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Determination of Usnic Acid Content in Some Lichen Species Found in Anatolia

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Abstract

85 species of lichens were collected from Anatolia (Ankara, Antalya, Bursa, Karabük, Giresun, Trabzon, Çankırı, Kayseri and Gümüşhane) in Turkey. Their usnic acid amounts were determined by HPLC in acetone extracts and the data obtained were evaluated statistically. Results showed that usnic acid contents of lichen species varied between 6.49 – 0.04 dry weight percentages.

Key words: Lichen, usnic acid, HPLC, Anatolia.

INTRODUCTION

As it is known, of more than 20.000 known species of lichens, only a few have been analyzed and identified as containing biologically active secondary compounds. A prime example is the antimicrobial compound, usnic acid [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl1,3(2H9βH)-dibenzofurandione], commonly found in the genus *Usnea* [1, 2]. Usnic acid is a yellowish pigment produced by several lichen species. Usnic acid has been documented to have antihistamine, spasmolytic, antiviral, and antibacterial activities. Two biologically active natural enantiomers of usnic acid, differing in the orientation of the methyl group at 9b, otherwise rigid molecule, have been identified as showing different biological activities and mechanisms of action [3]. Proska *et al.* (1996) reported that (-)-usnic acid inhibited urease and arginase activity. Several reports revealed that [4-6] that the (+)-enantiomer is a more effective antimicrobial agent, although no specific mode of action was determined [7]. Usnic acid as a pure substance has been formulated in creams, toothpaste, mouthwash, deodorants and sunscreen products, in some cases as an active principle, in others as a preservative. Also usnic acid is used as a preservative in cosmetic creams [3].

HPLC is an ideal tool for detecting trace substances, analysing small samples, quantifying phenolic lichen metabolites, and providing structural information from retention characteristics. HPLC has become more widely used as an effective analytical tool for the separation and identification of lichen substances. Early attempts to apply this method to lichen chemotaxonomy were performed using normal-phase silica columns [8,9], but better results were obtained by bonded reversed-phase columns.

In this study, usnic acid contents of 85 lichen samples collected from nine different regions of Turkey (Ankara, Antalya, Bursa, Karabük, Giresun, Trabzon, Çankırı, Kayseri, Gümüşhane) were detected by HPLC, and the data were evaluated statisticaly. The student-T test was concluded as the most appropriate statistic evoluation method. The correlation between usnic acid amount height and precipitation was evaluated. Beside this, the study constitutes a contribution to the determination of usnic acid content in some lichens from Turkey. In this respect this study is the first data from Turkey, and from this point of view it may show of the presence of variation in usnic acid content of lichen species growing in different geographical areas and ecologies.

MATERIALS and METHODS

Lichen materials

Usnic acid was isolated from 85 lichen samples collected from 28 different regions of Turkey, during 2002 and 2005. All of the lichen species were identified by Demet Cansaran Duman. The samples were dried at room temperature and foreign matters were removed prior to grinding. The lichen samples were stored in the herbarium of ANK (Ankara University, Department of Botany, Ankara, Turkey).

Determination of HPLC analysis of the lichen samples

Sample preparation for the HPLC analysis

HPLC analyses were performed as indicated previously Cansaran *et al.* [10]. Air-dried lichens were ground, and 0.05 gr sample was extracted in 10 ml acetone at room temperature (20 - 22 °C). The extracts were stored in a freezer until HPLC-analysis. Before the analysis, extracts were passed through 0.45 µm filters. After the filtration process, owing to solvent loss due to evaporation solutions were diluted to 10.0 ml with acetone, and then injected into the HPLC system in amounts of 20 µl.

Analytical Conditions

All other chemicals, including usnic acid, used in experiments were HPLC grade from Sigma of highest purity. 5 ml of usnic acid stock solution with 1 mg/ml concentration was prepared in acetone. An appropriate dilution of this stock solution was made with acetone and standard solutions with

Table 1. Collecting sites

Locations	Altitude (m)
1. Karabük-Yenice Yaylacık Forest Elmaören Locality N 45°43′523″ -E 45°38′411″	780
2. Karabük-Yenice Yaylacık Forest Sinekağzı Locality N 41°05′961″ -E32°19′483″	880
3. Karabük- Yenice Yaylacık Forest KaplıkayaTepe N 41° 06′849″- E 32°18′097″	500
4. Karabük- Yenice Yaylacık Forest Sarıçam Hill N 41° 05′961″- E 32°19′493″	990
5. Gümüşhane Örenkale village	1560
6. Antalya Termosos Natural Park Güllük Mountain	830
7. Antalya Beşkonak Locality	50
8. Antalya Konya altı Locality	10-15
9. Antalya Köprülü Kanyon Locality	160
10. Çankırı Yapraklı, Popirunkaşı Hill N 40°47′617″- E 33°46′737″	1750
11. Trabzon-Uzungöl-Soğanlı Locality UTM N 44°94′779″- E37°61′688″	1799
12. Trabzon-Uzungöl-Soğanlı Locality 37T 610821 E	1685
13. Giresun - Kümbet Area Merkez	1710
14. Giresun – Kümbet Salon Çayırı Locality	1600
15. Giresun Dereli – Kulakkaya Area N 40°45′090″- E 38°20′771″	1546
16. Giresun Dereli - Kulakkaya Area N 40°41′899″- E 38°20′771″	1300
17. Giresun Dereli Erimez Locality N 40°45′090″- E 38°21′298″	1134
18. Kayseri, Western slope of Erciyes Mountain, N 38°32′- E 35°30′	2550
19. Kayseri Erciyes Mountain; Western slope of Koç Mountain, N 38°32′- E 35°32′	2250
20. Kayseri Erciyes Mountain; North of Perikartın, N 38°35′- E 35°27′	2300
21. Ankara- Beynam Forest N 36°53′920″- E 32°55′005″	1381
22. Giresun- Şebinkarahisar Hatunkaya Area	1925
23. Giresun- Şebinkarahisar Tatar Locality	1950
24. Giresun- Şebinkarahisar Kabak Hill	2430
25. Giresun- Şebinkarahisar Çorak area	1750
26. Giresun- Şebinkarahisar Gamyalı Locality	1930
27. Giresun Castle Locality	150
28. Bursa-İnegöl- near the Oylat thermal spring	840

concentrations 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 mg/l were prepared. These standards were directly injected into the HPLC system, using the autosampler. Calibration curves for usnic acid were obtained from seven samples of various concentrations using linear regression analysis (Fig. 1). Since the relative standard deviation is higher than 10% the limit of quantification (LOQ) was determined according to Meyer V. R. [11] and the detection limit was determined to be 3.6 ppm. This value was found 0.072% for a 50 mg lichen sample. Results under 0.1% were evaluated as under detection limit values and not taken into account.

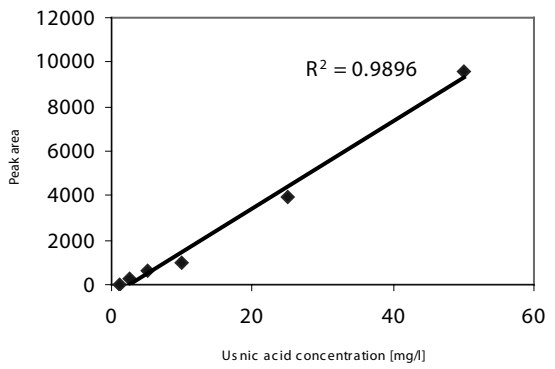

Figure 1. Calibration curve of usnic acid (Sigma)

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. Usnic acid was detected at 245 nm, with the methanol-phosphate buffer (pH 7.4) (70:30 v/v), because of that maximum absorbans were observed at 245 nm. 20 µl aliquots of the extracts were injected into the HPLC system. Each analysis was carried out in triplicate.

RESULTS and DISCUSSION

Quantitative analysis of usnic acid in 85 lichen samples was achieved using HPLC. Identification of peaks in chromatograms of lichen extracts was accomplished by comparison of retention times with that of standard usnic acid. A sample of representative chromatograms is shown in Fig. 2.

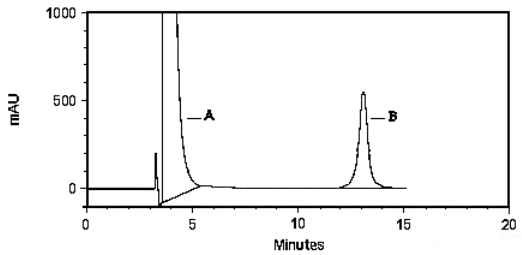

Figure 2. Analysis of usnic acid from *Rhizoplaca* by HPLC. (A) solvent (t_R=3.9 min); (B) usnic acid (t_R=13.1 min)

The results obtained from HPLC were displayed in Table 2. The usnic acid content could be as high as 6.49 %. On the other hand, the acid content remained under the detection limit for 28 species. As shown in Table 2, usnic acid content of the lichens found in Turkey varies within a very wide range. It alters from detection level to 6.5%. The wide interval of usnic acid content recorded in this study is consistent with the previous studies [2]. In literature, it has been reported that, for *Cladonia, Usnea, Lecanora, Ramalina, Evernia* and *Parmelia* the usnic acid content can be as high as 6%. On the other hand, the maximum possible seasonal variation was recorded as two fold, for an arctic lichen species [12].

According to the statistical analysis, the differences between collected species of genera were significant. The usnic acid content of *Usnea* is undoubtedly the highest among the species tested in the study. Moreover, two *Usnea* species *Usnea florida* and *Usnea barbata* yielded significant variation. On genus basis, statistical evaluation could be performed when all species contain more than four samples.

Table 2. Concentration of usnic acid in dry weight

Species	Number of the locality	% Usnic acid in dry weight and standart deviation
Anaptychia caspica	13,2	udl, udl
Aspicilia calcarea	22	udl
Aspicilia fruticulosa	22	udl
Bryoria chalybeiformis	25	udl
Bryoria nadvornikiana	11	udl
Cetralia cetraroides	13	udl
Cetraria islandica	22	udl
Cladonia conicria	12	udl
Cladonia fimbriata	13,11,28,6	udl, 0.14±0 udl, udl
Collema undulatum	27,4	0.46±0, udl
Dermatocarpon intestiniforme	14	Udl
Dermatocarpon luridum	26	Udl
Dermatocarpon miniatium	27	Udl
Evernia divaricata	1,7,25	Udl,Udl,0.35±0
Evernia illarica	2	0.21±0
Flavoparmelia caperata	12, 27, 1	1.29±0.03, 0.47±0.02, 2.38±0.02
Hypogymnia laminasorediata	8	0.63±0.03
Hypogymnia physodes	14, 10, 9, 4	0.73±0.08, 0.596±0.01, 0.55±0.02, 1.05±0
Hypogymnia tubulosa	9, 21, 10, 28, 2	2.4±0, 2.21±0.11, 0.826±0.05, 1.41±0.1, 1.24±0.09
Letharia vulpina	5, 25	2.897±0.08, 0.53±0.05
Lobaria amplissima	2, 13	Udl, Udl
Lobaria pulmanaria	2, 3	0.26±0.02, Udl
Lobarina scrobulata	1	Udl
Melanelia exasperatula	22	Udl
Parmelia conspersa	16	1.10±0.05
Parmelia elagantula	25	Udl
Parmelia saxatilis	23,23	0.135±0, 0.096±0.006
Parmelia sulcata	1,8	Udl, Udl
Parmelina tiliacea	9	0.101±0.01
Parmeliopsis ambigua	26	0.35±0.25
Peltigera canina	15	Udl
Peltigera didactyla	22	Udl
Peltigera praetextata	24,4,17	Udl, Udl, Udl
Plasmatia grabratula	1	Udl
Protoparmeliopsis muralis	22	0.55±0.02
Pseudevernia furfuracea	1, 5, 6, 10	0.23±0, Udl, Udl, 0.123±0.01
Ramalina capitata	18	1.25±0.29
Ramalina fastigiata	6, 19	3.23±0.16, 0.21±0.01
Ramalina fraxinea	19	0.13±0.01
Ramalina pollinaria	10	0.22±0.01
Ramalina polymorpha	20, 21	0.10±0, 0.27±0.02
Rhizoplaca chrysoleuca	19	4.0±0.07
Rhizoplaca melanopthalma	18	0.19±0.009
Rhizoplaca peltata	20	0.53±0.04
Umbilicaria cinereorufescens	25	Udl
Umbilicaria leiocarpa	19	0.27±0.05
Usnea barbata	11, 1,13	0.17±0.006, 2.16±0.67, 1.74±0.18
Usnea florida	1	2.36±3.7
Usnea hirta	10	0.68±0.04
Usnea longissima	13	1.12±0.11
Usnea rigida	15	0.22±0.007
Usnea subflorida	1, 6	6.49±0, 6.13±0.58
Xanthoria pariatina	6	0.127±0
Xanthoria ulophyllodes	23	Udl

udl; under detection limit

Table 3. Annual precipitation between the years 2000 – 2004 (mm H_2O)

Region	2000	2001	2002	2003	2004
Bursa	793.3	724	761.1	712.3	585.8
Ankara	346.6	328.1	455.2	308.3	251.1
Antalya	839.4	891.8	971.6	1773.6	1268.2
Karabük	540,7	464,0	412.8	367.6	294,4
Giresun	581.3	498.2	418.7	440.6	497.5
Trabzon	1030.6	972.1	832.5	870.4	972.7
Çankırı	404	358.2	342.7	384.8	538.1
Gümüşhane	453.6	478.4	454.7	459.6	407.5
Kayseri	356.2	257.9	444.1	287.4	359.4

These genera are *Usnea, Hypogymnia, Ramalina, Evernia, Cladonia, Peltigera, Pseudevernia, Lobaria, Parmelia* and *Flovoparmelia*. When the distributions were examined on genus basis, no significant variation was observed between *Usnea & Parmelia* and also between *Hypogymnia & Ramalina*, however when the genus average was calculated the clear distinction of *Usnea* could be easily noticed. When the genus averages were taken into consideration, the below order of the genara was generated. *Usnea > Hypogymnia > Ramaliana > Parmelia > Evernia > Pseudevernia > Lobaria > Cladonia > Peltigera.*

Although the under detection limit was 0.1% there was uncertainity with some species. For example, it was observed for two species of *Pseudevernia furfuraceae*, and it wasn't observed another two. Out of the 4 *Lobaria* samples, 3 did not show the presence of usnic acid. Only one sample showed a result. Actually usnic acid should not be present in *Lobaria*. This is probably the result of an experimental error. In general, there is direct correlation between the analysis results and species.

Statistical evaluations revealed minor differences between regions. The usnic acid contents of the lichens of Antalya, Eastern Blacksea and Western Blacksea showed significant differences but between Antalya and Middle Anatolia, also between Middle Anatolia and Western Blacksea and Eastern Blacksea, no significant variations were recorded. Beside these, the precipitation averages of the regions where the species have

been collected were evaluated but no clear correlation was found. The precipitation averages of the regions in Turkey where the lichens have been collected were displayed in Table 3.

As seen from Table 3, the highest precipitation receiving region is Antalya among the regions taking place in the study. There are almost no differences between the regions Bursa, Bolu and Giresun. On the other hand, Middle Anatolian provinces Ankara-Kayseri receive less precipitation.Fig 3 shows the correlation between altitude and usnic acid values of species. From the graph it could be inferred that the species collected from the altitudes between 700 – 1500 m yielded higher levels of usnic acid. Annually, the highest level of water where it remains liquid is above 700 m. Above 1500 m the weather is colder within a year and the probability of water remaining as ice is higher above this elevation. For this reason, it is expected to find higher levels of usnic acid between the altitudes 700m - 1500 m where the water preserved as liquid.

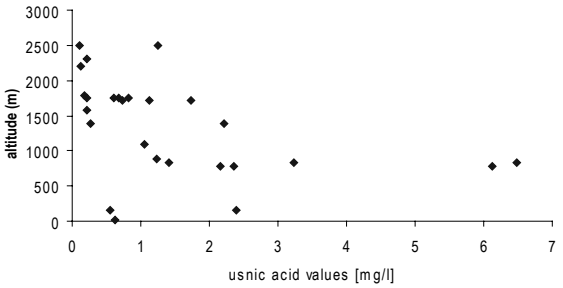

Figure 3. The altitudes of the regions where the species were collected and usnic acid values

Most researches have been focused on the influence of ecological factors on secondary metabolite concentrations. For usnic acid, it has indeed been shown that its concentration is strongly influenced by various ecological factors. Ravinskaya *et al.* [13] observed that light, temperature and humidity play an important role in the concentration of lichen acids (usnic acid and atranorin) [13]. Seasonal variations in the concentration of usnic acid have been detected in *Usnea aurantiaco-atra* in Antarctica [14]. Monthly measurements over 3 years showed that probably in relation to light intensity, concentrations of usnic acid are higher in winter and spring, and lower from summer to autumn,. In this study, significantly different results were found according to regional variations. Very important difference was recorded between Mediterranean and Eastern Black Sea regions. Although Mediterrannean region of Turkey has a warm climate, Eastern Blacksea has a climate with heavy precipitation. It is noticed that, the usnic acid contents of the lichen species, growing around these two regions show differences.

According to Proksa *et al.* [4], usnic acid is widely distributed in species of *Cladonia* (Cladoniaceae), *Usnea* (Usneaceae), *Lecanora* (Lecanoraceae), *Ramalina* (Ramalinaceae), *Evernia, Parmelia* (Parmeliaceae) and other lichen genera [4]. *Alectoria* (Alectoriaceae) species are often rich sources of usnic acid, and yields up to 6% have been reported [4]. In the current study *Usnea subflorida* yielded the highest usnic acid content with the value of 6.49%. This lichen species was collected from Karabük- Yenice located in Western Blacksea region. When they are ordered on genus level the below order was generated. *Usnea* > *Hypogymnia* > *Ramalina* > *Parmelia* > *Evernia* > *Pseudevernia* > *Lobaria* > *Cladonia* > *Peltigera.* Also the result obtained from this study is consistent with the results reported by Proska *et al.* [4].

Of the hundreds of known secondary lichen metabolites, the dibenzofuran derivative usnic acid without a doubt is the most extensively studied one. From this perspective the level of usnic acid content in lichens is very important for the medical and pharmaceutical application. In this regard, with the studies like this it is possible to determine the usnic acid contents of lichens and provide broader application areas for their usage.

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