Screening of Antimicrobial Activity and Cytotoxic Effects of Two *Cladonia* Species

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The present study explores the antimicrobial activity and cytotoxic effects in culture assays of two fruticose soil lichens, *Cladonia rangiformis* Hoffm. and *Cladonia convoluta* (Lamkey) Cout., to contribute to possible pharmacological uses of lichens. *In vitro* antimicrobial activities of methanol and chloroform extracts against two Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*), two Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus)*, and the yeast *Candida albicans* were examined using the paper disc method and through determination of minimal inhibitory concentrations (MICs). The data showed the presence of antibiotic substances in the chloroform and the methanol extracts of the lichen species. The chloroform extracts exhibited more significant antimicrobial activity than the methanol extracts. However, a higher antifungal activity was noted in the methanol extract of *C. rangiformis*. The maximum antimicrobial activity was recorded for the chloroform extract of *C. convoluta* against *E. coli.* The cytotoxic effects of the lichen extracts on human breast cancer MCF-7 cells were evaluated by the trypan blue assay yielding IC_{50} values of ca. 173 and 167 μg/ml for the extracts from *C. rangiformis* and *C. convoluta*, respectively.

Key words: Lichen, Antimicrobial Activity, MCF-7, *Cladonia*

Introduction

Lichens are well-known symbiotic associations between fungi and algae, usually an ascomycete as mycobiont partner and a green alga or a cyanobacterium as photosynthetic partner, so called "lichenized fungi", including over 20,000 species all over the world. They usually grow on rocks and soil as well as epiphytes on trees and leaves.

The use of lichens as herbal medicine is a traditional way to cure ailments; they have been applied for more than five millennia in several civilizations. Even today, in many developing countries plant materials continue to play a major role in primary health care as therapeutic agents. Most of the preliminary information on biological activity and potential use of lichen metabolites is derived from their ethno-botanical lore (Ingolfsdottir, 2002; Romagni and Dayan, 2002). These unique organisms are able to produce lichen-specific secondary compounds, which have been used in medicine, food, cosmetics, dye, and for other ethno-botanical purposes from ancient to recent times (Llano, 1950; Romagni and Dayan, 2002; Çobanoğlu and Yavuz, 2003; Yavuz and Çobanoğlu, 2010).

Lichen compounds have been shown to have a range of activities, depending on the species of lichen, concentration of the extract, type of the solvent, and the tested organisms. Many lichen species have antimicrobial (Esimone and Adikwu, 1999; Yılmaz *et al*., 2004; Ranković *et al*., 2009; Çobanoğlu *et al*., 2010), antifungal (Proksa *et al.*, 1996; Halama and Van Haluwyn, 2004; Schmeda-Hirschmann *et al.*, 2008; Zibbu and Batra, 2010), antioxidant (Aslan *et al*., 2006; Odabasoglu *et al*., 2006; Luo *et al*., 2009; Ranković *et al*., 2010), antiviral and cytotoxic (Karagöz and Aslan, 2005), as well as anticancer and anti-inflammatory (Shukla *et al*., 2010; Suleyman *et al.*, 2002) effects, respectively. Many herbal medicines and compounds isolated from natural products have potential

antitumour effects. Some lichen polysaccharides and glycoproteins are also known to exhibit antitumour activity and inhibition of HIV (Brodo *et al*., 2001). Some lichen extracts selectively inhibit human cancer cell lines (Bézivin *et al*., 2003). Also, the extract of *Lethariella zahlbruckneri* inhibited HT-29 human colon cancer cell proliferation (Ren *et al*., 2009).

In this study, antimicrobial activity and cytotoxic effects in culture assays of two fruticose lichen species, *Cladonia rangiformis* and *Cladonia convoluta*, are presented. These species are terricolous (living on the ground) and have potentially high medicinal and economical values. Many species of the genus *Cladonia* have been traditionally used in folk medicine to treat fevers, diarrhea, infections, pains, and wounds, also in traditional Chinese medicine (Hu *et al*., 1980). For instance, *C. pyxidata* is used to treat whooping-cough (pertussis), *C. stellaris* is used in powdered form to expel intestinal worms. *Cladonia* species were applied on the umbilical cord to control infections. By Ojibwas Indians in the USA, newborns were bathed in water in which reindeer moss (*C. rangiferina*) had been boiled (Brodo *et al*., 2001). The present study aims to screen *Cladonia* extracts for their antimicrobial activity and cytotoxic effects and thus contributes to the ethno-pharmacological uses of lichen contents.

Material and Methods

Lichen material and test microorganisms

Lichen materials were sampled from the Kandira district of Kocaeli province in the east Marmara region of Turkey, in April 2010. The lichen species were investigated under a stereomicroscope (Olympus SZ40; Olympus Medical Systems Corp., Tokyo, Japan) and identified by G. Çobanoğlu as *Cladonia rangiformis* Hoffm. and *Cladonia convoluta* (Lamkey) Cout. (Smith *et al*., 2009).

The test microorganisms, *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* ATCC 2592, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* ATCC 90028, were provided by the Medical Microbiology Department of the Medicine Faculty of Yeditepe University, Istanbul, Turkey.

The test microorganisms were grown in nutrient broth medium (NB-No. 3, for microbiology, 70149; Fluka, Munich, Germany) at 37 °C for 24 h, adjusted to 0.5 McFarland standard, approximately 10^8 cfu/ml for bacteria and 10^6 cfu/ml for *C. albicans*.

Preparation of lichen extracts

The air-dried samples were ground by means of a mortar and a pestle. Powdered lichen materials from the samples of *C. rangiformis* (5 g) and *C. convoluta* (3 g) were successively extracted in a Soxhlet extractor using each 270 ml of methanol and chloroform as solvents, respectively. The extracts were filtered through Whatman No. 1 filter paper (Whatman, Maidstone, England) and the solvents then evaporated to dryness by a rotary evaporator under reduced pressure to yield 322 mg of *C. rangiformis* and 166 mg of *C. convoluta* methanol extracts and 22 mg and 15 mg of *C. rangiformis* and *C. convoluta* chloroform extracts, respectively. The extracts were sterilized by membrane filtration using 0.45-μm Millipore filters (MF-Millipore, Billerica, MA, USA), and were kept at –20 °C until assay.

Antimicrobial assays

The disc diffusion susceptibility test was used for the tested microorganisms which were grown in nutrient broth medium (NB-No. 3) in incubators at 37 °C, overnight for bacterial strains and for 48 h for the yeast strain. They were diluted to 0.5 McFarland standards, and then the bacteria and *C. albicans* were spread on nutrient agar plates (Salubris, Istanbul, Turkey) and Muller Hinton agar (MHA) (Sigma-Aldrich, Munich, Germany), respectively. For the disc diffusion assay, the dried extracts were dissolved in the respective solvents methanol and chloroform, diluted 1:10, to give final concentrations of 16.6 mg/ml and 1.5 mg/ml respectively, and sterilized by filtration through 0.45-μm Millipore filters. Twenty μl of these methanol and chloroform solutions, respectively, were added onto Whatman filter paper discs (6 mm diameter) allowing the solvent to evaporate during the applications (Bauer *et al*., 1966).

Negative controls were prepared using the respective solvents employed to dissolve the lichen extracts. A number of antibiotics were used as positive reference standards for the bacteria, chloramphenicol, piperacillin/tazobactam, and vancomycin, and fluconazole for the yeast. Since all microbiological tests were made in laboratories with an International Quality Certification

(ISO-15189), a large antibiotic control panel recommended by CLSI (Clinical Laboratory Standards Institute) was used.

The bacterial inhibition zones on the test plates were measured under the bacterial colony counter Colony Star (Funke-Gerber, Berlin, Germany). The final concentrations of each solution, which exhibited relatively larger zones of inhibition, were diluted serially from one- to ten-fold to determine the minimal inhibitory concentrations (MICs).

The serial dilutions were tested on the microorganisms during overnight incubation. All experiments were done in triplicate and checked with the control plates.

Cell culture and cytotoxicity assay

The MCF-7 cell line was purchased from ATCC (The American Type Culture Collection, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium/nutrient F-12 Ham (DMEM-F12) medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Seromed, Istanbul, Turkey), penicillin (50 units/ml), and streptomycin (0.05 mg/ml) (Biological Industries, Beit-Haemek, Israel) in a humidified atmosphere under 5% CO_2 at 37 °C.

Cytotoxicity was determined by a trypan blue dye assay. MCF-7 cells were seeded at a concentration of $1 \cdot 10^5$ cells/well in 24-well tissue culture plates and incubated with various doses (1, 5, 10, 30, 50, 100, 200 μg/ml) of the chloroform extracts from *C. rangiformis* and *C. convoluta*, respectively, for 24 h. The cells were collected and dyed in trypan blue solution (0.4%, liquid, sterile-filtered, suitable for cell culture; Sigma) at room temperature for 5 min. A hemocytometer was used for cell counting under a light microscope, and stained non-viable cells were also observed.

Results

The antimicrobial activities of the methanol and chloroform extracts of *Cladonia convoluta* and *Cladonia rangiformis* were analysed against the microorganisms *E. coli* and *P. aeruginosa* (Gram-negative), *E. faecalis* and *S. aureus* (Gram-positive), and *C. albicans*, using the disc diffusion method. The diameters of the growth inhibition zones and the MIC values are indicated in Tables I and II. Extracts from both lichen species were

Table I. Antimicrobial activity of the extracts of *C. rangiformis* and *C. convoluta* in the disc diffusion assay.

Lichen species[a]		*E. coli*	*P. aeruginosa*	*E. faecalis*	*S. aureus*	*C. albicans*
C. rangiformis	M	–	–	–	–	17 ± 0.50
	C	16 ± 0.58	17 ± 0.58	–	14 ± 2.00	12 ± 0.00
C. convoluta	M	–	–	–	13 ± 1.73	–
	C	36 ± 0.58	7 ± 0.00	–	8 ± 0.58	12 ± 0.57
Antibiotics[b]	C				26 ± 0.58	
	FLU					25 ± 0.58
	TZP	26 ± 1.53	26 ± 1.52			
	Va			17 ± 1.15		

Values are mean inhibition zones ± SD (in mm) of three replicates; – no inhibition observed.
[a] C, chloroform extract (30 μg/disc); M, methanol extract (332 μg/disc).
[b] Antibiotics used as positive reference standards: C, chloramphenicol (30 μg/disc); FLU, fluconazole (25 μg/disc); TZP, piperacillin/tazobactam (110 μg/disc); Va, vancomycin (30 μg/disc).

Table II. Minimum inhibitory concentration (MIC) of the extracts of *C. rangiformis* and *C. convoluta* against the test organisms.

Lichen species[a]		*E. coli*	*P. aeruginosa*	*E. faecalis*	*S. aureus*	*C. albicans*
C. rangiformis	M	–	–	–	–	161 ± 0.00
	C	6 ± 0.58	6 ± 0.00	–	8.4 ± 1.00	9.6 ± 1.00
C. convoluta	M	–	–	–	99.6 ± 0.58	–
	C	9 ± 0.58	15 ± 0.58	–	12 ± 1.00	12 ± 1.00

Values are means ± SD (in μg/ml) of three replicates; – non-affective on the bacteria.
[a] C, chloroform extract; M, methanol extract.

found to have antibacterial activity to various degrees against three of the tested bacteria, but not against *E. faecalis*, and antifungal activity against *C. albicans*, the chloroform extracts being more active.

The chloroform extracts of *C. rangiformis* exhibited a moderate antibacterial activity against *P. aeruginosa*, *S. aureus*, and *E. coli* with the zones of inhibition ranging from 14 to 16 mm, while the methanol extracts of *C. rangiformis* had no antibacterial effect. On the other hand, both the chloroform and the methanol extract of *C. rangiformis* had moderate antifungal effects on *C. albicans*.

The chloroform extract of *C. convoluta* had the strongest antimicrobial activity, inhibiting the growth of *E. coli* (diameters of growth inhibition zones ranged above 30 mm and the MIC value was 9 μg/ml), but showed a low activity against *P. aeruginosa*. Both the methanol and chloroform extracts of this species exhibited a moderate antibacterial activity against *S. aureus.* On the other hand, the chloroform extract of *C. convoluta* had a weak effect on *C. albicans*, however, the methanol extract did no exhibit any antifungal activity.

In the negative controls no growth inhibition was observed.

The cytotoxicity of the *C. rangiformis* and *C. convoluta* extracts against the MCF-7 cells were examined using the trypan blue assay. Treatment for 24 h with the two extracts inhibited MCF-7 cell proliferation in a dose-dependent manner (Fig. 1). The IC_{50} values were estimated to be in the range of 173 μg/ml (*C. rangiformis*) and 167 μg/ml (*C. convoluta*).

Discussion

In this study, in addition to confirming the presence of antibiotic substances in lichen extracts, we also detected novel antimicrobial properties and effects on cell viability of the two lichen species examined. In particular, the chloroform extract of *C. convoluta* exhibited strong antimicrobial activity. To determine which substances in the lichen samples are effective against which type of microorganism, we first need to consider the chemical constituents of the species in the genus *Cladonia*. Most studies have focused on the activities of crude lichen extracts (Ranković *et al*., 2009; Saenz *et al*., 2006; Santiago *et al*., 2010). Compounds in *Cladonia* spp. that have previously been tested for antimicrobial activity include usnic, perlatolic, ursolic, and didymic acids, respectively, as well as strepsilin and atranorin, as discussed below.

In the present study, we determined that the chloroform extracts of both *Cladonia* species exhibited high activity against Gram-negative bacteria, particularly *E. coli* and *P. aeruginosa* (Table I). These results agree with those of some

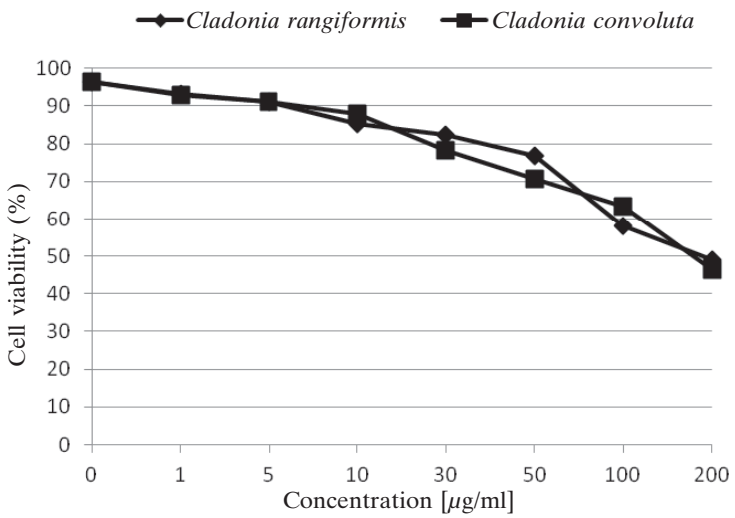

Fig. 1. Inhibition of MCF-7 cells by chloroform extracts from *C. rangiformis* and *C. convoluta*. Cells were treated with increasing concentrations of chloroform extracts of *C. rangiformis* and *C. convoluta* for 24 h. Viable cells were detected by the trypan blue assay.

previous reports, but contrast with others. Yılmaz *et al*. (2004) examined the antimicrobial effects of (–)-usnic acid, atranorin, and fumarprotocetraric acid, constituents obtained from an acetone extract of *C. foliacea*, and found that atranorin was the least active of these compounds, exhibiting high antimicrobial activity only against Gram-positive bacteria. Similarly, Ingolfsdottir (2002) reported that the (+)-and (–)-forms of usnic acid are inactive against *E. coli* and *P. aeruginosa.* On the other hand, usnic acid isolated from an acetone extract of *Parmelia* species showed activity against *E. coli* but not against *P. aeruginosa* (Cansaran Duman, 2009). In addition, Çobanoğlu *et al*. (2010) reported that acetone and chloroform extracts of some lichen species were active against several Gram-negative bacteria. As usnic acid is not active against Gram-negative bacteria, some other substances in the extracts must be responsible for the activity against these bacteria. Indeed, it is not possible to conclude that lichen substances in general are inactive against Gram-negative bacteria; further research is needed to clarify this point.

Usnic acid has long been known to be an important, unique lichen substance, which has been utilized for medicinal, perfumery, and ecological purposes. In a review by Ingolfsdottir (2002), the natural isomers of usnic acid, a constituent of lichen genera such as *Alectoria*, *Cladonia*, *Lecanora*, *Evernia*, *Ramalina*, and *Usnea*, were described as active substances against Gram-positive bacteria and some mycobacteria. Usnic acid also has antifungal properties. In particular, (–)-usnic acid isolated from *Alectoria ochroleuca* has been reported to have antifungal activity (Proksa *et al*., 1996). In *Cladonia* species, usnic acid appears to be more bioactive than other secondary lichen substances (Falk *et al*., 2008; Romagni and Dayan, 2002; Santiago *et al*., 2010; Shukla *et al*., 2010). Saenz *et al*. (2006) identified usnic acid and ursolic acid in an acetone extract of *C. convoluta*.

Ursolic acid possesses anti-inflammatory, antitumour, and antimycobacterial properties (Romagni and Dayan, 2002). Zibbu and Batra (2010) demonstrated that ursolic acid inhibits the growth of *C. albicans*. In the current study, the methanol extract of *C. rangiformis* was more effective in inhibiting the growth of *C. albicans* than that of *C. convoluta*, while chloroform extracts of both species had similar, moderate effects on the growth of this fungus.

Previous studies employing methods similar to those used in the current study have demonstrated the antibacterial properties of *Cladonia* species. Santiago *et al*. (2010) reported that an acetone extract of *C. gracilis* is active against *S. aureus*. In another study, acetone and methanol extracts of *C. furcata* showed no activity against *E. coli*, but were highly active against *S. aureus.* In addition, methanol extracts are generally more active against the organisms tested than acetone extracts (Ranković *et al*., 2009). However, our results indicate that chloroform extracts are much more active than methanol extracts.

Cladonia species have been used in folk medicine for the treatment of wounds and various infections since ancient times. Our results confirm the antimicrobial effectiveness of *Cladonia* species and support the notion that *Cladonia* has possible applications in modern medicine. In the present study, both methanol and chloroform extracts of both species examined were inactive against *E. faecalis*. Furthermore, the methanol extracts had no activity against *E. coli* or *P. aeruginosa*. Aslan *et al*. (2006) found that none of the methanol extracts of any of the lichen species examined, including *C. foliacea*, showed antibacterial activity against *P. aeruginosa*, *S. aureus*, or *E. faecalis*. Bézivin *et al.* (2003) found that some lichen extracts have cytotoxic activity against cancer cell lines *in vitro*. Also, Ren *et al*. (2009) observed that lichen extracts in various solvents have antiproliferative activity and induce apoptosis in cancer cell lines. Similarly, in the current study, extracts of *C. rangiformis* and *C. convoluta* had antiproliferative effects against MCF-7 cancer cells.

Studies of the antimicrobial effects of lichen extracts on the same or different microorganisms have sometimes yielded different results. In general, varying results of the antimicrobial activity of lichen extracts may be related to differences in extraction methods or differences in the collection sites of the lichen samples.

Conclusions

Antibiotic substances have been demonstrated in chloroform and methanol extracts of two lichen species. The antimicrobial activity of *C. convoluta* against *E. coli* and the particularly inhibitory effect of this species against *C. albicans* are reported here for the first time. In addition, we found that chloroform is a more effective solvent than methanol. The two lichen extracts were found to have cytotoxic effects on cancer cells,

which opens up a potential line of research. These lichens may represent novel candidates for cancer treatments.

Ethno-botanical studies have the potential for revealing new insights and providing new medicinal approaches through the evaluation of traditional uses of plants. To evaluate the effectiveness of each substance found in lichen extracts, these chemical compounds should be investigated in detail and subjected to further study.

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