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# Antimicrobial Activities of the Lichens *Hypogymnia vittata*, *Hypogymnia physodes* and *Hypogymnia tubulosa* and HPLC Analysis of their Usnic Acid Content

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The aim of present study is to determine the potential antibiotic properties of three lichen species from the Turkey. The acetone extracts obtained from the *Hypogymnia vittata*, *Hypogymnia physodes* and *Hypogymnia tubulosa* were evaluated for antimicrobial activity against 7 bacteria including *Escherichia coli* (ATCC 35218), *Enterococcus faecalis* (RSKK 508), *Proteus mirabilis* (Pasteur Ens. 235), *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus megaterium*, *Pseudomonas aeruginosa*. The inhibition zone diameter was determined for each extract using the agar diffusion method and quantitative analysis of usnic acid in these species was achieved using HPLC. Among the *Hypogymnia* species, maximum activity was observed in *H. tubulosa*. Usnic acid contents of tested *Hypogymnia* species was determined to vary between 0.63-2.40 % of dry weight.

**Key Words: *Hypogymnia*, Antimicrobial, Usnic acid, HPLC.**

## INTRODUCTION

Historically, a large portion of the world's medicine has been derived from plants and fungi. Lichens, which are the symbiotic associations between a fungus and a photosynthetic partner such as an alga or a cyanobacterium, were also used for ancient medicine in the past. For example, Nitinaht tribe of Native Americans used *Usnea longissima* as a dermatological aid for dressing wounds[1]. Investigation of traditional uses of lichens gives a basis for exploration of lichens chemical constituents and their usage for modern science. Lichens are well known for being rich in structurally unique phenolic derivatives with unclear biological roles[2-4]. Only 50-60 of the 630 known lichen secondary metabolites have been found in other fungi or higher plants[5]. These compounds represent usually 0.1-5.0 % of dry weight; however, they can comprise up to 20 % of thalli dry weight[6,7].

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Even though various activities of lichen metabolites (antibiotic, antimycobacterial, antiviral, antiinflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects) have now been recognized. Their therapeutic potential has not yet been fully explored and thus remains pharmaceutically unexploited[8]. Antibiotic properties of the lichens are of special interest to the scientists[4]. According to one estimate, 50 % of all lichens have antibiotic properties[9]. Burkholder *et al*.[10] was pioneer initiating research on lichens as antibacterial agents. They tested 42 lichens for antibiotic properties and 27 of them were reported to inhibit growth of *S. aureus* or *B. subtilis*, four species inhibited *P. vulgaris* or *Alcaligenes fecalis* bacteria[10]. Usnic, vulpinic, evernic, physodic, gyrophoric acids and atranorin, are some examples of lichen substances showing antimicrobial activity. Usnic acid is a wide-spectrum antibiotic characterized from the lichens[11-15].

Vulpinic and physodic acid has mild antibiotic property[16,17]. Atranorin has been found to be much less biologically active than usnic acid[17]. Usnic acid although showing differences in effectiveness against various species, is widely accepted as having strong antibacterial and antifungal activity[15,18].

*Hypogymnia* species are leaf-like with grayish-green colouration on top and is black below. It can be confused with *Parmelia*, but *Hypogymnia* has hollow, tube-like lobes. *Hypogymnia* is usually found on conifers (needle-leaf trees) and sometimes rocks. Information on the medicinal uses of *Hypogymnia* species is scattered. Although many lichens are known to have patent antibiotic properties, most of the *Hypogymnia* species are not known. They are reported to contain mainly physodic, 3-hydroxyphysodic, physodalic, 2'-O-methylphysodic, protocetraric acids, atronin and chloratranorin[19-21]. The aim of this study is to determine the potential antibiotic properties of the acetone extracts of these lichen species from the Turkey: *Hypogymnia vittata* (Ach.), *Hypogymnia physodes* (L.) Nyl. and *Hypogymnia tubulosa* (Schaer.) Havvaas and Hav. and to investigate the existence of usnic acid as a secondary compound in three lichen species by using HPLC.

## EXPERIMENTAL

All *Hypogymnia vittata*, *Hypogymnia physodes* and *Hypogymnia tubulosa* species were collected from the forests of Yaylacik and Termosos National Park, Turkey in the month of May to July 2006. Collection areas were as shown in Table-1. Lichen species was identified by Cansaran-Duman and voucher specimens were deposited in the Department of Botany, Ankara University. Collected samples (each one 0.05 g) were dried at room temperature and foreign matter was removed prior to grinding.

### Determination of antimicrobial activity

**Microorganisms:** The microorganisms *Escherichia coli* (ATCC 35218), *Entero-coccus faecalis* (RSKK 508), *Proteus mirabilis* (Pasteur Ens. 235), *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus megaterium*, *Pseudomonas aeruginosa* were obtained from Refik Saydam National Type Culture Collection (RSKK) and Ankara University, Faculty of Science, Department of Biology.

TABLE-1
LOCATIONS OF THE LICHEN SAMPLES

| Species | Locality name |
|---|---|
| *Hypogymnia vittata* | Antalya Termosos Natural Park Gulluk Mountain |
| *Hypogymnia physodes* | Karabuk-Yenice Yaylacik Forest Sinekagzi Locality N 41°05'961''-E32°19'483'', 880 m |
| *Hypogymnia tubulosa* | Antalya Koprulu Kanyon Locality, 160 m |

**Preparation of lichen extracts for antimicrobial activity:** Lichen extracts for antimicrobial activity was isolated from lichen material according to the method given by Cansaran *et al.*[22]. In brief the extraction procedure was as follows: From dried lichen samples 0.05 g were weighed and put into screw capped glass tubes. Extraction was performed by adding 10 mL of acetone with 1 h extraction at room temperature. Chemicals used for extraction were obtained from Sigma and were of the highest grade available. At the end of incubation period tubes were centrifuged to remove lichens from supernatants. These extracts were used in the experiments. To prevent evaporation of solvents screw capped glass tubes were kept in refrigerator and all discs were prepared from each lichen extract at one time to prove consistency of concentrations.

**Antimicrobial activity assays:** For screening of antimicrobial activity the agar disc diffusion method was used. The extracts (50 µL) were dried on 6 mm filter paper discs. In addition control discs were prepared with solvents free of lichen extract in order to determine the antimicrobial activity of solvent acetone. Tetracycline (30 µg/disc) and usnic acid were used as references. Usnic acid discs were prepared by dissolving 0.05 g pure usnic acid (Aldrich, 329967-56) in 10 mL of acetone and soaking 50 µL of solution into filter paper discs. For antimicrobial assays, all bacterial strains were grown in Nutrient Broth medium (Oxoid) for 24 h at 37 °C. Then 0.1 mL of each culture of bacteria was spread on nutrient agar plate surfaces. After that, discs were placed onto agar petri plates and incubated. The inhibitory activity was indicated by inhibition zone and diameters were measured in mm[22]. The diameters of zone of inhibition produced by extracts were compared with those of standard antibiotics. All tests were performed in triplicate.

**Determination of HPLC analysis of the lichen samples**

**Sample preparation for HPLC analysis:** HPLC analysis was performed according to the protocol defined by Cansaran *et al.*[22]. In particular: air-dried lichens were ground and extracted in 0.05 g amount of 10 mL acetone at room temperature (20-22 ºC). The extracts were taken to darkness and stored at 4 ºC until HPLC analysis. Before analysis extracts were passed through 0.45 µm filters and then injected into the HPLC system in amounts of 20 µL.

**Standard and solvents:** All of the chemicals used in experiments were of HPLC grade from Sigma of highest purity. A stock solution of 1 mg/mL usnic acid was prepared in acetone. An appropriate dilution of this stock solution was made with

acetone. All of the standards were placed in an autosampler and analyzed. Calibration curves for usnic acid were obtained with seven samples of various concen-trations using linear regression analysis (Fig. 1).

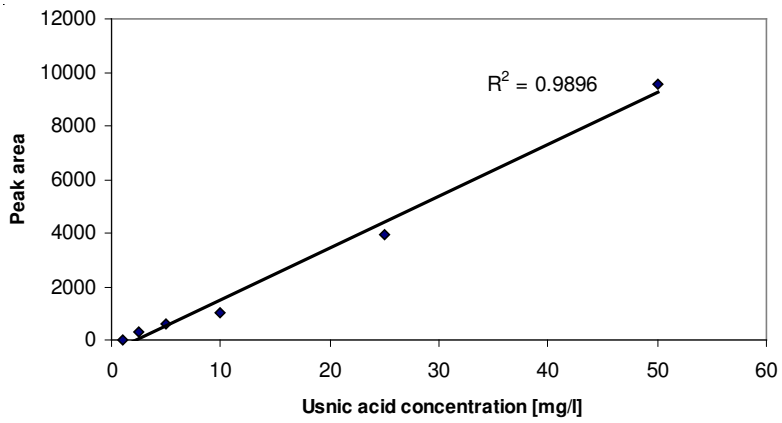


Fig. 1. Calibration curve of usnic acid (Sigma)

**Analytical conditions and apparatus:** A thermo Finnigan HPLC system equipped with a Surveyor LC pump, Surveyor photodiode array detector, Surveyor autosampler and data processor (ChromQuest 4.01) was used. Reverse phase Shim-pack CLC-ODS (M), 5 µm particle size, in a 250 mm × 4.6 mm i.d. stainless steel column was used. Flow-rate was 0.8 mL/min. For usnic acid detection at 245 nm, a mixture of methanol and phosphate buffer (pH 7.4) (70:30 v/v) was used as a mobile phase. Aliquots of the extracts (20 µL) were injected into the HPLC system. Each analysis was carried out in triplicate.

## RESULTS AND DISCUSSION

The antimicrobial activity of different crude extracts of *Hypogymnia* species was determined against seven bacterial strains which are reported in Table-1. Acetone extract of *Hypogymnia* species showed promising antibacterial activities against some bacterial strains. Among the *Hypogymnia* species, maximum activity was observed in *H. tubulosa* whereas *H. vittata* showed the least activity against all the bacterial strains. In this study, among gram positive bacteria *B. subtilis* was the most susceptible with inhibition zones of 16, 14 and 22 mm in acetone extracts of *H. vittata*, *H. physodes* and *H. tubulosa* species, respectively. Whereas in case of gram negative bacteria, *Escherichia coli* was the most susceptible bacteria with inhibition zones of 10, 12 and 16 mm in acetone extracts of *H. vittata*, *H. physodes* and *H. tubulosa* species, respectively.

According to present results both gram positive and gram negative bacteria were susceptible to the lichen extracts, but lichen extracts were more active against gram positive microorganisms than the gram negative ones. Resistances of gram

negative bacteria to several plant and lichen extracts were reported by some workers[23-25]. The MIC values of lichen acids are generally higher for gram negative bacteria[16,26].

In literature, diversity of bacterial species and strains as well as lichen species used in different researches affect the results of antimicrobial activity of plant extracts[27-33]. Choice of extractant is also another factor affecting the results[34,35]. The aqueous extracts of the lichens generally have no antimicrobial activity and extraction with acetone, ethanol or methanol was preferred in the investigations. Halama and van Haluwin[36] investigated antifungal activity of acetone extracts of the *Hypogymnia physodes*, *Evernia prunastri* and *Cladonia portentosa* against eight plant pathogenic fungi and found that *H. physodes* and *E. prunastri* exhibit total or strong inhibition on *Pythium ultimum*, *Ustilago maydis* and *Phytophthora infestans* growth.

Rankovic *et al.*[17] showed that the secondary metabolites of *Hypogymnia physodes*, *Physcia aipolia*, *Umbilicaria polyphylla* and *Parmelia caperata* have antimicrobial effect on some of gram negative and gram positive bacteria. They isolated the physodic acid as lichen substance from *Hypogymnia physodes*. They found that application of physodic acid at high concentration (1 mg/mL) was necessary to inhibit tested bacteria and fungi.

Atranorin which is also a metabolite of *Hypogymnia* was shown to bear antimicrobial properties[17,37]. Yilmaz *et al.*[29] investigated the antimicrobial activity of extracts of *Hypogymnia tubulosa* and its 3-hydroxyphysodic acid constituent. They reported that 3-hydroxyphysodic acid isolated from *Hypogymnia tubulosa* (Schaerer) *Havaas* (Parmeliaceae) showed antimicrobial activity against *Aeromonas hydrophila*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Proteus vulgaris*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Candida albicans*[37]. However, no antifungal activity of the extracts has been determined against ten filamentous fungi.

In a recent study, Rankovic *et al.*[38] investigated antibacterial and antifungal activities of acetone, methanol and aqueous extracts of the lichens *Hypogymnia physodes*, *Cladonia furcata*, *Parmelia caperata*, *Parmelia pertusa* and *Umbilicaria polyphylla*. Acetone extracts of *Hypogymnia physodes* manifested strong antimicrobial activity and acted on all of the bacteria and fungi tested. Diameters of inhibition zones based on disc-diffusion assay for *B. subtilis*, *E. coli* and *S. aureus* are more than present findings for the corresponding bacteria (Table-2). The reasons for these differences may be usage of the harsh extraction procedure or the higher extract concentrations soaked to discs.

In this study, quantitative analysis of the antimicrobial substance usnic acid in the acetone extracts of *Hypogymnia* species were achieved using HPLC. A sample of representative chromatograms is shown in Fig. 2. Identification of peaks in chromatograms of lichen extracts was accomplished by comparison of retention times with that of standard usnic acid. Usnic acid amounts and retention times in the

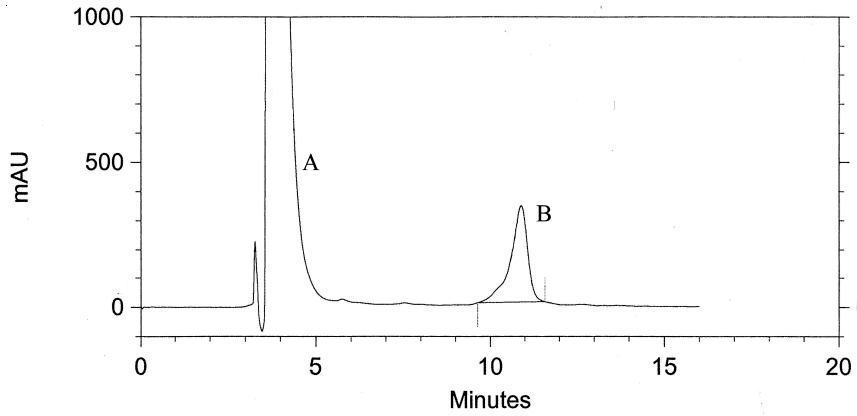


Fig. 2. Analysis of usnic acid from *Hypogymnia tubulosa* by HPLC. (A) solvent ($t_R$ = 5.5 min); (B) usnic acid ($t_R$ = 11.1 min)

TABLE-2
ANTIMICROBIAL ACTIVITES OF ACETONE EXTRACTS OF *Hypogymnia vittata*, *Hypogmnia physodes* AND *Hypogymnia tubulosa* AGAINST DIFFERENT GRAM-POSITIVE *cocci*, *bacilli* AND GRAM-NEGATIVE *bacilli* TESTED BASED ON DISC-DIFFUSION ASSAY

| Strains | Inhibition zones in diameter mm | | | | |
|---|---|---|---|---|---|
| | *Hypogymnia vittata* | *Hypogymnia physodes* | *Hypogymnia tubulosa* | Tet | Usnic acid |
| Bacteria | | | | | |
| *Escherichia coli* (ATCC 35218) (G-) | 10 ± 0.01 | 12 ± 0.01 | 16 ± 0.01 | 12 | 16 |
| *Proteus mirabilis* (Pasteur Ens. 235) (G-) | 10 ± 0.01 | – | 14 ± 0.01 | 8 | 19 |
| *Pseudomonas aeruginosa* (G-) | – | – | – | 20 | – |
| *Enterococcus faecalis* (RSKK 508) (G+) | 7 ± 0.01 | – | 8 ± 0.01 | 30 | 12 |
| *Staphylococcus aureus* (G+) | – | – | – | 40 | – |
| *Bacillus subtilis* (G+) | 16 ± 0.01 | 14 ± 0.01 | 22 ± 0.01 | 26 | 26 |
| *Bacillus megaterium* (G+) | 12 ± 0.01 | 16 ± 0.01 | 20 ± 0.01 | 20 | 22 |

*Includes diameter of disc (6 mm). Tet: Tetracycline; (–) no inhibition; G+ gram positive; G: Gram negative.

TABLE-3
USNIC ACID CONTENT AND RETENTION TIMES OF LICHEN SPECIES

| Species | Percentage of usnic acid in dry weight | Retention time (min) |
|---|---|---|
| *Hypogymnia vittata* | 0.63 ± 0.03 | 11.1 |
| *Hypogymnia physodes*[44] | 1.05 ± 0.00 | 10.8 |
| *Hypogymnia tubulosa*[44] | 2.40 ± 0.00 | 11.1 |

acetone extracts of *Hypogymnia vittata*, *Hypogymnia physodes* and *Hypogymnia tubulosa* are given in Table-3. The highest amount of usnic acid was found to be about 2.4 % of the dry lichen weight in *Hypogymnia tubulosa*. Usnic acid is extensively distributed in species of *Cladonia*, *Usnea*, *Leconora*, *Ramalina*, *Evernia*, *Parmelia*, *Xanthoparmelia* and other lichen genera[39,40]. *Alectoria* species are rich in terms of usnic acid and yields of up to 6 % have been reported[41]. Cansaran *et al*. showed that the usnic acid content in *Rhizoplaca* species varied between 0.19-4.00 % dry lichen weight[42]. Usnic acid contents of *Ramalina* species was reported to be varied between 0.13 and 3.23 % of dry weight[22]. Usnic acid contents of *Usnea* species ranges from 0.22-6.49 % of dry weight[43]. In this study usnic acid contents of tested *Hypogymnia* species was determined to vary between 0.63-2.40 % of dry weight. To our best of knowledge, existences of usnic acid in the presented *Hypogymnia* species together with their antimicrobial activities were not evaluated in previous studies. This may be due to the relatively low concentration of usnic acid in extracts[44] compared to other *Hypogymnia* metabolites like physodic acid, 3-hydroxyphysodic, physodalic acids, atronin, *etc*. Thus in antimicrobial activity of these species, usnic acid with other bioactive acids may be involved in a synergistic manner.

## Conclusion

Lichens are suitable for studies that evaluate antibiotic properties. Antimicrobial activities of *Hypogymnia* species were assessed and their usnic acid contents determined by HPLC were given. The present study is devoted to determine the potential antibiotic properties of the acetone extracts of these *lichen* species from the Turkey. To our best of knowledge the current research will clarify the usnic acid concentration and antimicrobial activities of these species for the first time.

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