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Original Article

Antioxidant and Antimicrobial Potential, and HPLC Analysis of Stictic and Usnic Acids of Three *Usnea* Species from Uludag Mountain (Bursa, Turkey)

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Abstract

In this study, antioxidant and antimicrobial potentials of *Usnea intermedia, U. filipendula,* and *U. fulvoreagens* and their stictic and usnic acid contents were investigated. Antioxidant activity and total phenolic contents were evaluated in acetone, ethanol, and methanol extracts of these three species. Antioxidant activity was measured by ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] method and total phenolic contents were measured by Folin-Ciocalteu method. High-performance liquid chromatography (HPLC) was used for the determination of lichen acids. It can be concluded from stictic and usnic acids contents that the order of solvent efficiency is acetone > ethanol > methanol and acetone > methanol > ethanol, respectively. Broth microdilution method was performed to determine the minimum inhibitory concentration (MIC) of the methanol extracts of three *Usnea* species. The MIC values of all the extracts ranged from 64 µg/mL to 512 µg/mL for all the bacterial strains that were tested in this study, and all the Fluoro quinolone-resistant *Escherichia coli* isolates (except for E101) were sensitive to the methanol extracts of the three *Usnea* species. This paper is the first study to determine the stictic acid content in *U. intermedia* and *U. filipendula.* Our findings indicate that these three *Usnea* species could be used as antioxidant and antimicrobial agents.

Keywords: Stictic acid; *Usnea filipendula; Usnea fulvoreagens*; *Usnea intermedia*; Usnic acid.

Introduction

Lichens or lichenized fungi are symbiotic organisms consisting of fungi, algae and/or cyanobacteria (1). These associations synthesize a great variety of secondary metabolites that are typical of these groups. As a result of the development of analytical techniques and experimental methods, more than one thousand lichen substances have been identified (2). Lichens and their natural products have been used as medicine, food, fodder, perfume, spice, dyes for different purposes throughout the world (3). Lichen metabolites have several potential biological activities, such as antibiotic, antimycobacterial, antiviral, antitumor, antiherbivore, antiprotozoal, antimutagenic, antiinflammatory, antioxidant, allelochemical, allergenic, analgesic, antipyretic, antiproliferative and cytotoxic effects (1-4).

Lichens produce a large number of phenolic compounds, such as depsides, depsidones, and dibenzofurans. Phenolic compounds have strong

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antioxidant properties because they act as hydrogen donors and singlet oxygen quenchers and, therefore, have redox capacity (5).

Many lichen species from different parts of the world have been investigated in terms of their potential antioxidant properties, and some have shown very strong antioxidant activities (6-13). However, these studies were made with lichen extracts. In such studies, it is not clear which compounds are responsible for the antioxidant activity. Only, in recent years, a few studies have determined which lichen secondary metabolites have antioxidant effect (5, 14-18).

Burkholder *et al.* have started pioneering research on lichens as antibacterial agents in 1944 (19, 20). The antimicrobial activity of lichen species was also demonstrated in other studies (21-25). The antimicrobial activity of lichens is variable, depending on the species of lichen, the concentrations of the extract and the tested organisms (26). Most studies have indicated that the lichen species are more effective against Gram-positive bacteria than against Gram-negative ones (27, 28).

The genus *Usnea* belongs to the family Parmeliaceae (29). The dibenzofuran usnic acid is found in all *Usnea* species and it is one of the most common, abundant and studied lichen metabolites. Usnic acid as a pure substance has been used in creams, toothpastes, mouthwashes, deodorants and sunscreen products as an active ingredient or as a preservative because of its antimicrobial properties. Usnic acid has many biological activities, such as antibiotic, antibacterial, antifungal, antiviral, antiprotozoal, antiproliferative, anti-inflammatory, analgesic, antipyretic, anti-tumor, antimitotic, antigrowth, antiherbivore and anti-insect effects (30-32). Moreover, the phenolic depsidon stictic acid is found in some *Usnea* species and has apoptotic, cytotoxic, and antioxidant activities (14, 15, 33 and 34).

The main objective of this study was to determine the stictic and usnic acid content of *U. filipendula, U. fulvoreagens* and *U. intermedia* by high performance liquid chromatography-diode array detection (HPLC-DAD) and to examine the efficiency of different solvent systems for the extraction of phenolic compounds. The total antioxidant activity and total phenolic contents of the extracts of three *Usnea* species were determined by the ABTS and Folin-Ciocalteu methods, respectively. In this study, we also aimed to determine the antimicrobial activity of these species using broth microdilution method.

Experimental

Materials

Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], an HPLC grade methanol, ethanol and usnic acid were purchased from Sigma–Aldrich (Steinheim, Germany). Stictic acid was purchased from ChromaDex (California,USA). Analytical-grade acetone, dimethyl sulfoxide (DMSO), tetrahydrofuran and orthophosphoric acid (89%) were purchased from Merck (Merck, Darmstadt, Germany).

Lichen collection and identification

Lichen samples were collected in November 2012 from the trunks of *Abies* sp. from Uludag Mountain, Bursa, Turkey. The samples were identified by Seyhan Oran using standard methods and information from the literature (34, 35). A voucher specimen of each species (*U. filipendula* 16444, *U. fulvoreagens* 16445, *U. intermedia* 16446) was deposited at the Herbarium of Uludag University (BULU), Bursa, Turkey, for future reference.

Extraction

Air-dried and cleaned lichen materials were ground in a household blender. The lichen samples (1 g) were separately blended with 40 mL of organic solvent (acetone, methanol, or ethanol) at room temperature in the dark for 4 h with a magnetic stirrer. The samples (total volume 40 mL) were separated from the solid matrix by filtration through sheets of filter paper (Whatman No. 1). The extracts were used to determine the total antioxidant activity and total phenolic contents by the ABTS and Folin-Ciocalteu methods, respectively. The organic solvent extracts (5 mL) were evaporated to dryness. Finally, residues were dissolved in 2 mL of DMSO. The DMSO extracts were used for the HPLC analysis.

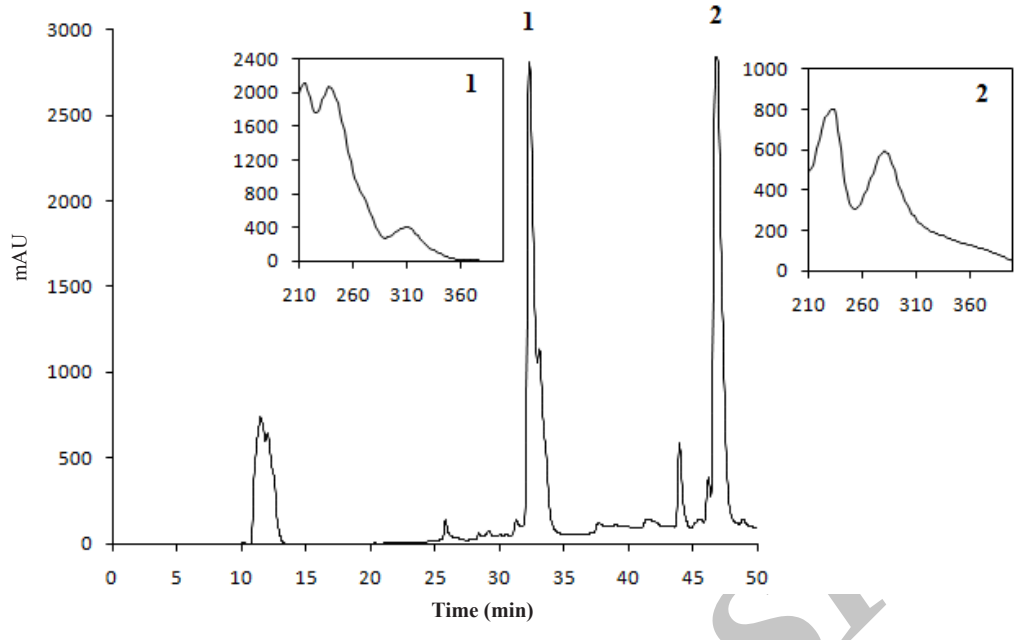

Figure 1. The chromatogram of *U. filipendula* acetone extract at 240 nm (1; stictic acid, 2; usnic acid).

HPLC

An Agilent 1200 HPLC system (Waldbronn, Germany), consisting of a vacuum degasser, binary pump, autosampler and a diode-array detector was used. Chromatographic separations were carried out using an XBridge C18 (3.5 µm, 4.6 x 250 mm) column from Waters. The mobile phase consisted of 0.25 % orthophosphoric acid and 1.50 % tetrahydrofuran in water (solvent A) and methanol (solvent B). The gradient conditions are as follows; 0-15 min 30-70 % A, 15-30 min 70-100 % A, 30-35 min 100 % A, 35-36 min 100-30 % A, and 36-50 min 30 % A with a total run time of 50 min. The column was equilibrated for 10 min prior to each analysis. The flow rate was 0.25 mL/min, and the injection volume was 10 µL. The data acquisition and preprocessing were performed with ChemStation for LC (Agilent). The monitoring wavelength was 240 nm.

Antioxidant activity assay

The total antioxidant activity of *Usnea* species was determined with the ABTS method as described by Sarıburun and others (36). The absorbance was measured by spectrophotometry (Varian Cary 50, Australia) at 734 nm against a blank after 6 min. The results were expressed as mg of trolox equivalent (TE) per 100 g of dried weight.

Folin-Ciocalteu method

The total phenolic content as determined by the Folin-Ciocalteu reagent was carried out according to the procedure reported by Sarıburun and others (36). The absorbance was measured by spectrophotometry (Varian Cary 50, Australia) at 750 nm. The total phenols were expressed as mg of gallic acid equivalent (GAE) per 100 g of dried weight.

Bacterial strains

The bacterial strains that were used in this study were *Escherichia coli* ATCC 25922, *Escherichia coli* O157:H7, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 33591 and eight FQ-resistant *E. coli* isolates.

Determination of Minimum Inhibitory Concentration (MIC)

Broth microdilution testing was performed to determine the MICs of the lichen species according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2003). The bacterial cultures were prepared in

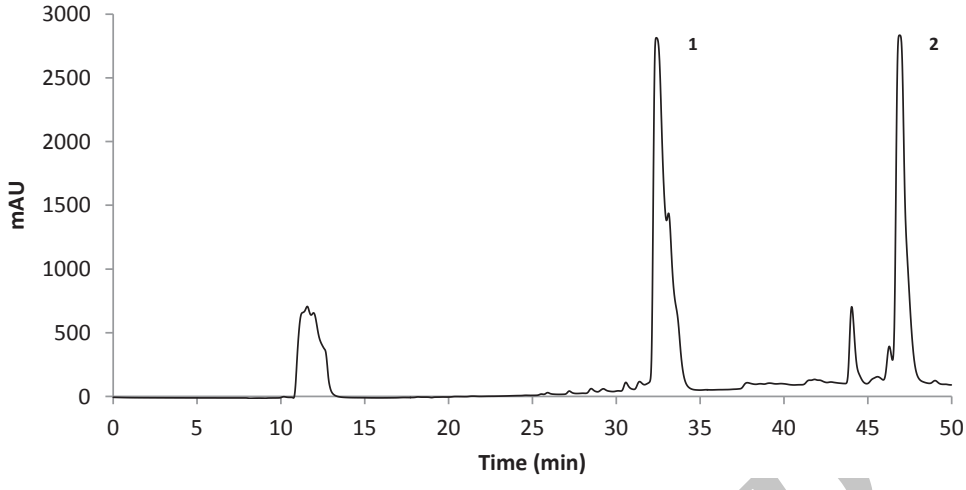

Figure 2. The HPLC chromatogram of *U. fulvoreagens* acetone extract at 240 nm (1; stictic acid, 2; usnic acid).

Mueller-Hinton Broth (MHB) at 37 ºC for 16-20 h. Powdered lichens were extracted with methanol due to common use of methanol as a solvent for *in-vitro* susceptibility tests. The methanol extracts were dissolved in 20:80 methanol:PBS (phosphate buffered saline) (v/v). Freshly prepared stock solutions were sterilized using 0.20-µm single-use filter units (Minisart, Sartorius Stedim Biotech.). Dilutions ranging from 0.008 to 256 mg/L were prepared in MHB, and inocula with a density equivalent to 0.5 McFarland turbidity were added to tubes containing the extracts' dilutions. After incubating at 37 ºC for 16-20 h, the MICs were defined as the minimum concentration of extract that inhibited the growth of the organism. The optical densities (ODs) of the cultures were measured at a wavelength of 595 nm (Bio-Rad, iMark).

Results

Identification of Stictic and Usnic acids in Usnea species

The concentrations of stictic and usnic acids in the three different extracts of *U. filipendula, U. fulvoreagens,* and *U. intermedia* were determined by HPLC-DAD, and the results

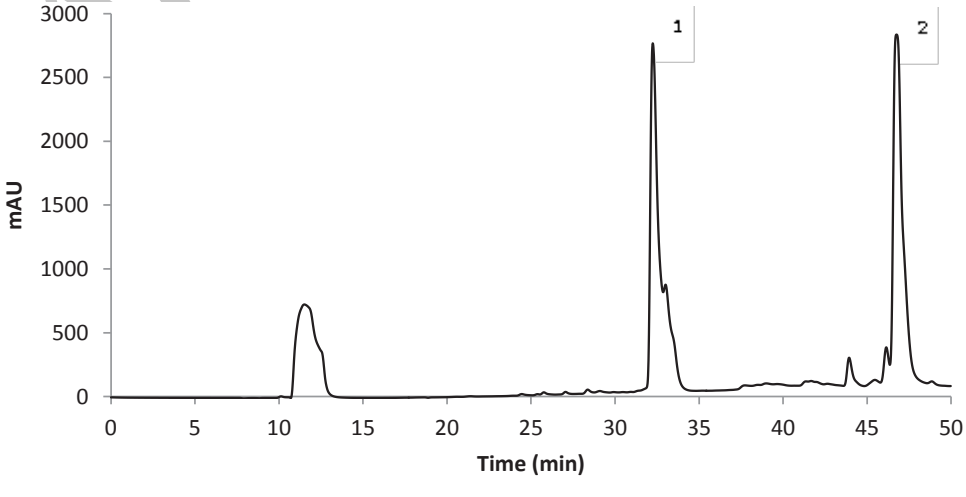

Figure 3. The HPLC chromatogram of *U. intermedia* acetone extract at 240 nm (1; stictic acid, 2; usnic acid).

Table 1. The amounts of stictic and usnic acids extracted from *Usnea* species using different solvents (milligrams per gram dried lichen).

	Extraction solvent	Stictic acid	Usnic acid
Usnea filipendula	Acetone	9.85±0.49	1.10±0.16
	Ethanol	5.72±0.06	0.97±0.03
	Methanol	5.37±0.48	1.07±0.09
Usnea fulvoreagens	Acetone	7.60±0.28	1.19±0.01
	Ethanol	5.33±0.09	1.01±0.01
	Methanol	4.77±0.51	1.04±0.07
Usnea intermedia	Acetone	5.72±0.14	0.97±0.05
	Ethanol	4.04±0.18	0.72±0.02
	Methanol	2.98±0.28	0.94±0.04

Mean of two determinations±SD

are shown in Table 1. Identification of these compounds was achieved by comparison of their retention time values with the standard substance purchased from ChromaDex and Sigma-Aldrich. The HPLC chromatograms of the acetone extracts of *U. filipendula, U. fulvoreagens* and *U. intermedia* are shown in Figures 1-3

The highest concentrations of stictic and usnic acids were found in the acetone extract of *U. filipendula* (9.85±0.49 mg of stictic acid/g of dried lichen) and *U. fulvoreagens* (1.19±0.01 mg of usnic acid/g of dried lichen) respectively. The smallest concentrations of stictic and usnic acids were found in the methanol extract (2.98±0.28 mg of stictic acid/g of dried lichen) and ethanol extract (0.72±0.02 mg of usnic acid/g of dried lichen) of *U. intermedia.* The stictic acid concentrations that were obtained by the HPLC method indicate that the order of solvent efficiency is acetone > ethanol > methanol, while the usnic acid concentrations that were obtained by the HPLC method indicate that the order of solvent efficiency is acetone > methanol > ethanol for the three *Usnea* species. The solvents with a relatively lower polarity were more efficient in general in extracting phenolic compounds in the three *Usnea* species. Because of the different solubility of the lichen substances in different solvent, hexane, diethyl eter, acetone and methanol were used for the extraction of lichen substances. Acetone is very useful solvent for the extraction, as the most lichen substances are soluble in this solvent. Also acetone was used for the extraction of lichen acids from *Usnea* species in the literature (37).

Antioxidant activity and total phenolic content of Usnea species

The antioxidant activity and total phenolic contents of the three *Usnea* species are displayed in Tables 2 and 3. The highest antioxidant activity and total phenolic contents were found in the methanol extract of *U. fulvoreagens* (181.0±4.4 mg of TE/100 g of dried lichen) and the acetone extract of *U. filipendula* (329.7±18.5 mg of GAE/100 g of dried lichen), respectively.

Table 2. Total antioxidant capacity evaluated by ABTS assay of the organic extracts of three *Usnea* species.

Extraction solvent	*U. filipendula* (mg TE 100 g^{-1} of dried lichen)	*U. fulvoreagens* (mg TE 100 g^{-1} of dried lichen)	*U. intermedia* (mg TE 100 g^{-1} of dried lichen)
Acetone	125.2±6.4	73.4±0.5	52.2±2.0
Ethanol	65.4±0.5	61.0±1.7	115.8±11.3
Methanol	174.8±6.9	181.0±4.4	141.6±6.0

The values are the mean of two lichen extracts.
TE: Trolox equivalent

Table 3. Total phenolic content evaluated by Folin Ciocalteu method of the organic extracts of three *Usnea* species.

Extraction solvent	*U. filipendula* (mg GAE 100 g^{-1} of dried lichen)	*U. fulvoreagens* (mg GAE 100 g^{-1} of dried lichen)	*U. intermedia* (mg GAE 100 g^{-1} of dried lichen)
Acetone	329.7±18.5	135.5±10.4	170.2±1.1
Ethanol	197.4 ±16.0	174.4±1.3	194.7±2.2
Methanol	291.5±12.1	229.9±14.0	239.7±0.3

The values are the mean of two lichen extracts.
GAE: gallic acid equivalent

The antioxidant activity measured by the ABTS method indicate that the order of solvent efficiency is methanol > acetone > ethanol except for *U. intermedia.* The total phenolic contents as determined by the Folin method indicate that the order of solvent efficiency is methanol > ethanol > acetone except for *U. filipendula.* As seen from these results, in general, methanol could recover the highest yield of antioxidant activity and total phenolic contents.

Validation of the the analytical method

The linearity of the HPLC-DAD method was investigated for phenolic compounds in the range of 1-100 mg/L for stictic acid and 1.4-140 mg/L for usnic acid at nine concentration levels. Two calibration plots with $R^2 \geq 0.999$ correlation coefficient were obtained by reporting the peak areas as a function of the concentrations of each phenolic compound (Table 4). The validation of the quantitative determination of the phenolic compound concentration in the *Usnea* samples was performed by estimating the limits of detection (LOD, 3 s/m), limits of quantification (LOQ, 10 s/m) and recovery (%) of stictic and usnic acids (Table 4), where s is the sample standard deviation for the replicates, and m is the slope of the calibration curve. The LOD ranged from 0.49 to 0.039 mg/L, and the LOQ ranged from 1.64 to 0.13 mg/L for stictic and usnic acids, respectively. The extraction efficiency of the lichen standards of stictic and usnic acids was evaluated by spiking the samples with the mixture of standards and extracting using acetone, ethanol and methanol. The recovery study was carried out only for usnic acid as identified by HPLC-DAD. The mean percentage recoveries ranged from 92.22±1.72 to 96.37±2.13 (Table 4). All of the other recoveries are within the experimental error range. In the calculation of the final results, the recoveries of the pure phenolic standards were considered..

Antimicrobial activity of the lichen extracts

In this study, the broth microdilution method was used to test for antimicrobial susceptibility. The MICs of the methanol extracts of the lichen species ranged from 64 µg/mL to ≥ 512 µg/mL for all of the bacterial strains that were tested in this study (Table 5). The highest antimicrobial activity was observed for the methanol extracts of *U. filipendula*, *U. fulvoreagens* and *U. intermedia* against *E. coli* E245 and for the methanol extracts of *U. filipendula* and *U. intermedia* against *E. coli* O157:H7 with 64 µg/mL MIC.

Table 4. Validation parameters and recovery of stictic and usnic acids in *Usnea* extracts.

Validation parameters	Stictic acid	Usnic acid
LOD (mg L^{-1})	0.490	0.039
LOQ (mg L^{-1})	1.640	0.130
R^2	0.999	0.999
Recovery (%)		
Acetone	96.37±2.13	92.22±1.72
Ethanol	95.97±1.77	94.34±1.56
Methanol	95.07±1.61	95.38±2.38

LOD limits of detection, *LOQ* limits of quantification

Table 5. Minimum inhibitory concentration of three *Usnea* species methanolic extracts.

Isolates ID	MIC (µg/mL)		
	U. filipendula	***U. fulvoreagens***	***U. intermedia***
E. coli E101	512	≥512	256
E. coli E103	128	128	128
E. coli E121	128	128	128
E. coli E224	128	128	128
E. coli E245	64	64	64
E. coli E246	128	128	128
E. coli E248	128	128	128
E. coli E300	128	128	128
E. coli 25922	128	512	128
E. coli O157:H7	64	512	64
S. aureus 25923	128	512	128
S. aureus 33591	256	256	≥512

Discussion

The depsidone stictic acid is an aromatic organic compound and a central subject of medical research. Stictic acid has a significant apoptotic effect (33). To the best of our knowledge, this study is the first to establish the stictic acid concentration in three different solvent extracts of *U. intermedia* and *U. filipendula*.

Usnic acid is found in several lichens and is especially abundant in the genera *Alectoria*, *Cladonia*, *Usnea*, *Lecanora, Ramalina* and *Evernia.* The biological activity of usnic acid has been studied previously (32, 38). Many lichens and extracts containing usnic acid have been utilized for medicinal, perfumery, and cosmetic areas (30). Our results indicate that the acetone extracts of three species of *Usnea* could be used for these applications because of their contents of usnic acid. The contents of usnic acid in our species were lower than the content of usnic acid with these of *Usnea longissima*, *Usnea hirta* and *Usnea rigida* in the literature (37).

Folin Ciocalteu analysis and ABTS data evidenced no correlation between the total phenolic contents and the antioxidant activities of the *Usnea* extracts. The presence of phenolic groups in lichen metabolites is considered as a key element for their antioxidative effect, but antagonistic or synergistic effects of different chemicals when interacting with each other should be considered. Moreover, only a few studies have considered the possible interactions between phenolics, whereas a potent regeneration of an antioxidant by another antioxidant can increase or decrease the activity of a mixture of antioxidants (39).

Previous reports on the antimicrobial properties of lichens have demonstrate a low antimicrobial activity against Gram-negative bacteria, but in this study, FQ-resistant *E. coli* isolates (except for E101) were sensitive to the three *Usnea* species as reported previously (22, 37, 40). Furthermore, our results demonstrate that there is no difference between the antimicrobial sensitivity of *S. aureus* strains and *E. coli* strains and isolates.

Conclusion

In conclusion, our findings indicate that the three *Usnea* species could be used as antioxidant and antimicrobial agents. Lichen metabolites are poorly studied as a new source of active compounds. Therefore, further studies are required to isolate the lichen substances that exhibit potent biological activity.

Acknowledgments

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