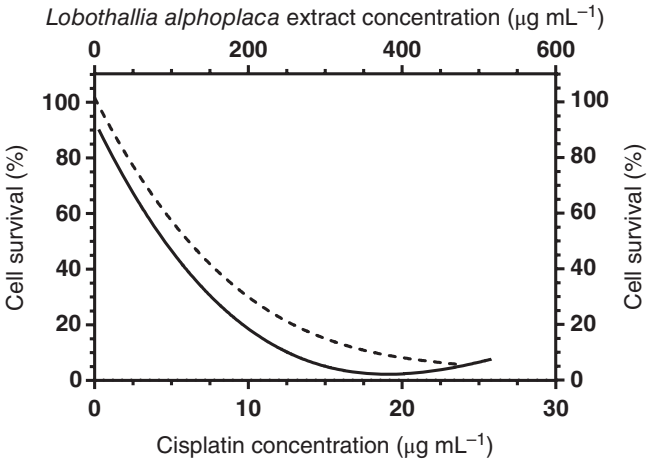

Figure 4. Concentration–effect curve representing MCF-7 cell sensitivity to cisplatin (dashed line) and *L. alphoplaca* acetone extract (solid line).

of specific components of the crude extract.[29] For example, usnic acid extracted from *Xanthoparmelia farinosa* appeared to have some biological activity.[29]

Anticancer activity

The anticancer activity of acetone extract from *L. alphoplaca* against MCF-7 cells showed promising results (Fig. 3), though the extracts of *A. socialis* and *X. mexicana* did not show significant anticancer activity (data not shown). The effective concentration where 50% of cells are killed (EC_{50}) for *L. alphoplaca* was recorded at a lichen metabolite concentration of 87 μg mL^{-1}, whereas the cisplatin positive control had an EC_{50} of 5 μg mL^{-1} (Fig. 3). This led to the conclusion that there is a statistically significant difference between the lichen treatment and the cisplatin control ($P < 0.05$). Furthermore, acetone solvent alone caused a 5% decrease in viability at the maximum concentration used (data not shown). Although the effective concentration of the acetone lichen extract was significantly higher than cisplatin, the similarity in the concentration–effect curve (Fig. 4) is evidence to suggest that the lichen extract may contain constituent(s) that act via a similar mechanism of action to cisplatin. With the use of UV–visible, fluorescence and linear dichroism spectroscopic techniques, it has been shown that a series of lichen secondary metabolites (parietin, atranorin, usnic and gyrophoric acid) could bind to DNA with high affinity.[30] Preliminary evidence also showed that the MCF-7 cell cycle is arrested in the G2 phase, and that DNA synthesis may also be inhibited by the acetone extracts of *L. alphoplaca* (Table 2). Currently, we have undertaken experiments to isolate the active compound(s) from the lichen acetone extract in order to delineate its antitumor potency and toxicity as compared to efficacy and toxicity of the existing chemotherapeutic agents.

In summary, the results of this study showed that extracts from the lichens *A. socialis*, *L. alphoplaca* and *X. mexicana* possess significant biological activity: (i) ethanol (or methanol) extracts of *A. socialis* were effective in scavenging free radicals; (ii) acetone or ethyl acetate extracts of *X. mexicana* inhibited growth of three species of Gram-positive bacteria; and (iii) acetone extracts of *L. alphoplaca* were effective in inhibiting the growth of cancerous MCF-7 cells. Our work extends the list of lichen species with known biological activities, and we are encouraged by the fact that lichens are progressively found to be an effective source of biomolecules useful in therapeutics and as food additives.

Table 2. Flow cytometry data[a] for MCF-7 cells treated with *L. alphoplaca* acetone extracts

Phase	acetone control	Acetone extract
G1	65.72 ± 1.07	52.51 ± 0.96
S	14.01 ± 0.51	5.40 ± 0.34
G2	22.33 ± 1.95	48.58 ± 2.33

[a] G1: cells in Gap 1 phase of the cell cycle; S: cells in the Synthesis phase of the cell cycle; G2: cells in the Gap 2 phase of the cell cycle.

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