In the category of medicinal plants with remarkable therapeutic properties, lichens are also included. An important representative of this group is the Usnea Adans. genus, with over 650 species spread throughout the globe. Representative species of this genus were also found in Romania, so the studies were conducted on Usnea barbata (L.) F. H. Wigg., harvested from the Calimani Mountains, Suceava County. The objectives of this research are: obtaining the dry extract of the Usneae lichen followed by the identification and determination of the usnic acid content by ultra-high performance liquid chromatography (UHPLC) method. Usnic acid content determined in dry extract of Usnea barbata (L.) F. H. Wigg. was significant, of 16.53 ± 6.53% (mean ± RSD), which supports the continuation of the research with the evaluation of biological effects (antibacterial, antioxidant and antitumoral).

Keywords: Usnea barbata (L.) F. H. Wigg dry extract, usnic acid, UHPLC

In recent years, there has been an increasing tendency to supplement the action of synthetic drugs, whose therapeutic benefits are doubled by multiple adverse effects, with natural remedies. Many plants have antioxidant properties, especially due to the content of polyphenols; these constituents have an important role in the normal growth and the development of plants, as well as in the development of the oxidative stress defense abilities [3, 4, 8, 13]. Among the plants with remarkable therapeutic properties, there are also the lichens [7, 12, 17]. Structurally, the lichens represent a symbiosis between a fungus (mycobiont) and a green alga or a cyanobacteria (photobiont); the resulting thallus is completely different from the structure of each component. The special dual structure of the lichens and the specific conditions in which they live, determine the synthesis of many metabolic products, which provide them with optimal protection against disruptive, physical and biological factors [1, 12, 16, 17]. The metabolites produced by lichens are divided into two major categories: primary and secondary metabolites. The primary metabolites are produced by intracellular metabolism (proteins, aminoacids, polysaccharides); the secondary metabolites are the majority of organic compounds that characterize the lichen phytochemistry, being synthesized exclusively by mycobiont (polyphenols, terpenes, dibenzofurans, depsides, carotenoids, steroids) [1, 12, 17, 19]. The secondary metabolites are extracellular and they are stored in the crystal form on the surface of the hyphae of mycobiont; more than 800 such compounds have now been isolated. They have a very low solubility in water, and their extraction is realized with organic solvents. Usually, the secondary metabolites content may vary between 0.1 - 10%, but, sometimes, it can reach up to 30%, relative to the dry vegetable mass [12, 16].

An important representative of this group is the Usnea Adans. genus, with over 650 species spread throughout the globe. The scientific literature shows that the lichens of the genus Usnea Adans. are used in traditional medicine for thousands of years to treat various diseases [12, 14, 15]. For all lichens of this genus, the common secondary metabolite is the usnic acid, an organic compound with dibenzofuranic structure [10, 16, 17, 19]. Representatives of the Usnea Adans. genus are found in Romania, so that the present study was conducted on Usnea barbata (L.) F. H. Wigg., harvested from mountain areas (Calimani Mountains); this region is characterized by a low degree of pollution.

Experimental part

Material

Usneae lichen is Usnea barbata (L.) F. H. Wigg dried thallii; this lichen was identified based on the anatomical characters described in the literature [8, 12]. The freshly harvested lichen thalli were cleaned from impurities and dried at constant temperature below 25°C in an airy room, sheltered from the sun. The dry thallii (Usneae lichen) have been properly preserved under the above-mentioned conditions. To obtain the dry extract, the Usneae lichen was milled as a powder and held for 8 h with acetone in a Soxhlet continuous reflux system at 70°C; for evaporating the solvent, it was used the rotary evaporator TURBOVAP 500 Caliper [2, 11, 20]. The dry acetone extract was transferred to a sealed-glass bottle and stored in the freezer at below -20°C until processing [17].

Ultra-High Performance Liquid Chromatography (UHPLC) method

Identification and determination of usnic acid content was performed by the UHPLC method [9]. This method had the following features: UHPLC Perkin-Elmer FLEXAR, binary pump, PDA plus detector, thermostatic compartment for the column, degassing system,
autosampler. Working conditions consisted of: C18 column, 150 mm / 4.6 mm; 5 µm (Perkin-Elmer); mobile phase in an isocratic system: methanol: water: glacial acetic acid = 80: 15: 5; detection: UV = 282 nm; flow = 1.5 mL / min; temperature in the column compartment = 25° C; injection volume = 20 µL; analysis time: 6 min. Sample: the dry Usnea barbata extract was solubilized in DMSO in 0.2% concentration, diluted 1 to 10, 1 to 20 and 1 to 50. Reference substance: usnic acid in DMSO at concentrations of 10, 20, 50, 100, 200 µg / mL, with which the calibration curve was drawn (y = 4.84629E + 04x - 4.01679E + 04; r²: 0.999877), shown in fig. 1.

The following parameters were evaluated for validation of the method: specificity, accuracy, repeatability, precision, limit of detection (LOD) and limit of quantification (LOQ) [9]. The linearity of the method was verified by the method of least squares, on the 10 µg / mL - 200 µg / mL range, for a value of R²> 0.99.

Identification and quantification of the usnic acid were based on the reference substance for which the retention time was determined at RT = 3.64 ± 0.07 min (fig. 2).

Results and discussions
Obtaining of Usneae extract

From 100 g of Usneae lichen, 7.97 g of dry extract (Usneae extract) was obtained (fig. 3). The dry extract has a greenish-brown coloration, a strong acetone odour and a spicy taste. It was stored in a freezer, at a temperature below -20°C until it was used.

UHPLC analysis

The sample to be analysed has a peak at retention time RT = 3.70 min; it was identified based on the reference substances, as usnic acid. The method calibration is linear with r² = 0.999877; the accuracy is 2.26%; the method precision, calculated as repeatability at the concentration 100 µg / mL, is 1.16%; the limit of quantification (LOQ) was found to be 0.20µg / mL with a signal-to-noise ratio = 14:1 and the limit of detection (LOD) was estimated at 0.04 µg / mL [9, 22].

Determination of the Usneae extract composition was performed on three dilutions of 0.2% extract in DMSO: 1 to 10, 1 to 20 and 1 to 50 (fig. 4).

The usnic acid content determined in Usnea barbata dry extract, by UHPLC method, was 16.53 ± 6.53% (mean ± RSD). In the chromatograms of diluted 1:10, 1:20, 1:50 solutions, the presence of other, yet unidentified, organic compounds, that may contribute to the therapeutic potential of the Usneae extract, was also observed.

In the accessed scientific literature, the obtaining of dry lichen extracts is a common method used for: chromatographic determinations of chemical constituents, isolation of active compounds and determination of biological actions [8, 12, 16]. The provided methods are similar to those used in this study. The amount of 7.97 g dry extract obtained from 100 g Usneae lichen (harvested from Calimani Mountains) is proportional to the same quantity mentioned in the literature: from 400 g Usnea flexuosa
The presence of a high amount of usnic acid justifies further research with biological studies to evaluate the antimicrobial, antioxidant and antitumoral activities of Usnea extract.

Conclusions

The UHPLC analysis of Usnea lichen dry acetone extract reveals an 8-fold higher content of usnic acid than cold acetone macerated extract.

This study provides an important basis for further research in order to identify the other relevant organic compounds, followed by the exploration of their therapeutic actions.

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