

Squamarina lentigera türlerinde usnik asit konsantrasyonunun antimikrobiyal aktivitesi

Antimicrobial activity of usnic acid on *Squamarina lentigera* lichen species

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ÖZET

Amaç: *Squamarina lentigera* liken türlerinden ekstre edilen usnik asitin *Bacillus megaterium*, *Bacillus subtilis*, *Enterococcus faecalis* (RSKK 508), *Escherichia coli* (ATCC 35218), *Proteus mirabilis* (Pasteur Ens. 235), *Pseudomonas aeruginosa* ve *Staphylococcus aureus* olmak üzere yedi bakteri türüne karşı antimikrobiyal aktivitesi test edilmiş ve bu bakteriler üzerinde inhibisyon etkisi olan iki likenin usnik asit miktarları belirlenmiştir.

Yöntem: *S. lentigera*'ya ait 0,05 g tallusa 10 mL aseton eklenip bir saat oda sıcaklığında bekletilmiştir. Her iki liken türünde bulunan usnik asidin antimikrobiyal aktivitesinin tespiti için agar difüzyon yöntemi uygulanmıştır. Buna ek olarak, HPLC yöntemi kullanılarak bu liken türünün usnik asit miktarları belirlenmiştir. Ayrıca, liken ekstraktının kromatogramlarında alıkonma sürelerine göre oluşan pikler, standart usnik asidin alıkonma süreleri ile karşılaştırılmıştır.

Bulgular: Bu çalışmada, *S. lentigera* türünün usnik asit ekstraktının aktif olduğu ayrıca *B. megaterium* ile *B. subtilis* bakterilerine karşı daha yüksek inhibisyon etkisi gösterdiği belirlenmiştir. *Squamarina lentigera*'dan elde edilen inhibisyon zon çapı standart

ABSTRACT

Objective: Usnic acid extracted from *Squamarina lentigera* was tested for antimicrobial activities against seven bacteria including *Bacillus megaterium*, *B. subtilis*, *Enterococcus faecalis* (RSKK 508), *Escherichia coli* (ATCC 35218), *Proteus mirabilis* (Pasteur Ens. 235), *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and usnic acid concentrations of this lichen species was determined.

Method: 0.05 g of thalli of *S. lentigera* was added into 10 mL acetone and left for extraction at room temperature for 1 h. We used agar diffusion method for screening antimicrobial activity of usnic acid in this lichen species. In addition, the quantitative analysis of usnic acid in this lichen species was achieved by using HPLC. Identification of peaks in chromatograms of lichen extract is achieved by comparison of retention times with that of standart usnic acid.

Results: The usnic acid extracts of *S. lentigera* were effectively active lichen extracts, and showed the highest inhibition effect on *B. megaterium* and *B. subtilis*. When the inhibition zones obtained from *S. lentigera* was compared with that of standart antibiotic, *E. coli* seems to be more susceptible

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antibiyotik ile karşılaştırıldığında; liken ekstraktına en duyarlı bakterinin *E. coli* olduğu belirlenmiştir. Çalışılan liken türünün aseton ekstraktı, *S. aureus* hariç test edilen tüm Gram pozitif bakterilerin gelişmesini inhibe etmiştir. *B. subtilis*'in çalışılan liken türüne karşı duyarlı olduğu gözlenmiştir. Kontrol olarak kullanılan aseton çalışmada kullanılan hiçbir bakteriye karşı etki göstermemiştir. *S. lentigera*'nın aseton ekstraktının usnik asit miktarları %2,47 olarak bulunmuştur.

Sonuç: Çalışmamızın sonucunda; *S. lentigera* türlerinin önemli antimikrobiyal etki gösterdikleri belirlenmiştir. Bu da bize usnik asit miktarı ile antimikrobiyal aktivite arasında bir korelasyon olduğunu ve usnik asit konsantrasyonunun artmasıyla antimikrobiyal aktivitenin de arttığını göstermiştir. Bu çalışma, medikal ve farmakolojik ürün olarak kullanılabilir *S. lentigera* türünün antimikrobiyal aktivitesinin ve usnik asit derişimlerinin belirlediği literatürdeki ilk makaledir.

Anahtar Sözcükler: Antimikrobiyal aktivite, HPLC, liken, usnik asit.

to the lichen extract. Acetone extract of examined lichen species inhibited the growth of all tested Gram positive bacteria, with the exception of *S. aureus*. *B. subtilis* seems to be sensitive to the acetone extracts of examined lichen species. The solvent controls did not show any activities against the bacteria. The amounts of usnic acid in the acetone extracts of *S. lentigera* were determined as 2.47%.

Conclusion: Our results demonstrating significant antimicrobial effects of *S. lentigera*, which are a contribution to the literature. This might explain the correlation between usnic acid concentration and antimicrobial activity. Results show an increase of antimicrobial activities by the increase of the amount of usnic acid concentration. The present study is the first paper on usnic acid concentration and antimicrobial activity of *S. lentigera*, which could be attracting for medicinal or pharmacological products.

Key Words: Antimicrobial activity, HPLC, lichen, usnic acid.

INTRODUCTION

Lichens are products of symbiotic associations between a mycobiont (fungus) and photobiont(s) (algae and/or cyanobacteria) that comprise about 17,000 species. The organism consists of thalli-shaped fungal tissue in which the algal cells are located and, therefore, it can grow photosynthetically. There is specificity and selectivity in the association, and the thallus shape, physiology and nutritional dependencies are defined and stable (1). Lichens produce a diverse range of secondary metabolites such as depsides, depsidones, dibenzofurans and pulvinic acids. Lichens and their metabolites exert a wide variety of biological functions and have been used in perfumery, cosmetics, ecological applications, and pharmaceuticals. These compounds have attracted much attention in investigations because of their antimicrobial, antimycotic, antiprotozoal, antiproliferative, anti-inflammatory, analgesic and

antipyretic, antiviral, antibiotic, antioxidant, antitumor, allergenic and plant growth inhibitory activities (2, 3).

Usnic acid was isolated as a prominent secondary lichen metabolite by the German scientist Knop in 1844 (4). When extracted from the lichen, it is yellow and crystalline in appearance. Usnic acid [2,6 - diacetyl - 7,9 - dihydroxy - 8, 9b - dimethyl-dibenzofuran - 1,3 (2H, 9bH) - dione] exists in two enantiomers; (+) D-usnic acid and (-) L-usnic acid, indicating an R or S projection of the angular-CH₃ group at position 9b. The enantiomers have been identified as showing different biological activities (5). In addition, two other natural isomers-(+) and (-) isousnic acids [2,8 - diacetyl - 7,9 - dihydroxy - 6,9b - dimethyl-dibenzofuran - 1,3 (2H, 9bH) - dione] are also found in lichens (5).

Usnic acid has been identified in several species of such genera as *Alectoria*, *Cladonia*, *Dermatocarpon*, *Evernia*, *Flavoparmelia*, *Hypogymnia*, *Lecanora*, *Letharia*, *Parmelia*, *Platismatia*, *Protoparmeliopsis*, *Punctelia*, *Ramalina*, *Rhizoplaca*, *Squamarina*, *Xanthoparmelia*, *Umbilicaria* and *Usnea*. Usnic acid is a prominent secondary lichen metabolite that has been used for various purposes worldwide. Crude extracts of usnic acid or pure usnic acid have been marketed in the United States as dietary supplements to aid in weight loss. However, the USA Food and Drug Administration (FDA) received 21 reports of liver toxicity related to the ingestion of dietary supplements that contain usnic acid; this was prompted by the FDA to issue a warning about one such supplement, LipoKinetix, in 2001 (6).

The *in vitro* antimicrobial activities of usnic acid were evaluated in combination with five therapeutically available antibiotics, using checkerboard microdilution assay against methicillin-resistant clinical isolates strains of *S. aureus*. MIC90, MIC50, as well as MBC90 and MBC50. A synergistic action was observed in combination with gentamicin, while antagonism was observed with levofloxacin. The combination with erythromycin showed indifference, while variability was observed for clindamycin and oxacillin. Data from checkerboard assay were analysed and interpreted using the fractional inhibitory concentration index (FICI) and the response surface approach using the E-model. Discrepancies were found between both methods for some combinations. These could mainly be explained by the failure of FIC approach, being too much subjective and sensitive to experimental errors. These findings, beside confirm the well known antimicrobial activity of usnic acid, suggest, however, that this substance might be a good candidate for the individuation of novel templates for the development of new antimicrobial agents or combinations of drugs for chemotherapy (7).

Usnic acid has several interesting biological traits. It is an antibiotic and it also seems to exert

an antimetabolic action. It has even been postulated that usnic acid can play a role as an environmental indicator, since its concentration varies according to the presence of toxic agents. A series of tests have been run on different biological systems such as fungi, yeasts, plant cells and neoplastic human cell cultures in order to make a general evaluation of the properties of usnic acid and to highlight any analogy between its effects on phylogenetically distant organisms. The obtained results confirm some of known properties of usnic acid and identify concentration ranges that are active against cells from different organisms. Furthermore, at low concentrations, the acid displays a capacity to stimulate cell metabolism in some of the biological systems tested (6).

In order to make a general evaluation of the biological activity of usnic acid, Cardarelli et al. investigated its effects on a number of widely different biological systems (8). These study concludes to interesting results concerning usnic acid's capacity to act as 1) an activator of the respiratory capacity of immobilized *Saccharomyces cerevisiae* Meyen yeasts, 2) a cytotoxic and anti-mitotic agent against mesophyll leaf protoplasts and cultured plant cells of *Nicotiana tabacum* L. (tobacco), and 3) a cytotoxic and antimetabolic agent against human tumour cells. The inhibition of growth of the parasitic fungi *Fusarium moniliforme* J. Sheld. and of the germination of *Nicotiana tabacum* plantules has also been confirmed (8).

Usnic acid has also recently been discovered to have antiviral properties. In a cancer chemoprevention assay, (+)-usnic acid isolated from *Usnea longissima* Ach. was found to be significantly effective against tumor-promoter-induced Epstein-Barr virus with an ED50 of 1.0 µg/mL (9). (+)-Usnic acid also inhibited the cytopathologic effects of herpes simplex type I and polio type1 viruses in the infected kidney cells of the African green monkey (10). In a clinical trial, the effect of an intravaginal formulation containing usnic acid and zinc sulfate as an adjuvant therapy to

radio surgical treatment was evaluated in 100 women with genital infections of human papilloma virus. The treatment significantly improved the time of re-epithelization one month after the radio surgery (10).

The study of lichens and lichen substances, in point of antibiotic view started in 1944, when Burkholder and Evans (11) published the first qualitative study of the antibiotic properties of lichens (12). Although several detailed studies on the lichen from Turkey concerning with its antimicrobial activities have recently been published (13-22), knowledge on the antimicrobial activities of several lichens are still necessary.

The aim of this study is to evaluate in vitro antimicrobial activities of the acetone extracts obtained from lichen *Squamarina lentigera* (Weber) Poelt against seven test bacteria and determine usnic acid concentration of them. To our knowledge there are no published reports on antimicrobial activity of the usnic acid content from *S. lentigera* against these seven test bacteria.

MATERIAL AND METHODS

Lichen material

Squamarina lentigera was collected Mersin, Çamlıyayla, east of Sebil village, 1200 m, 37° 08' 40" N, 34° 34' 36" E, 08/ 08/ 2009. Collected samples (each one 0.05 g) were dried at room temperature and foreign matter was removed prior to grinding.

Determination of antimicrobial activity

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

Preparation of lichen extracts for antimicrobial activity: Lichen extracts for antimicrobial activity

was isolated from lichen material according to protocol given by (22). Briefly, 0.05 g of dried thalli were putted into screw capped glass tubes. Extraction was performed by adding 10 ml of acetone and incubating for 1 h at room temperature. Chemicals used for extraction were obtained from Sigma and were of the highest purity. At the end of incubation period, tubes were centrifuged to remove lichen from supernatants. This extract was 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: The agar disc diffusion method was used to screen antimicrobial activity of usnic acid in lichen species. 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 the solvent acetone. Tetracycline (30 µg/disc) was used as reference. 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 clear zones around the discs and inhibition zone diameters were measured in mm after incubation for 24 h at 37°C (4). 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-Duman (22). In particular, air-dried lichen was ground and 0.05 g sample was extracted in 10 ml acetone at room temperature. The extract was taken to darkness and stored at 4°C until HPLC analysis. Before analysis, extract was 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 concentrations using linear regression analysis (Figure 1).

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) were used. Reverse phase Shim-pack CLC-ODS (M), 5 µm particle size, in a 250 mm x 4.6 mm ID stainless steel column was used. Flow-rate was adjusted to 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 the mobile phase. Aliquots of the extracts (20 µl) were injected into the HPLC system. Each analysis was carried out in triplicate.

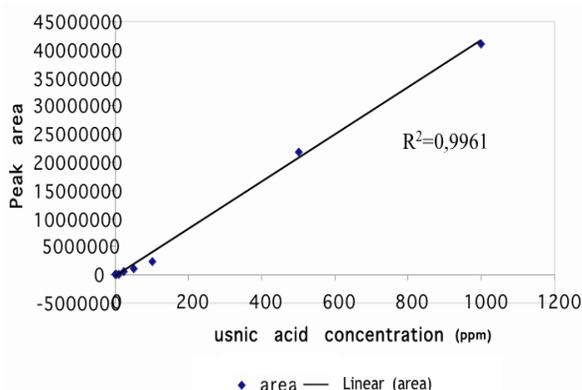


Figure 1. Standard calibration for the quantification of usnic acid by HPLC.

RESULTS

In this study, *S. lentigera* was analysed by HPLC analysis and for its antimicrobial activities. For this purpose, usnic acid concentration of *S. lentigera* was screened for their antimicrobial effects on seven test bacteria. Table 1 summarizes the inhibition results

obtained with *S. lentigera* extract against the tested bacteria. The solvent controls did not show any activities against the bacteria.

The usnic acid extracts of *S. lentigera* were effectively active lichen extracts, and showed the highest inhibition effect on *B. subtilis* and *B. megaterium*. Extracts of *S. lentigera* were more susceptible to *E. coli* when compared to standard antibiotic. Acetone extract of examined lichen species inhibited the growth of all tested Gram positive bacteria, with the exception of *S. aureus*. *B. subtilis* seems to be sensitive to the acetone extracts of both lichen species.

Quantitative analysis of usnic acid in species *S. lentigera* was performed by using HPLC. Identification of peaks in chromatograms of lichen extracts was achieved by comparison of retention times with that of standart usnic acid. *S. lentigera* of representative chromatograms in shown in Figure 2. The amounts of usnic acid and retention times in the acetone extracts of examined lichen species is given in Table 2. The amount of usnic acid was found as 2.47% of the dry lichen weight in *S. lentigera*.

Table 1. Inhibition diameter zones (mm) on the tested bacteria of acetone extract of *Squamarina lentigera*

Tested Bacteria	Inhibition Diameter Zone	Control (Tetracycline)
<i>E. coli</i>	14±0.01	12±0.00
<i>E. faecalis</i>	7±0.01	30±0.00
<i>P. mirabilis</i>	13±0.01	8±0.00
<i>S. aureus</i>	-	40±0.00
<i>B. subtilis</i>	25±0.01	26±0.00
<i>B. megaterium</i>	23±0.01	20±0.00
<i>P. aeruginosa</i>	-	20±0.00

-: no inhibition

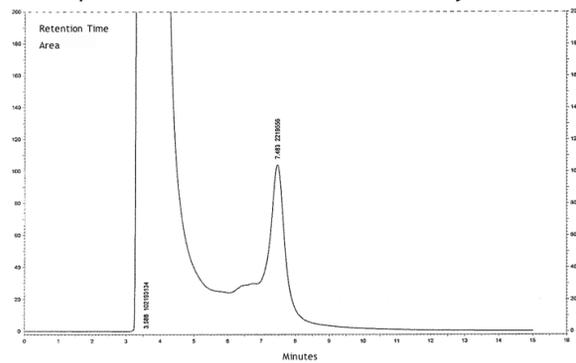
Tablo 2. Usnic acid content and retention time of acetone extracts of *Squamarina lentigera*

Acetone extracts of <i>Squamarina lentigera</i>	
Retention time (min.)	7.48
% of Usnic Acid in Dry Weight	2.47±0.01

DISCUSSION

During the 1980s, interest in usnic acid as an antimicrobial agent was renewed because of increasing experience of multidrug resistance caused by overuse of synthetic antibiotics (23). It has been shown that both the optical enantiomers of usnic acid are active against Gram-positive bacteria and mycobacteria (24), and several research studies and clinical trials have confirmed the antibacterial properties of usnic acid (24). Usnic acid has been shown to suppress the growth of Gram-positive organisms that are mainly responsible for body odor. Ethoxydiglycol extracts of lichens containing 10% usnic acid on a wet weight basis have been demonstrated to have preservative potential in moisturizing cream (24). Other recent studies have shown that usnic acid is active against methicillin-resistant *S. aureus* (25, 26), and its potential use in the sterilization of surgical implants is being investigated (6, 27).

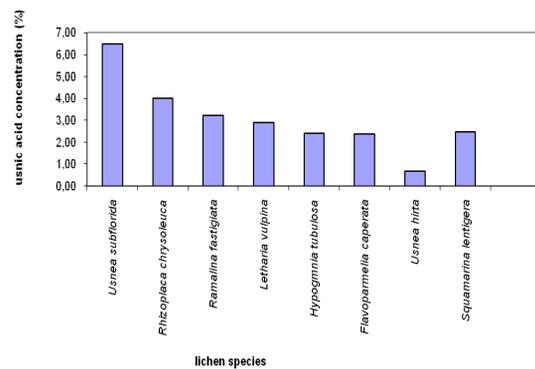
In our laboratory, several studies were accomplished on the antimicrobial activity of lichens

**Figure 2.** Analysis of usnic acid from *Squamarina lentigera* by HPLC. (A), solvent ($t_R = 3.5$ min.); (B), usnic acid ($t_R = 7.48$ min.)

collected from Turkey (16-22). According to these results, the highest usnic acid concentration was recorded in *Usnea subfloridana* Stirt. (6.49%) (19), *Rhizoplaca chrysoleuca* (Sm.) Zopf (4.00%) (21), *Ramalina fastigiata* (Pers.) Ach. (3.23%) (17), *Letharia vulpina* (L.) Hue (2.89%) (18), *Hypogymnia tubulosa* (Schaer.) Hav. (2.40%) (16), *Flavoparmelia caperata* (L.) Hale (2.38%) (22) and *Usnea hirta* (L.) Weber ex F.H. Wigg. (0.68%) (20) (Figure 3). Comparing with the the present study, the usnic acid amounts of *S. lentigera* is found as 2.47% (Figure 3).

Compared with the antimicrobial activity of *R. chrysoleuca* (21), which shows the highest inhibition effects on *B. megatarium*, the inhibition zones of *S. lentigera* was less, those of *B. megatarium* was found to be more sensitive to *R. chrysoleuca* extracts.

Usnic acid and three other secondary lichen metabolite were isolated from *Punctelia subrudecta* (Nyl.) Krog collected on Palma of the Canary Islands in Spain. Compounds were purified by solvent extraction, silica gel column chromatography, and preparative high-performance liquid chromatography (HPLC) consecutively. The structures of these four compounds were elucidated by one and two-dimensional nuclear magnetic resonance (NMR) experiments and mass spectrometric investigations. As a result, these compounds showed activity against important Gram-positive and Gram-negative

**Figure 3.** Usnic acid concentration (%) of some lichen species.

pathogens like mycobacteria and multiresistant staphylococci. This activity was combined with antiproliferative activity and cytotoxicity (28).

Several records are available on the studies of the antimicrobial activity of lichens in some provinces of Turkey. For example, according to Güllüce et al. (29) methanol extracts of *Parmelia saxatilis* (L.) Ach., *Platismatia glauca* (L.) W.L. Culb. & C.F. Culb., *Ramalina polymorpha* (Lilj.) Ach. and *Umbilicaria nylanderiana* (Zahlbr.) H. Magn. were shown to have antimicrobial and antioxidant activities on some test bacteria, fungi and yeast. As a result, all of the extracts from five different lichens species have an antimicrobial activity against at least some of these

bacteria species, and the extract of *R. pollinaria* was shown to have the highest antimicrobial activity, while *P. saxatilis* has the highest inhibition effect on *B. megaterium* and *B subtilis*.

As conclusion, the present study might explain a correlation between usnic acid concentration and antimicrobial activity. The higher the usnic acid concentration, the increased the antimicrobial activities. According to the best of our knowledge, this is the first report showing that usnic acid concentration and antimicrobial activity determination of both lichen species for medicinal or pharmacological products.

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