



EVALUATION OF ANTIMICROBIAL ACTIVITY OF THE LICHENS *PHYSICIA AIPOLIA*, *XANTHORIA PARIETINA*, *USNEA FLORIDA*, *USNEA SUBFLORIDANA* AND *MELANOHALEA EXASPERATA*

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Abstract. The present study aimed to evaluate the antimicrobial activity of methanol and chloroform extracts of five lichen species, *Melanohalea exasperata*, *Physcia aipolia*, *Usnea florida*, *U. subfloridana* and *Xanthoria parietina*. Antimicrobial activity in culture assays of these foliose and fruticose lichen extracts were examined against two Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*), two Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*), and the yeast *Candida albicans* using the paper disc method through determination of minimal inhibitory concentrations (MICs). The obtained results indicated the existence of different levels of antibiotic substances in the chloroform and the methanol extracts of the examined lichen species. The chloroform extracts of *Usnea subfloridana* showed the highest activity against *Escherichia coli* and *Pseudomonas aeruginosa* while the methanol extracts of this species were not active against these microorganisms. The chloroform extracts of the examined species exhibited more significant antimicrobial activity than the methanol extracts. None of the species were active against *Enterococcus faecalis* and *Staphylococcus aureus*. Most of the lichen extracts indicated a moderate antifungal activity against *Candida albicans*, except for *Physcia aipolia*, which was not active.

Key words: antimicrobial activity, lichen, MIC

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Introduction

Lichens are successful symbiotic associations between fungi and algae, usually an ascomycete as a mycobiont partner and a green alga or a cyanobacterium as a photosynthetic partner, so called “lichenized fungi”, and includes over 20,000 species all over the world. These unique organisms are able to produce lichen-specific secondary metabolites, which comprise phenolic compounds, anthraquinones, dibenzofurans, depsides, depsidones, depsones, γ -lactones, and pulvinic acid derivatives, with currently about 1,000 substances identified (MUGGIA *et al.* 2009). The potential of secondary lichen metabolites in pharmaceutical sciences is derived from their traditional uses. Lichens have been utilized in folk medicine, food, cosmetics, dyes, and for other ethnobotanical purposes for more than five millennia in several civilizations (LLANO 1950; ROMAGNI

& DAYAN 2002; MALHOTRA *et al.* 2007; YAVUZ & ÇOBANOĞLU 2010). Through their synthesis of biologically-active substances, the lichens as much as plant materials have potential healing power and continue to play a major role in herbal medicine for both traditional and modern treatments (INGOLFSDOTTIR 2002; ROMAGNI & DAYAN 2002; YAVUZ 2013).

In recent years there has been a rising interest in the discovery of new antibiotic compounds to control bacterial diseases. The antimicrobial activity of many lichens have been reported (ASLAN *et al.* 2001; CROCKETT *et al.* 2003; ÇOBANOĞLU *et al.* 2010; SANTIAGO *et al.* 2010; RANKOVIĆ *et al.* 2011; AÇIKGÖZ *et al.* 2013). A number of lichens also have antifungal effects (HALAMA & VAN HALUWYN 2004; SCHMEDA-HIRSCHMANN *et al.* 2008), antioxidant capacity (WEISSMAN *et al.* 2005; LUO *et al.* 2006, 2009; KEKUDA *et al.* 2009; ATALAY *et al.* 2011), antiviral, cytotoxic and antigenotoxic activity

(MANOJLOVIĆ *et al.* 2010; TURKEZ *et al.* 2012; AÇIKGÖZ *et al.* 2013), as well as anticancer and anti-inflammatory effects (SULEYMAN *et al.* 2002; RUSSO *et al.* 2008; TRIGGIANI *et al.* 2009).

The following study presents the *in vitro* antimicrobial activity of chloroform and methanol extracts of five lichen species: *Melanohalea exasperata* (De Not.) O. Blanco, A. Crespo, Divakar, Essl., D. Hawksw. & Lumbsch, *Physcia aipolia* (Ehrh. ex Humb.) Fürnr., *Usnea florida* (L.) Weber ex F.H. Wigg., *U. subfloridana* Stirt. and *Xanthoria parietina* (L.) Th.Fr. Each has potential medicinal and economic value as a candidate for contribution to pharmacological use of secondary lichen metabolites. For instance, *Xanthoria parietina* was reported as being used to cure jaundice (HUNECK 1999). A strong anticancer activity was stated for *Xanthoria parietina* by TRIGIANI *et al.* (2009). Various colours were obtained from *Xanthoria parietina* as well as some other lichen species in natural dying by ŞEN *et al.* (2014). A number of studies showed that many *Usnea* species have strong antimicrobial activity (İNGOLFSDOTIR 2002; FRANCOLINI 2004; THIPPESWAMY 2011). *Usnea* has often been mentioned in the history of ethno-medicine (LLANO 1950; MALHOTRA *et al.* 2007; YAVUZ & ÇOBANOĞLU 2010; YAVUZ 2013). However, the biological activities of the other species contained in the present study have not been previously investigated.

Material and methods

Lichen samples. Lichen materials for the present antimicrobial tests were collected from the provinces of Isparta and Bolu in Turkey. Five lichen species were identified by examination under a stereomicroscope (Olympus SZ40; Olympus Medical Systems Corp., Tokyo, Japan) by G. Çobanoğlu (SMITH *et al.* 2009). These species, including *Melanohalea exasperata*, *Physcia aipolia*, *Usnea florida*, *U. subfloridana* and *Xanthoria parietina*, are epiphytic-living (on tree bark) and morphologically foliose and fruticose.

Preparation of lichen extracts. Air-dried thalli of the samples were finely ground by means of a mortar and a pestle. Powdered lichen

materials from samples of *Physcia aipolia* (5 g), *Xanthoria parietina* (5 g), *Usnea subfloridana* (5 g), *Melanohalea exasperata* (3 g) and *Usnea florida* (3 g), were extracted in a Soxhlet extractor apparatus using 270 ml of methanol and chloroform as solvents. The extracts were filtered through Whatman No. 1 filter paper (Whatman, Maidstone, England). The solvents were then evaporated by a rotary evaporator. In this way, the dry methanol extracts; 135 mg for *Melanohalea exasperata*, 351 mg for *Physcia aipolia*, 237 mg for *Usnea florida*, 417 mg for *Usnea subfloridana*, 252 mg for *Xanthoria parietina* and the dry chloroform extracts for the same species; 10 mg, 20 mg, 10 mg, 15 mg, 10 mg, respectively, were obtained. The extracts were sterilized by membrane filtration using 0.45 µm pore-sized Millipore filters (MF-Millipore, Billerica, MA, USA), and were kept at -20 °C until assay.

Microorganisms and media. The following microorganisms were used as test organisms in this study: *Escherichia coli* ATCC 2592 and *Pseudomonas aeruginosa* ATCC 15442 (Gram-negative bacteria), *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 (Gram-positive bacteria), and *Candida albicans* ATCC 90028 (Fungus). All the bacteria and fungus 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 a 0.5 McFarland standard, approximately 10⁸ cfu/ml for bacteria and 10⁶ cfu/ml for *Candida albicans*.

Antimicrobial activity assay. A paper disc test was used to test microorganisms that were grown in nutrient broth (NB-No. 3, as above) in incubators at 37 °C, overnight for bacterial strains and for 48 h for the yeast strain. For the disc diffusion assay, the samples were diluted to 0.5 McFarland standards, and then the bacteria and *Candida albicans* were spread on nutrient agar plates (Salubris, Istanbul, Turkey) and Muller Hinton agar (MHA) (Sigma-Aldrich, Munich, Germany), respectively. At the same time, methanol and chloroform

Tab. 1. Antimicrobial activities of extracts of *Melanohalea exasperata*, *Physcia aipolia*, *Usnea florida*, *U. subfloridana*, and *Xanthoria parietina* in the disc diffusion assay.

Lichen species ^a		<i>E. coli</i>	<i>P. aeruginosa</i>	<i>E. faecalis</i>	<i>S. aureus</i>	<i>C. albicans</i>
<i>M. exasperata</i>	M	-	-	7 ± 0.58	-	13 ± 0.58
	C	17 ± 0.58	19 ± 0.58	-	10 ± 0.58	-
<i>P. aipolia</i>	M	-	-	-	-	-
	C	23 ± 0.58	12 ± 0.58	-	-	-
<i>U. florida</i>	M	-	-	7 ± 0.57	10 ± 0.00	13 ± 0.58
	C	13 ± 0.00	9 ± 0.00	9 ± 0.00	10 ± 0.00	7 ± 0.58
<i>U. subfloridana</i>	M	-	-	-	10 ± 0.58	11 ± 0.00
	C	32 ± 0.58	31 ± 0.00	-	10 ± 0.58	-
<i>X. parietina</i>	M	-	-	-	-	10 ± 0.57
	C	9 ± 0.58	21 ± 0.00	-	8 ± 0.58	-
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 Extracts: **C** – chloroform extract; **M** – methanol extract.

^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).

extracts were diluted in the respective solvents. The final concentrations of the methanol extracts were 13.5 mg/ml for *Melanohalea exasperata*, 35.1 mg/ml for *Physcia aipolia*, 23.7 mg/ml for *Usnea florida*, 41.7 mg/ml for *U. subfloridana* and 25.2 mg/ml for *Xanthoria parietina*. Alike for the same species 1 mg/ml, 2 mg/ml, 1 mg/ml, 1.5 mg/ml and 1 mg/ml final concentrations were obtained from the chloroform extracts, respectively. The solutions were sterilized by filtration through 0.45-µm Millipore filters. Finally methanol extracts of 270 µg for *Melanohalea exasperata*, 702 µg for *Physcia aipolia*, 474 µg for *Usnea florida*, 834 µg for *Usnea subfloridana* and 504 µg for *Xanthoria parietina*, and respectively, 20 µg, 40 µg, 20 µg, 30 µg, and 20 µg chloroform extracts of the same species were added onto Whatman filter paper discs (6 mm diameter) allowing the solvent to evaporate during application (BAUER *et al.* 1966). The respective solvents employed to dissolve the lichen extracts were

used as negative controls. Also, for the bacteria; chloramphenicol (C), piperacillin/tazobactam (TZP), vancomycin (Va) and for the yeast; fluconazole (FLU) were preferred as positive reference standards. A bacterial colony counter, Colony Star (Funke-Gerber, Berlin, Germany), was used to measure bacterial inhibition zones on the test plates. A large antibiotic control panel recommended by CLSI (Clinical Laboratory Standards Institute) was used for all microbiological tests, which were done in triplicate at laboratories with an International Quality Certification (ISO-15189).

To determine minimal inhibitory concentrations (MIC), the final concentrations of *Usnea subfloridana* chloroform solution that exhibited relatively larger zones of inhibition than antibiotics, were diluted serially from one-to ten-fold. The serial dilutions were tested on the *Escherichia coli* and *Pseudomonas aeruginosa* during overnight incubation.

Results, discussion and conclusions

The antimicrobial activities of five lichen extracts against four tested microorganisms were evaluated in this study. The diameters of growth inhibition zones in the disc diffusion assay are shown in the Tab. 1. Antimicrobial activity was compared with antibiotics that were used as positive reference standards: chloramphenicol, piperacillin/tazobactam, vancomycin for bacteria and fluconazole for fungi.

Maximum antimicrobial activity manifested in the chloroform extracts of the lichen *Usnea subfloridana* against Gram-negative bacteria *Escherichia coli* (MIC = 3 µg/ml) and *Pseudomonas aeruginosa* (MIC = 27 µg/ml). This lichen showed stronger activity than standard antibiotics (Tab. 1). In comparison, the methanol extracts of the same lichen species were not active against any bacteria. Both extracts of the other *Usnea* species, *U. florida*, were not as active against the tested microorganisms as *U. subfloridana*. Similarly, the chloroform extracts of *Physcia aipolia* and *Melanohalea exasperata* markedly inhibited these two Gram-negative bacteria (in concentrations close to the activity of the standard antibiotics) (Tab. 1), but not the Gram-positive bacteria (*Enterococcus faecalis*, *Staphylococcus aureus*) and the fungus. Except for *Usnea subfloridana*, which had the highest activity, the chloroform extract of *Physcia aipolia* was more active against *Escherichia coli* in diameter of the growth inhibition zone at 23 mm than the other lichen species, while the chloroform extract of *Xanthoria parietina* exhibited higher antimicrobial activity against *Pseudomonas aeruginosa* in terms of the diameter of an inhibition zone at 21 mm (Tab. 1).

The methanol extracts of the lichen species had no or weak effects on the tested bacteria compared to the chloroform extracts. In contrast, the results of antifungal activity showed that the tested methanol extracts of the lichens had moderate effects on *Candida albicans*, except for *Physcia aipolia*, which had no effect. The methanol extracts of *Melanohalea exasperata* and *Usnea florida* were more active against *Candida albicans* than the other lichen

species, with a smaller diameter inhibition zone (13 mm) compared to fluconazole (25 mm). In the negative controls, there was no observed growth inhibition.

The results of the present study demonstrated that the chloroform extracts of five lichen species (but not their methanol extracts) exhibited strong antimicrobial activity against Gram-negative bacteria, particularly *Escherichia coli* and *Pseudomonas aeruginosa* (Tab. 1). The findings are in line with a part of the results from earlier papers. While the chloroform extracts of *Physcia aipolia* and *Xanthoria parietina* significantly inhibited two Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* in this study, ASLAN *et al.* (2001) reported that neither acetone nor chloroform extracts of lichen *Physcia aipolia* exhibited activity against these Gram-negative bacteria. Also, in another study by KARAGÖZ *et al.* (2009), aqueous extracts of *Xanthoria parietina* were inactive against *Escherichia coli* and *Pseudomonas aeruginosa*, but active against *Staphylococcus aureus*. In comparison, DÜLGER *et al.* (1997) determined antimicrobial effects in *Usnea florida* and found that chloroform, ethanol, acetone and ethyl acetate extracts were all active against *Escherichia coli* to a degree greater than a standard antibiotic. Our findings, particularly on the antimicrobial activity of the chloroform extracts of the same lichen species in the present study coincide with their results. Moreover, the acetone and chloroform extracts of some lichen species were reported as active against these two Gram-negative bacteria by a previous study of ÇOBANOĞLU *et al.* (2010). KARTHIKAIDEVI *et al.* (2009) also reported on the antimicrobial activity of the chloroform extracts of lichens against these two Gram-negative bacteria, while the methanol extracts were inactive. It is concluded that solvent types and their concentrations are very effective on the level of activity of the same lichen species.

Although the antimicrobial activities of crude lichen extracts have been examined in many studies (KARTHIKAIDEVI *et al.* 2009; ÇOBANOĞLU *et al.* 2010; SANTIAGO *et al.* 2010; RANKOVIĆ *et al.* 2011; AÇIKGÖZ *et al.* 2013; BASILE *et al.* 2015), the chemical constituents

of the lichen species need to be analysed more specifically to determine the antibiotic activity of an individual lichen substance. Diversity at the level of antimicrobial activity of lichen species may be related to where the species are collected, the type of solvent, and the tested organisms. However, expressive results in many recent studies encourage further studies that concentrate on lichen substances.

Thus, the highest antibiotic activity was reported in lichens extracted with a chloroform solvent. The chloroform extract of *Usnea subfloridana* showed the maximum inhibitory effect against *Escherichia coli* and *Pseudomonas aeruginosa*, which is a significant finding in this study and is reported here for the first time. In addition, chloroform was found to be a more effective solvent than methanol in the present study. With respect to this, one finding is that solvent type is important to the level of activity of the same lichen species. Further, the grade of activity or effectiveness depends on species of the lichen in relation to the kinds of secondary metabolites involved. A range of different results in biological activity studies may be due to different habitat conditions and neighbouring species of the collected lichen material, which may affect the production of secondary metabolites in a lichen body. The other variables influencing outcomes of the studies included: type of the solvent, concentration of the extract and the tested microorganism.

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