ORIGINAL ARTICLE

DETERMINATION OF THE CONTENT IN USNIC ACID AND POLYPHENOLS FROM THE EXTRACTS OF *USNEA BARBATA* L. AND THE EVALUATION OF THEIR ANTIOXIDANT ACTIVITY

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Abstract

The aim of this study was the identification and quantification of the main active compounds from different Romanian *Usnea barbata* L. extracts and evaluation of their antioxidant activity. The vegetal product *Usneae lichen* was harvested from Călimani Mountains, Suceava County, Romania, in March 2016. We prepared 10% extracts in: acetone, 96% ethanol and water. The quantitative analysis of the identified constituents was performed using an HPLC method. The antioxidant activity was evaluated by the scavenger DPPH (2,2-diphenyl-1-picrylhydrazyl) method. For this analysis, we prepared 20% extracts in: acetone, 96% ethanol and water. For each extract, we prepared two dilutions: 1:2 and 1:4. Our results showed that each extract contains usnic acid and polyphenols. The intensity of the antioxidant activity varied with the different solubility of polyphenols in each solvent.

Rezumat

Lucrarea prezintă identificarea și determinarea conținutului în principii active cu acțiune terapeutică din extractele obținute din *Usnea barbata* L. din Romania, în diferiți solvenți, precum și evaluarea acțiunii antioxidante a acestora. Produsul vegetal *Usneae lichen* a fost recoltat din munții Călimani, județul Suceava, România, în martie 2016. Au fost preparate extracte 10% în diferiți solvenți: apă, acetonă și etanol 96%. Identificarea și determinarea conținutului în acid usnic și polifenoli s-a realizat prin metoda HPLC. Determinarea activității antioxidante s-a realizat prin metoda de captare a radicalilor liberi DPPH (2,2-difenil-1-picrilhidrazil). Pentru aceasta determinare, am preparat extracte 20% in apă, acetonă și alcool etilic 96%. Pentru fiecare extract am realizat 2 diluții, 1:2 și 1:4 și am determinat activitatea lor antioxidantă. Rezultatele obținute au demonstrat faptul că toate extractele conțin acid usnic și polifenoli, în concentrații diferite, în funcție de solubilitatea lor în solvenții utilizați. Activitatea antioxidantă, datorată în special polifenolilor, a fost prezentă la toate extractele luate în lucru, dar a variat, în mod corespunzător cu solubilitatea polifenolilor în solvenții utilizați la extracție.

Keywords: *Usneae lichen* extracts, usnic acid, polyphenols, antioxidant activity

Introduction

Phytotherapy research aims to find herbal products with bioactive compounds for the treatment of various diseases. *Usnea barbata* L. is a lichen with a large habitat, used in traditional medicine [13-15]. A lichen is a symbiotic organism, the symbiosis being realized between a *fungus* (*Ascomycetes* or *Bazidiomycetes*) and an *algae* or a *cyanobacteria*, [3, 9, 10]. The most important natural compounds from *Usnea spp* are secondary metabolites, especially usnic acid (dibenzofuran derivative) and polyphenols [9, 13-16]. Usnic acid is the most prominent secondary lichen metabolite in all approximately 500 species of *Usnea* genus [12-14]. Usnic acid has many pharmacological activities: antibacterial, antifungal, antiviral, antioxidant, antiinflammatory, analgesic-antipyretic, anticancer, antigenotoxic, antimutagenic, antiplatelet/antithrombotic, antiulcerogenic [9, 10, 14-16] with important effects in various diseases [14-16]. Polyphenols have a significant antioxidant action [2, 7, 8-15] which can be used in the treatment of oro-dental diseases [2, 3].

Materials and Methods

General methodology included work methods of natural compounds from medicinal plants presented in Romanian Pharmacopoeia Xth Edition and European Pharmacopoeia 9th Edition [20, 21].

Obtaining the vegetal product Usneae lichen

Usneae lichen, the *Usnea barbata* L. dry *thallus*, was harvested from Călimani Mountains, România, in March 2016, when the lichen presents the highest content in bioactive compounds [9,13]. The lichen was cleaned and dried at a constant temperature below 25°C in an airy room, protected from the sunlight and brought to the degree of crushing required for loose tissues (sieve 3) [20].

HPLC analysis of phenolic compounds

For the separation, identification and quantification of phenolic compounds, a standardized HPLC method described by the USP 30-NF25 monograph, has been adapted [24]. *Apparatus*: Agilent 1200 HPLC, quaternary pump, DAD detector, thermostat, degassing system, auto sampler.

Working conditions: C18 column, 150 mm 4.6 mm; 5 µm (Zorbax XDB). Mobile phase: solution A: 0.1% phosphoric acid, solution B: acetonitrile, with gradient elution. Temperature: 35ºC; flow: 1.5 mL/ min; detection: UV 310 nm; injection volume: 20 µL; analysis time: 22 min.

Extracts preparation

Three samples of 10 g vegetal product were extracted each with 100 mL solvent (water, acetone and 96% ethanol). The three resulting extractive solutions were filtered and then made up to 100 mL volumetric flask with each solvent used in the extraction.

The reference substances (70% methanolic solutions) were: E-resveratrol = 0.37 mg/mL, Z-resveratrol = 0.22 mg/mL (obtained by exposing the E-resveratrol solution to UV radiation at λ = 254 nm for 12 h), caffeic acid = 0.36 mg/mL, chlorogenic acid = 0.37 mg/mL, cinnamic acid = 0.58 mg/mL, vanillin = 0.42 mg/mL, gallic acid = 0.39 mg/mL, ferulic acid = 0.38 mg/mL, 3-methyl gallic acid = 0.51 mg/mL, ellagic acid = 0.40 mg/mL. The reference substances were injected 6 times (20 µL) in the chromatographic system. The identification and quantitative determination of the natural compounds of the solution to be analysed, was performed by comparing the chromatogram of the standards and the solution to be analysed.

HPLC analysis of usnic acid

For the separation, identification and quantification of usnic acid, a standardized HPLC method was adapted [22].

Working conditions: C18 column chromatograph, 150 mm/4.6 mm; 5 µm (Zorbax XDB); mobile phase: methanol:water:acetic acid = 80:15:5, detection: UV = 282 nm, flow rate = 1.5 mL/min; temperature = 25ºC, injection volume = 20 µL; analysis time: 6 minutes.

Reference substance (solution in acetone): usnic acid = 50 µg/mL (ppm). The reference substance was injected 6 times (20 µL) in the chromatographic system. The identification and quantitative determination of the active principles in the assayed solutions was performed by comparing the chromatogram of the standard and the solution to be analysed. Unic acid presented a retention time of 4.463 ± 0.008053 min, with a correlation coefficient r^2 of 0.9998.

Antioxidant activity assay - DPPH-method

The antioxidant capacity was determined on a Jasco V630 UV-Vis spectrophotometer, using the DPPH method [1, 4-7, 10-12, 14-19]. The principle of this method is that antioxidants react with the free radical DPPH (purple coloured solution) and by hydrogen donating, transform it into a clear DPPH-H, where the degree of discoloration indicates the antioxidant potential [19]. The DPPH solution was prepared by dissolving DPPH (Sigma Aldrich) in methanol to obtain an absorbance value of 0.8 ± 0.02; 0.1 mL for each extract was vortexed for 30 s with 3.9 mL of DPPH solution and left to react for 30 min, after which the absorbance at 515 nm was recorded. The DPPH solution with no added extract was used as control [4]. Gallic acid was used as reference standard, dissolved in water, in order to obtain a solution with a similar concentration as the *Usneae lichen* solutions [4]. Methanol was used as blank [4]. Four extractive solutions were analysed: S_1 = *Usneae lichen* extract 200 mg/mL in ethanol 96%, S_2 = *Usneae lichen* extract 200 mg/mL in water, S_3 = *Usneae lichen* extract 200 mg/mL in acetone, S_4 = reference usnic acid solution 50 µg/mL (0.05 mg/mL). For each extract 200 mg/mL (S_1, S_2, S_3) 1:2 (100 mg/mL) and 1:4 (50 mg/mL) dilutions were obtained.

The scavenger activity was calculated as follows:

$$\% \text{ scavenger DPPH} = [(A_{control} - A_{sample})/A_{control}] \times 100,$$

where: $A_{control}$ and A_{sample} are the absorbances at 515 nm for DPPH methanol solution and samples [4, 13].

Results and Discussion

The quantitative determination of the bioactive compounds:

Using different extraction methods and different solvents, extracts with different content of bioactive compounds were obtained.

HPLC analysis of phenolic compounds

Retention times for the reference substances and correlation coefficients of the calibration curves are shown in Table I.

In order to determine the polyphenolic compounds from *Usnea barbata* L. extracts, an optimised HPLC method was used. We identified 6 polyphenols in the extracts: caffeic acid, p-coumaric acid, ellagic acid, chlorogenic acid, gallic acid, cinnamic acid. We found polyphenols especially in the aqueous and ethanolic extracts**.**

Three polyphenols were found in the ethanolic and the aqueous extracts: chlorogenic acid, gallic acid and p-coumaric acid, with higher amounts in aqueous extract. The amounts of polyphenols

identified in the analysed extracts, expressed as mg% are presented in Table II.

Table I

Retention times of phenolic compounds (reference substances) and square of correlation coefficients

No	Phenolic compound	Retention time (min)	r^2
1.	Gallic acid	0.990 ± 0.025	0.99537
2.	3-methyl gallic acid	2.606 ± 0.008	0.99563
3.	Chlorogenic acid	3.501 ± 0.015	0.99999
4.	Caffeic acid	4.598 ± 0.036	0.99619
5.	Vanillin	6.919 ± 0.051	0.99691
6.	p-Coumaric acid	7.187 ± 0.019	0.99798
7.	Ferulic acid	8.565 ± 0.058	0.99863
8.	E-Resveratrol	14.467 ± 0.017	0.99965
9.	Ellagic acid	15.303 ± 0.027	0.99885
10.	Z-resveratrol	15.751 ± 0.058	0.99729
11.	Cinnamic acid	15.867 ± 0.007	0.99845

Table II

Total polyphenols contents in the extracts

Extract	Polyphenols	Contents (mg%)
Ethanolic extract	Caffeic acid	0.46
	p-Coumaric acid	0.35
	Ellagic acid	255.99
	Chlorogenic acid	0.56
	Gallic acid	30.24
	Cinnamic acid	19.87
Aqueous extract	p-Coumaric acid	0.82
	Chlorogenic acid	0.86
	Gallic acid	66.18

In the acetonic extract we did not identified any polyphenol from the reference mixture.

HPLC analysis of usnic acid

Usnic acid has the greatest content in acetonic extract; we can observe that the usnic acid content is the smallest in the aqueous extract from *Usnea barbata* L. (Table III).

Table III

Usnic acid content in *Usnea barbata* L. extracts

***Usnea barbata* L. extract**	**Usnic acid content (mg%)**
Acetonic extract	2115.29
Ethanolic extract	256.56
Aqueous extract	44.60

Cansaran D *et al.* studied six species of *Usnea genus* (*U. florida, U. barbata, U. longissima, U. rigida, U. hirta and U. subflorida*), collected from different areas of Anatolia¸ Turkey [6]. The usnic acid content in acetone extracts was determined by HPLC and varied between 220 mg% and 6490 mg% [6]. The amount of usnic acid in the acetonic extract of *Usnea barbata* L. from Anatolia, was 2160 mg% [6], comparable to the results obtained in this study (2115.29 mg%).

Antioxidant activity assay

Usnea barbata L. extracts reduced DPPH radical with different degrees of scavenging activity. It was found that the ethanolic extract has the greatest antioxidant activity, followed by the aqueous extract and acetonic extract. The results of the antioxidant activity evaluated by DPPH method are presented in Figure 1.

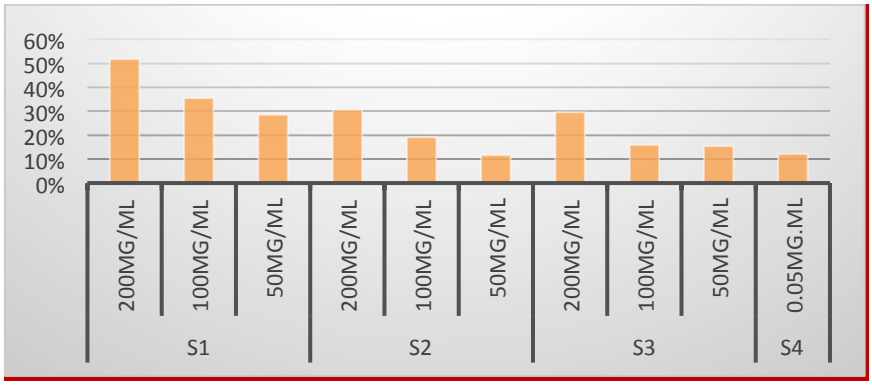

Figure 1.

DPPH scavenger activity of the *Usneae lichen* different extracts (S_1 - S_3) and of the usnic acid standard solution (S_4), where S_1 = *Usneae lichen* ethanolic extract; S_2 = *Usneae lichen* aqueous extract; S_3 =*Usneae lichen* acetonic extract; S_4 = usnic acid 50 µg/mL (0.05 mg/mL) in acetone

The scavenging effect was higher with the increase of the extracts concentration. It was found that *Usneae lichen* 200 mg/mL extract in ethanol had a DPPH scavenger activity over 50%.

The IC 50 value was 189.94 mg/mL for the ethanolic extract, calculated by linear interpolation, using the generated equation:

inhibition = 0.1561 x concentration + 20.35.

The acetonic and aqueous extracts in the present study presented a scavenger activity under 50%, IC 50 was not calculated.

Rankovic B *et al.* evaluated the antioxidant potential of the *Usnea barbata* L. acetonic extract. They used the DPPH method, with ascorbic acid as reference substance and they showed an IC 50 value of 667.97 µg/mL, for the acetonic extract [16].

Zugic *et al.* evaluated the antioxidant activity of *Usnea barbata* L. using the FRAP (ferric reducing ability of plasma) assay for 4 extracts of *Usnea barbata* L.: supercritical CO_2 extract (SCE), compared to the extracts (etheric, ethanolic and aqueous) obtained with conventional techniques. The obtained results showed that SCE had the best antioxidant potential among the investigated extracts with FRAP value, being almost twice higher in comparison to the etheric fraction of Soxhlet extract, followed by a lower antioxidative potential of ethanolic fraction of Soxhlet extract and, finally, aqueous extract [21].

Conclusions

Our research on *Usnea barbata* L. from Călimani Mountains, Suceava County, România, showed a 2115.29 mg% usnic acid content in the acetonic extract.

All the analysed extracts in different solvents of *Usnea barbata* L. showed antioxidant activity. The results indicate that *Usnea barbata* L. presents a potential to be use in the treatment of oro-dental conditions or other diseases related to oxidative stress.

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