TÜRKİYE'DE BAZI LİKEN TÜRLERİNDEKİ USNİK ASİTİN HPLC YÖNTEMİ İLE DEĞERLENDİRİLMESİ VE ANTİMİKROBİYAL AKTİVİTELERİ

Evaluation of Usnic Acid in Some Likens of Turkey by HPLC Analysis and Screening of their Antimicrobial Activity

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ÖZET

Amaç: Bu çalışmanın amacı, *Parmeliacea* familyasına ait *Parmelia saxatilis* (L.) Ach., *Parmelia sulcata* Taylor, *Parmelina tiliacea* (Hoffm.) Hale, *Xanthoparmelia conspersa* (Ach.) Hale, ve *Flavoparmelia caperata* (L.) Hale liken türlerinin aseton ekstraktlarının *Escherichia coli* (ATCC 35218), *Enterococcus faecalis* (RSKK 508), *Proteus mirabilis* (Pasteur Ens. 235), *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus megaterium*, *Pseudomonas aeruginosa* türlerini içeren yedi farklı bakteri türüne karşı antimikrobiyal aktivitelerinin değerlendirilmesidir.

Yöntem: İnhibisyon zon çapları her bir ekstrakt için agar difüzyon yöntemi ile belirlenmiştir. Daha sonra, bu türlerdeki usnik asit miktarları HPLC yöntemi kullanılarak tespit edilmiştir.

Bulgular: Bu çalışmada elde edilen verilere göre, Türkiye'den toplanan beş liken türünün geniş bir aralıkta değişen oranlarda antimikrobiyal aktivite gösterdikleri sonucuna ulaşılmıştır. En yüksek usnik asit miktarı % 2.38'lik bir oran ile *Flavoparmelia caperata* liken türünde tespit edilmiştir. İncelenen liken türlerinin tümünün, *S. aureus ve P. aeruginosa* hariç; *E. coli*, *B. subtilis* ve *B. megaterium* bakterilerine karşı antimikrobiyal aktivite gösterdiği bulunmuştur. *F. caperata* 'nın aseton ekstraktının *B. subtilis* ve *B. megaterium*'a karşı en yüksek antimikrobiyal aktivite gösterdiği belirlenmiştir.

Sonuç: Araştırmada likenlerde usnik asit miktarı arttıkça antimikrobiyal aktivitenin de arttığı belirlenmiştir. Bu araştırmanın, Türkiye'de bulunan bazı *Parmelia* liken türlerinin usnik asit kompozisyonu ve antimikrobiyal aktivitesi üzerine yapılan ilk çalışma olması nedeni ile önemli olduğu düşünülmektedir. Ayrıca, *F. caperata* başta olmak üzere çalışılan liken türleri tedavi amaçlı ilaç içerisinde antimikrobiyal ajan olarak kullanılabilir.

Anahtar Sözcükler: Liken, usnik asit, HPLC

ABSTRACT

Objective: The aim of this study was to evaluate the antimicrobial activity of acetone extracts obtained from the *Parmelia saxatilis* (L.) Ach., *P. sulcata* Taylor, *Parmelina tiliacea* (Hoffm.) Hale, *Xanthoparmelia conspersa* (Ach.) Hale, and *Flavoparmelia caperata* (L.) Hale belonging to family *Parmeliaceae* against seven different bacterial species including *Escherichia coli* (ATCC 35218), *Enterococcus faecalis* (RSKK 508), *Proteus mirabilis* (Pasteur Ens. 235), *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus megaterium*, *Pseudomonas aeruginosa*.

Method: The inhibition zone diameters were determined for each extract by the agar diffusion method. Afterwards, quantitative analyses of usnic acid amounts found in these species were determined by HPLC method.

Results: Based on the data obtained in this research, it is possible to conclude that five species of lichen collected from Turkey exhibited a broad range of antimicrobial activity at varying degrees. In the study, highest amount of usnic acid was determined in *Flavoparmelia caperata* as; 2.38% of the dry lichen weight. All of the examined lichen species in this study, with the exception of the *S. aureus* and *P. aeruginosa*, were found to show antimicrobial activities against *E. coli*, *B. subtilis* and *B. megaterium*. *F. caperata* was determined have the highest inhibition effect on *B. subtilis* and *B. megaterium*.

Conclusion: In this research it was determined that, as the amount of usnic acid concentrations were higher, the antimicrobial activities of them also increased. This study is thought to be important as it is the first report on the usnic acid composition and antimicrobial activity of some *Parmelia* lichen species found in Turkey. Lichens species especially *F. caperata* could be used as antimicrobial agents in new drugs for therapy.

Key Words: Lichen, usnic acid, HPLC

INTRODUCTION

Lichens are slow growing associations which consist of two partners living in symbiotic association; an alga (the phycobiont) and a fungus (the mycobiont). Medical uses of lichens have been confirmed by studies showing that some lichen metabolites like depsides, depsidones, and usnic acid are active against mycobacteria and Gram positive bacteria (1).

Usnic acid is a naturally occurring compound which can be obtained from different kinds of lichens. Both the R-(+) and S-(-) forms are known. Usnic acid [C18H16O7] which is a yellow crystal substance of natural origin is a dibenzofuran derivative [2,6 - diacetyl - 7,9 - dihydroxy -8,9 b - dimethyl-1,3 (2H, 9 bH)- dibenzo - furandione. Usnic acid is known to show antimicrobial (2), antifungal (*Penicillium frequentas* and *Verticillium albo-atrium, Fusarium moniliforme*) (3), antiviral (*Herpes simplex, Polio virus, Epstein-Barr virus*) (4,5), antiproliferative (cytotoxic, cytostatic activity against malignat cells K-5629) (6-8), anti-inflammatory and analgesic activity (9,10). It can also be used against several skin infections (11).

Antimicrobial activity of both optical isomers of usnic acid is approximately the same against Gram positive bacteria (*Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus, Streptococcus mutans, Streptococcus pyogenes*), anaerobic bacteria (*Bacteroides fragilis, Bacteriodes acteriodes vulgatus, Propionibacterium acnes*) and mycobacteria (*Mycobacterium aurum, Mycobacterium tuberculosis var.* bosis) (12). Bearing in mind certain pharmacodynamical activities (antiseptic and anesthetic effects) (13), and the fact that usnic acid can be taken without any side effects in daily doses of 3-6 g per oral, the investigated substance finds its application in the production of pharmaceutical (septa-linguale, tooth paste) and cosmetic preparations (preservative in hydrated creams, inhibitor of Gram positive microorganisms in deodorant sprays) (14). However, from the technological point of view there is a permanent problem of aceutical-cosmetic industry so its sodium salt (Na-usninate) is used often.

In the last few years, in modern medicine, artificial devices are being used for repair or replacement of damaged parts of the body, delivery of drugs, and monitoring the status of critically ill patients. However, artificial surfaces are often susceptible to colonization by bacteria and fungi. Once microorganisms have adhered to the surface, they can form biofilms, resulting in highly resistant local or systemic infections. At this time, the evidence suggests that (+) usnic acid, a secondary lichen metabolite, possesses antimicrobial activity against a number of planktonic Gram positive bacteria, including *S. aureus, E. faecalis,* and *E. faecium*.

Since lichens are surface-attached communities that produce antibiotics including usnic acid, to protect themselves from colonization by other bacteria, the mode of action of usnic acid may be utilized in the control of medical biofilms (15).

In literature, extended number of patented and described procedures for isolation and characterization of usnic acid from various lichens are described in detail (8,11,16-18).

The aim of this study was to determine the usnic acid concentration and to evaluate in vitro antimicrobial activities of the acetone extracts obtained from *Parmelia saxatilis* (L.) Ach., *Parmelia sulcata* Taylor, *Parmelina tiliacea* (Hoffm.) Hale, *Xanthoparmelia conspersa* (Ach.) Hale, and *Flavoparmelia caperata* (L.) Hale species grown in Turkey.

MATERIALS AND METHODS

Lichen material

The lichen species were collected from various parts of Turkey in July 2006. Five lichen species were collected from different local and the collection areas are shown in Table 1. The lichen species used in this research were identified in a previous study (19).

Collected samples; 0.05 g of each, were dried at room temperature and foreign matters were removed prior to grinding. The lichen samples of this study were stored in the herbarium of Ankara University-ANK (Ankara University, Department of Botany, Ankara, Turkey).

Tablo 1. Locations of the lichen samples.

Species	Locality name
Parmelia saxatilis	Giresun- Şebinkarahisar Kabak Hill
Parmelia sulcata	Antalya Termosos Natural Park Güllük Mountain
Parmelina tiliacea	Antalya Termosos Natural Park Güllük Mountain
Xanthoparmelia conspersa	Giresun Dereli - Kulakkaya Area
Flavoparmelia caperata	Karabük- Yenice Yaylacık Forest Sarıçam Hill

Determination of antimicrobial activity

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

Preparation of lichen extracts for antimicrobial activity : Lichen extracts for antimicrobial activity were isolated from lichen materials according to protocol given by Cansaran D. et al (18, 23). Briefly the extraction protocol 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 and extraction at room temperature for 1 h. Chemicals used for extraction were obtained from Sigma (Germany) and were of the highest grade available. At the end of incubation period, tubes were centrifuged to remove lichens from supernatants. The extracts obtained with this procedure were used in the experiments. To prevent evaporation of solvents screw capped glass tubes were kept in refrigerator and to prove consistency of concentrations, all disks were prepared from each lichen extract at one time.

Antimicrobial activity assays : For screening of antimicrobial activity the agar disc diffusion method was used (4). The extracts (50 µl) were dried on 6 mm filter paper discs. Additionally, control discs were prepared with solvents free of lichen extract in order to determine the antimicrobial activity of solvent acetone. Tetracycline (30 µg/disc) was used as reference. For antimicrobial assays, all bacterial strains were grown in Nutrient broth (Oxoid, UK) at 37°C for 24 h. Then 0.1 ml of each culture of bacteria

were spread onto the surfaces of plates containing Nutrient agar (Oxoid). Afterwards, discs containing extracts were placed onto agar petri plates and incubated at 37°C for 24 h. After incubation, the inhibitory activities were indicated by clear zones around the discs and inhibition zone diameters were measured in mm (4). All tests were performed in triplicate.

Determination of HPLC analysis of the lichen samples

Sample preparation for HPLC analysis : HPLC analyses were performed according to the protocol defined by (19). In particular: 0.05 g of air-dried lichens were ground and extracted in 10 ml acetone at room temperature (20-22ºC). The extracts were taken to darkness and stored at 4ºC until HPLC analysis. Before analysis, extracts were passed through 0.45 µm filters and then 20 µl of them were injected into the HPLC system .

Standard and solvents : All of the chemicals used in experiments were of HPLC grade from Sigma (USA) 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 (Fig. 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) was used. Reverse phase Shim-pack CLC-ODS (M), 5 µm particle size, in a 250 mm x 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 (Figure. 2).

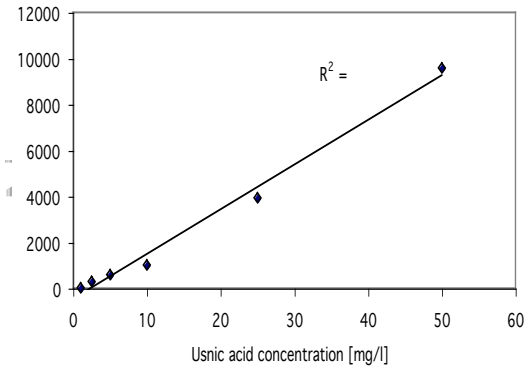

Figure 1: Standard calibration curve for the quantification of usnic acid by HPLC

RESULTS

A total of five species of lichens were studied in the HPLC analysis and antimicrobial activity. The usnic acid concentrations of *P. saxatilis, P. sulcata, P. tiliacea, X. conspersa,* and *F. caperata* species and their antimicrobial effects against seven tested bacteria were screened (Table 2). The solvent controls did not show any activities against the microorganism.

The usnic acid extracts of *F. caperata* were determined to be the most active lichen extracts, and showed the highest inhibition effects on *B. subtilis* and *B. megaterium*. When the inhibition zones obtained from *F. caperata* were compared with that of standard antibiotic, it was determined that *E. coli* and *P. mirabilis* were more susceptible to the lichen extract. In the study, it was observed that acetone extracts of examined lichen species inhibited the growth of all tested Gram positive bacteria except *S. aureus*. Besides, *B. megaterium* strain was found to be sensitive to the acetone extracts of all tested lichen species.

After that, the quantitative analysis of usnic acid in *P. saxatilis, P. sulcata, P. tiliacea, X. conspersa*, and *F. caperata* species were performed by using HPLC. Identification of peaks in chromatograms of lichen extracts were achieved by comparison of

retention times with that of standart usnic acid. A sample of representing these chromatograms is shown in Figure 2. The amounts of usnic acid and retention times in the acetone extracts of lichens are given in Table 3. The highest amount of usnic acid was found as nearly 2.38 % of the dry lichen weight in *F. caperata*.

DISCUSSION

Antimicrobial and antifungal properties of especially usnic acid and other components of various extracts of lichens have recently been of great interest in both research and pharmaceutical industry. Owing to strong antimicrobial and antifungal features exhibited in agar diffusion test, the extracts of these lichens could be considered as a natural herbal source that could be freely used in pharmaceutical industry. Several records are available on the studies of the antimicrobial activity of lichens in some provinces of Turkey.

It was reported by Gulluce et al. (2006) that; methanol extracts of *P. saxatilis, Platismatia glauca, Ramalina polymorpha* and *Usnea nylanderiana* were shown to have antimicrobial, antifungal and antioxidant activities (20). Especially the extract of *Ramalina pollinaria* was shown the highest antimicrobial activity. Also extracts of *P. saxatilis* were also found to possess antimicrobial activity against some of the tested bacteria, fungi and yeasts. As the data found in the mentioned study were compared with the data obtained in this research, and our results for the antimicrobial activity showed similarities. In both of the studies, *P. saxatilis* was determined have the highest inhibition effect on *B. subtilis* and *B. megaterium*.

The antimicrobial activities of the diethyl ether, acetone, chloroform, petroleum ether, and ethanol extracts of the lichen *Xanthoparmelia pokornyi* and its gyrophoric acid and stenosporic acid constituents which were also screened against some foodborne

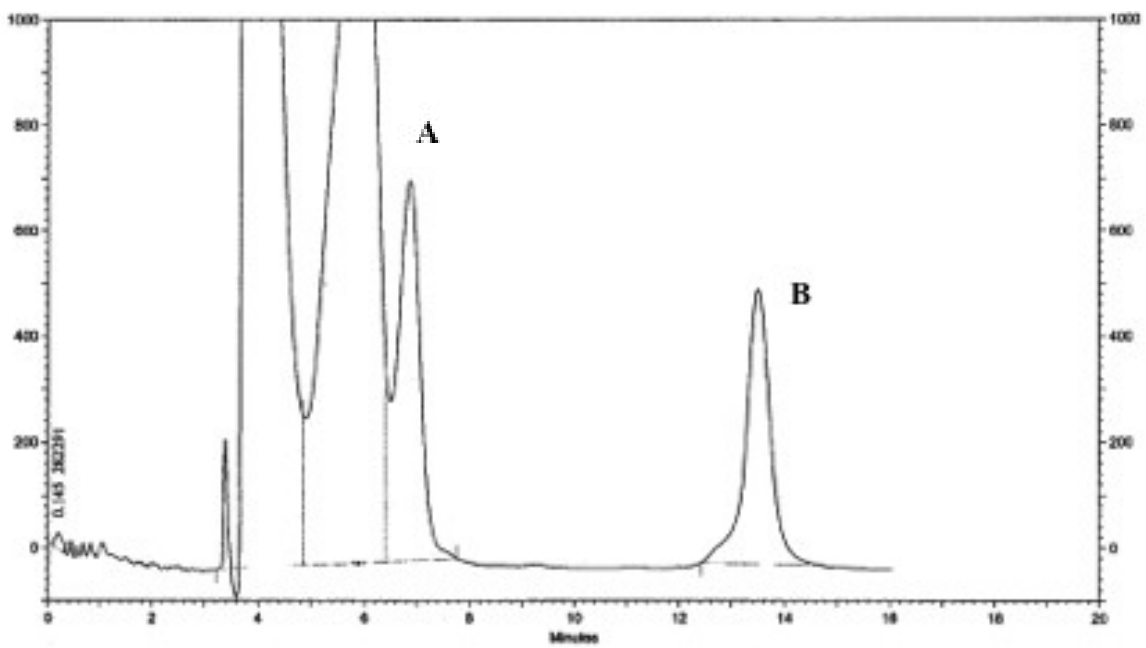

Figure 2: A sample chromatogram for the analysis of usnic acid from *Xanthoparmelia conspersa* by HPLC. (A) solvent (tR=5.5 min); (B) usnic acid (tR=13.4 min).

Tablo 2. Antimicrobial activity of acetone extracts of *P. saxatilis, P. sulcata, P. tiliacea, X. conspersa* and *F. caperata* against different Gram-positive cocci, bacilli and Gram-negative bacilli tested

	Mean inhibition zone diameter (mm)a					
	Xanthoparmelia conspersa	***Parmelia saxatilis***	***Parmelia tiliaceae***	***Parmelia sulcata***	***Flavoparmelia caperata***	**Tet**
Escherichia coli (ATCC 35218)	13±0.01	10±0.01	10±0.01	-	16±0.01	12
Enterococcus faecalis (RSKK 508)	7±0.01	-	-	-	7±0.01	30
Proteus mirabilis (Pasteur Ens. 235)	12±0.01	-	-	-	18±0.01	8
Staphylococcus aureus	-	-	-	-	-	40
Bacillus subtilis	20±0.02	9±0.01	10±0.01	10±0.01	24±0.01	26
Bacillus megaterium	17±0.01	12±0.01	12±0.01	12±0.01	21±0.02	20
Pseudomonas aeruginosa	-	-	-	-	-	20

*Includes diameter of disc (6 mm). Tet: Tetracycline; (-) no inhibition.

bacteria and fungi were investigated (21). Both the extracts and the acids showed antimicrobial activity against *Aeromonas hydrophila, Bacillus cereus, Bacillus subtilis, Listeria monocytogenes, Proteus vulgaris, Staphylococcus aureus, Streptococcus faecalis, Yersinia enterocolitica, Candida albicans* and *Candida glabrata*. In another study showed that the antimicrobial activities of acetone, chloroform, diethyl ether, methanol, and petroleum ether extracts of the lichen *P. sulcata* and its salazinic acid constituent which were screened against twenty eight foodborne bacteria and fungi (22). All of the extracts, with the exception of the petroleum ether extract, were found to show antimicrobial activity against *A. hydrophila, B. cereus, B. subtilis, L. monocytogenes, P. vulgaris, Y. enterocolitica, S. aureus, S. faecalis, C. albicans, C. glabrata, Aspergillus niger, Aspergillus fumigatus*, and *Penicillium notatum*. Our results which demonstrated significant antimicrobial effects of *P. saxatilis, P. sulcata, P. tiliacea, X. conspersa*, and *F. caperata* species were found to be in agreement with the data reported in the above mentioned reports.

Tablo 3. Usnic acid content and retention times of acetone extracts of *P. saxatilis, P. sulcata, P. tiliacea, X. conspersa* and *F. caperata*

Species	**% of usnic acid in dry weight**	**Retention time [min]**
Flavoparmelia caperata	2.38±0.02	11.1
Xanthoparmelia conspersa	1.10±0.05	13.4
Parmelia saxatilis	0.13±0.01	9.8
Parmelia tiliacea	0.10±0.01	11.2
Parmelia sulcata	0.07±0.02	10.8

Usnea species are often rich sources of usnic acid, and it was reported to have yields of up to 6.49 % (23). Comparing with the mentioned study, usnic acid amount of *F. caperata* was found 2.38% in the present research.

The antimicrobial activity of acetone, methanol and aqueous extracts of lichens *Cladonia furcata, P. caperata, P. pertusa, Hypogymnia physodes* and *Umbilicaria polyphylla* were assessed by Rankovic et al (24). The extracts were tested on six species

of bacteria and 10 species of fungi using the disk-diffusion method besides, broth tube dilution method was used for the determination of minimal inhibitory concentration (MIC). The strongest activity was recorded for the methanol extract of *P. pertusa* with the 0.78 mg/mL MIC value *P. caperata* was determined as the least active species showing its highest MIC value as; 50 mg/mL. A study was performed to found the effects of physodic acid, usnic acid, atranorin and gyrophoric acid on *Hypogymnia physodes, Parmelia caperata, Physcia aipolia* and *Umbilicaria polyphylla* (25). In the mentioned research, assessment for the antimicrobial effects were performed against six bacteria (three Gram positive and three Gram negative) and MIC were determined by using broth tube dilution method. It was reported that the lichen which was studied for the antimicrobial activity inhibited the growth of all tested microorganisms. The tested bacteria showed a higher sensitivity than the tested fungi. It was found that the usnic acid of the *P. caperata* lichen showed the highest antimicrobial activity, while the lowest MIC was found as 0.0037 mg/ml against Klebsiella pneumoniae (even lower than the one given by the streptomycin standard). In keeping with the finding of (25), acetone extracts of *F. caperata* were found more active against *B. subtilis* and *B. megaterium*.

Finally, the correlation of the usnic acid contents of the *P. saxatilis, P. sulcata, P. tiliacea, X. conspersa,* and *F. caperata* species with their antimicrobial activities is thought to be interesting in terms of finding new medicinal or pharmacological products. It appears that this ambiguity is linked to the very high stacks of the usnic acid concentrations of lichens. This is thought to be an explanation for the correlation between usnic acid concentration and antimicrobial activity. In this study; it was determined that the higher amount of usnic acid concentration increased the antimicrobial activities. Only a few studies have applied the usnic acid amount and their antimicrobial activity produced by *P. saxatilis, P. sulcata, Parmelina tiliacea, X. conspersa,* and *F. caperata* species in Turkey. According to the best of our knowledge, this is the first study which was conducted to determine the usnic acid concentrations and antimicrobial activites of these lichen species for research and also commercial purposes .

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