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Full Length Research Paper

Antibacterial activity of some lichen extracts

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The aqueous and ethanol extracts prepared from some lichens species were evaluated for antibacterial activity against six standard strains (*Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Klebsiella pneumoniae, Staphylococcus aureus* and *Staphylococcus epidermidis*) and two environmental strains (Aeromonas) that were isolated from different lakes. The aqueous and ethanol extracts showed a variable range of antibacterial activity to both standard strains and environmental strains. Ethanol extracts showed better antibacterial activity than aqueus extracts. It was found that the inhibition zone of tested bacteria against extracts have changed between 07 - 15 mm (diameter of inhibition zone). Some lichen extracts have moderate antibacterial effect. Both ethanol and aqueous extracts have inhibited the growth of three bacteria. Neither aqueous extracts nor ethanol extracts were inhibited the growth of five bacteria. The aqueous extract of Peltigera polydactyla and the ethanol extract of the Ramalina farinacea exhibited potent antibacterial activities.

Key words: Antibacterial activity, lichens, MTT method.

INTRODUCTION

The challenge for today's pharmaceutical industry lies in the discovery and development of new pharmacological active molecules (Behera et al., 2005). Similar to higher plants, lichens were used since antiquity as natural drugs. Lichens, together with some marine organism and frog venom, are important sources of biologically active compounds (Barnes, 2000). These organisms produce secondary metabolites and many of them are known for presenting biological and/or pharmacological activities. Lichens slow-growing organisms and their secondary metabolites are mainly depsides, depsidones, dibenzofurans, xanthones and terpene derivatives. They have been used by humans for centuries as food in periods of famine (that is, during the Leningrad siege), as a source of dye (from the early 1300s) and for their therapeutic properties in traditional medicine. Their efficacy is due to the synthesis of unique secondary compounds, a number of which have important biological roles (Perry et al., 1999).

The introduction of new analytical methods (thin layer chromatography, high-performance liquid chromatography, ultraviolet, infra red, magnetic resonance spectroscopy, mass spectrometry, X-ray crystallography) has led to the isolation of many new lichen substances, which by today number over 800. In recent years methods for cultivating lichen mycobionts and lichen tissue have been developed and these have given rise to hopes for the production of metabolites which are otherwise difficult to obtain. Another promising technique may prove to be the transfer of genes which are responsible for the synthesis of lichen substances into other rapidly growing organisms. These considerations explain the increasing interest of the pharmaceutical industry in these remarkable natural products (Huneck, 1999).

Plant product drugs and herbal remedies have been employed since prehistoric times to treat human and animal diseases and several countries still rely on plants and herbs as the main sources of drugs (Ogbonnia et al., 2008). Plants are known to produce certain bioactive molecules which react with other organism in the environment, inhibiting bacterial or fungal growth or modulating the development of other vegetables. Lichens which are the symbiotic organisms of fungi and algae, synthesize numerous metabolites, the "lichen sub-stances," which comprise amino acid derivatives, sugar alcohols, aliphatic acids, macrocyclic lactones, mono-cyclic aromatic compounds, quinones, chromones, xanthhones, dibenzo-

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furanes, depsides, depsidones, depsones, terpenoids, steroids, carotenoids and diphenyl ethers (Clix et al., 1984; Fiedler et al., 1986).

Lichens and their metabolites have manifold biological activity: antiviral, antibiotic, antitumor, allergenic, plant growth inhibitory, antiherbivore, ecological roles and enzyme inhibitory (Huneck, 1999; Aslan et al., 2001; Dülger et al., 1997; 1998). Host preference and performance of lichenivorous Eilema spp. larvae have been related to lichen secondary metabolites (Pöykkö and Hyvarinen, 2003). Usnic acid which is a very active lichen substance is used in pharmaceutical preparations. Usnic acid and vulpunic acid (produced by mycobiont) are cell division regulators of autotrophic partner of lichen symbiosis-photobiont (Backor et al., 1998). Turkey has a richly diverse and well-developed lichen flora (Aslan, 2000; Aslan et al., 2001, 2002; Yazici and Aslan, 2003; Yazici et al., 2004). The present study describes the evaluation of the antibacterial potency of 11 lichens species from Turkey.

MATERIALS AND METHODS

Collection and identification of lichen samples

Following lichen specimens Anaptychia ciliaris (L.) Körb. Ex A. Massal., Cetrelia olivetorum (Nyl.) W.L. Culb. & C.F.Culb, Lecanora muralis (Schreb.) Rabenh., Peltigera polydactyla (Neck.) Hoffm., Peltigera praetextata (Sommerf.) Zopf, Ramalina farinacea (L.) Ach., Rhizoplaca melanohpthalma (DC.) Leuckert and Poelt, Umbilicaria vellea (L.) Hoffm., Xanthoria elegans (Link) Th.Fr., Xanthoria parietina (L.) Th.Fr Xanthoparmelia tinctina (Maheu and A. Gillet) were collected from Artvin, Giresun and Trabzon provinces in year 2003. Various flora books were used for identification of samples (Poelt and Vězda, 1981; Aslan, 2000; Aslan et al., 2001; 2002; Yazıcı and Aslan, 2003; Yazici et al., 2004). Samples were dried at room temperature for 48 h. Herbarium was prepaered and kept at plant collection centre, Atatürk University, Erzurum.

Preparation of extracts

Two separate samples of the air-dried and powdered of the plant material (10 g of each) were extracted with distilled water (80 mL) and 70% ethanol (75 mL) at room temperature using a waring blender. Plant residues were removed by centrifugation (15.000 rpm, 30 min, 4°C) and the supernatant was filtered and evaporated to dryness under reduced pressure and/or lyophilized. In this way, two different crude extracts were obtained: aqueous extract (AE), 70% ethanol extract (EE). AE was dissolved in the medium (Eagle's mimimum essential medium, EMEM) and EE was dissolved in dimethyl sulfoxide (DMSO). They were then prepared at various concentrations in EMEM.

Cell culture

Vero cells were grown and maintained in Eagle's minimum essential medium (EMEM) with Earle's saline, supplemented with an antibiotic-antimycotic mixture [penicillin - 100 U/mL), streptomycin - 100 µg/mL), amphotericin B - 0.25 µg/mL)], and 10% fetal calf serum. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Cytotoxicity assay

The cytotoxic assays were performed according to the microculture MTT method (Mosmann, 1983; Quintero et al., 1999; Mantani et al., 2001; Kawase et al., 2003). The cells were harvested (5 x 10^4 cells/well) and inoculated in 24 well microtiter plates. The cells were washed with phosphate buffered saline (PBS) and the cultured cells were then inoculated with and without the extract. The final concentration of DMSO did not exceed 0.2% (v/v), a concentration without effect on cell replication. After 72 h incubation, the medium is aspirated. 150 µL of MTT solution (5 mg/mL in PBS, pH 7.2) is added to each well and the plates incubated for 4 h at 37°C. After incubation, 800 µL of DMSO was added to each well of plates, followed by gentle shaking to solubilize the formazan dye for 15 min. Absorbance was read at 540 nm using a photometer and surviving fraction calculated.

Bacterial strains

In this study, 6 standard strains were used (Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 9027, Bacillus subtilis ATCC 6633, Klebsiella pneumoniae ATCC 4352, Staphylococcus aureus ATCC 6538 and Staphylococcus epidermidis ATCC 12228) and 2 environmental strains (Aeromonas) that are isolated from different lakes.

Inhibitory effect by the agar-well diffusion method

The determination of the inhibitory effect of plant extracts against bacterial strains was carried out according to the agar-well diffusion method by using Mueller Hinton Agar. 6 mm in diameter Wells were punched into the agar and filled with extracts and appropriate solution. The plates were then incubated at 37°C for 18 - 24 h. The antimicrobial activity was evaluated by measuring the inhibition zone diameter observed (National Committee For Clincal Laboratory Standards Nccls Document, 1997). The standard antibacterial agents (cephalothin and tobramycin) were carried out all tested bacteria (Table 4).

RESULTS

The results of screening some lichens extracts for antimicrobial and cytotoxic activity are summarized in Tables 1 - 3. The cytotoxic effect of each extract was examined with the cell viability of Vero cells (Table 1). Cytotoxicity of extracts was determined by MTT assay in the Vero cell culture. As shown in Table 1, the MTT assay showed that the EEs were more toxic than the AEs. Lichen extracts have cytotoxic activity in different degrees. The concentrations selected for antimicrobial activity tests were noncytotoxic concentration.

We found that the extracts showed antibacterial activity against some of the tested bacteria. Antibacterial activities were produced to different extents by the aqueous and ethanol extracts of lichen species. Aqueus extracts showed better antibacterial activity than ethanol extracts. The aqueous extract of *P. polydactyla* and the ethanol

Table 1. Cytotoxicity of some lichen extracts.

Lichen species	Aqueous extract	Ethanol extract
	CC_{50} (µg/mL)	CC_{50} (µg/mL)
Anaptychia ciliaris	>500	125 ± 38
Cetrelia olivetorum	>500	20 ± 4
Lecanora muralis	>500	20 ± 2
Peltigera polydactyla	>500	100 ± 22
Peltigera praetextata	>500	250 ± 45
Ramalina farinacea	>500	25 ± 6
Rhizoplaca melanophthalma	>500	150 ± 10
Umbilicaria vellea	200±32	100 ± 28
Xanthoria elegans	>500	300 ± 45
Xanthoria parietina	500±38	250 ± 55
Xanthoparmelia tinctina	>500	450 ± 60

CC_{50} : The concentration of extract, which reduced the viability of the Vero cells by 50%. Values CC_{50} are averages and standard deviations for 3 independent experiments

Table 2. Results of antibacterial screening of the different concentration of crude aqueuous extracts of lichens.

Lichen species	Extract Concentration (µg/ml)	Extract solution	Bacterialnhibition zone (mm)							
			Bacillus subtilis	Staphylococcus aureus	Pseudomonas aeruginosa	Staphylococcus epidermidis	E. coli	Klebsiella pneumonia	Aeromonas 1(izolat)	Aeromonas 2 (izolat)-
Anaptychia ciliaris	500	H_2O	9	9	-	-	12	-	-	-
Cetrelia olivetorum	250	H_2O	-	-	-	-	-	-	-	-
Lecanora muralis	500	H_2O	12	-	-	-	-	-	-	-
Peltigera polydactyla	500	H_2O	14	8	-	-	15	-	-	-
Peltigera praetextata	500	H_2O	10	10	-	-	11	-	-	-
Ramalina farinacea	500	H_2O	12	10	-	-	11	-	-	-
Rhizoplaca melanophthalma	500	H_2O	9	9	-	-	-	-	-	-
Umbilicaria vellea	100	H_2O	10	7	-	-	8	-	-	-
Xanthoria elegans	500	H_2O	12	8	-	-	12	-	-	-
Xanthoria parietina	250	H_2O	-	11	-	-	-	-	-	-
Xanthoparmelia tinctina	500	H_2O	12	9	-	-	9	-	-	-

extract of the *R. farinacea* exhibitedpotent antibacterial activities. It was found that the inhibition zone of tested bacteria against extracts have changed between 07 - 16 mm. Both EEs and AEs have inhibited the growth of 3 bacteria. Neither AEs nor EEs were inhibited the growth of 5 bacteria (Tables 2 and 3). Although some of the AEs have specifically inhibited the growth of *E. coli* bacteria (Table 2), their EEs did not inhibit these bacteria. Some of the EEs have inhibited only the growth of S. Epidermidis bacteria (Table 3).

Furthermore, some of the EEs have inhibited the growth of S. epidermidis, their AEs didnot inhibit these bacteria.

The inhibition zones of tested bacterial strains against cephalothine and tobramycine have been found as 15 - 40 mm except *P. aeruginosa* (Table 4).

Table 3. Results of antibacterial screening of the different concentration of crude ethanol extracts of lichens.

Lichen species	Extract Concentration (µg/ml)	Extract solution	Bacteria Inhibition zone (mm)							
			Bacillus subtilis	*Staphylococcus aureus*	*Pseudomonas eruginosa*	*Staphylococcus pidermidis*	*E. coli*	*Klebsiella pneumonia*	Aeromonas 1(izolat)	Aeromonas 2 (izolat)-
Anaptychia ciliaris	50	EtOH	10	-	-	-	-	-	-	-
Cetrelia olivetorum	10	EtOH	10	10	-	11	-	-	-	-
Lecanora muralis	10	EtOH	9	8	-	13	-	-	-	-
Peltigera polydactyla	50	EtOH	-	-	-	-	-	-	-	-
Peltigera praetextata	100	EtOH	8	-	-	-	-	-	-	-
Ramalina farinacea	10	EtOH	12	16	-	10	-	-	-	-
Rhizoplaca melanophthalma	50	EtOH	-	-	-	16	-	-	-	-
Umbilicaria vellea	50	EtOH	-	-	-	-	-	-	-	-
Xanthoria elegans	100	EtOH	-	-	-	-	-	-	-	-
Xanthoria parietina	100	EtOH	-	-	-	-	-	-	-	-
Xanthoparmelia tinctina	250	EtOH	-	-	-	-	-	-	-	-

Table 4. The inhibition zones of tested bacterial strains against cephalothine and tobramycine.

Antibiotics	BacterialInhibition zone (mm)							
	E.coli	*P. aeruginosa*	*B. subtilis*	*K. pneumoniae*	*S. aureus*	*S. epidermidis*	*Aeromonas spp (1)*	*Aeromonas spp (2)*
Tobramycin	15	22	17	20	15	18	20	20
Cephalothin	21	0	22	15	40	20	28	34

DISCUSSION

Plant product drugs and herbal remedies have been employed since prehistoric times to treat human and animal diseases and several countries still rely on plants and herbs as the main sources of drugs (Ogbonnia et al., 2008). Plants are known to produce certain bioactive molecules which react with other organism in the environment, inhibiting bacterial or fungal growth or modulating the development of other vegetables. Lichens which are the symbiotic organisms of fungi and algae, synthesize numerous metabolites, the “lichen substances,” which comprise amino acid derivatives, sugar alcohols, aliphatic acids, macrocyclic lactones, mono-cyclic aromatic compounds, quinones, chromones, xanthhones, dibenzofuranes, depsides, depsidones, depsones, terpenoids, steroids, carotenoids and diphenyl ethers (Clix et al., 1984; Fiedler et al., 1986).

Lichens and their metabolites have manifold biological activity: antiviral, antibiotic, antitumor, allergenic, plant growth inhibitory, antiherbivore, ecological roles and enzyme inhibitory (Huneck, 1999; Aslan et al., 2001; Dülger et al., 1997, 1998). Host preference and performance of lichenivorous Eilema spp. larvae have been

related to lichen secondary metabolites (Pöykkö and Hyvarinen, 2003). Usnic acid which is a very active lichen substance is used in pharmaceutical preparations. Usnic acid and vulpunic acid (produced by mycobiont) are cell division regulators of autotrophic partner of lichen symbiosis-photobiont (Backor et al., 1998).

In this study, the effects of the lichen extracts were examined both on the growth of some bacteria and the viability of Vero cells. The results of the cytotoxicity and antibacterial activity of some lichen extracts are shown in Table 1 - 3. As shown in Table 1, the most potent extracts affecting Vero cell viability were the ethanol extracts. Especially, *C. olivetorum, L. muralis and R. farinacea* extracts showed more toxic effect than other lichens.

The noncytotoxic concentrations of plant extracts were used for antimicrobial activity tests. Our findings suggest that the antimicrobial activity is not due to the cytotoxic activity of extracts. Antibacterial activities were produced to different extents by extracts of lichens. According to results, it was found that the inhibition zones of some lichen extract were smaller than those of cephalothine and tobramycine antibiotics (Table 4). These results have shown that some lichen extracts have moderate anti-bacterial effect. All lichen extracts were examined totally and all experiments were done with their water and ethanol extracts.

According to the results of screening some lichen extracts for antimicrobial activity, we think that the bacterial inhibition can vary with the lichen extract, the solvent used for extraction and the bacteria tested. The antimicrobial properties of several naturally occuring compounds have been known for decades. Recently, many plants have received attention as sources of antibiotics (Basile et al., 2000). Dülger et al. (1997, 1998) have found that although 4 diferent extracts of a Macro-fungus have inhibition effect against *B. subtilis* ATCC 6633 and other some Gram (+) ve Gram (-) bacteria, they have not this effect against E.coli ATCC 11230, *S. epidermidis* NRRL B-4377, S. aureus ATCC 6538P (Dülger et al., 1998). Gücin et al. (1997) reported that lichen *P. furfuracea* (L.) Zopf have revealed antimicrobial activities on some Gram (+) bacteria and yeast. It has been determined that lichens have showed the inhibition effect against a lot of bacteria such as *Bacillus, Pseudomonas, E. coli, Streptococcus, Staphylococcus, Enterococcus, Mycobacterium* (Esimone and Adikwn, 1999; Perry et al., 1999).

Behera et al. (2005) reported that the acetone, methanol, and light petroleum extracts of lichen Usnea ghattensis were effective against *Bacillus licheniformis, B. megaterium, B. subtilis and S. aureus*. Yam et al. (1997) showed that aqueous extracts of teas (*Camellia sinensis*) of different types and from various sources inhibited a wide range of pathogenic bacteria, including methicillin-resistant *S. aureus.* The aqueous extracts and the ethanol extracts prepared from some lichen species were evaluated for antiviral activity against human parainfluenza virus type 2 (HPIV-2). The aqueous extract of *Xanthoria parietina* (L.) Th. Fr. and the ethanol extract of the *X. tinctina* (Maheu and A. Gillet) exhibited attractive antiviral activities (Karagöz and Aslan, 2005).

In summary, some of aqueous extracts and ethanol extracts that had been prepared from lichens appear to have a potential towards antibacterial activity. Further investigations on the antibacterial activity as well as the economical and fast isolation of the metabolite from the lichen are needed. Consequently, the antibacterial effect of plants tested can be explained with new studies by using different solvents for extraction and other bacteria, accurately. Future research will search for new lichen metabolites, investigate in greater detail the action of lichen substances and synthesize new and possibly more active derivatives for their application.

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