Studies on the Antitumor Action Mechanisms of *Usnea barbata* (L.) F.H.Wigg from Romania

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Abstract

Our previous studies it has been shown that the Usnea barbata (L.) extract has antitumor effect and also we tried to elucidate the mechanisms of the cancer cells death. Given the fact that proteins are fundamental elements for maintaining life, it is estimated that the decrease in the amount of total protein leads to cell death. The working hypothesis of our study is that different concentrations of Usnea barbata (L.) extract induces death of tumor cells through this mechanism. Analysis of obtained results, showed that at the concentration of 100 µg / mL lichen extract, the amount of soluble proteins is similar to the value recorded in the control. In contrast, at the maximum dose tested (400 µg / ml), the protein content decreased significantly, being almost 3 times lower compared to the control, thus proving the cytotoxic effect of the tested Usnea barbata (L.) extract.

Key words: Usnea barbata (L.), antitumor potential, CAL27 tumor cells, proteins
J.E.L. classification: I10

1. Introduction

Cancer is a heterogeneous class of diseases, characterized by the uncontrolled division of cells and their ability to invade, from close to close, neighboring tissues and to metastasize, by lymphatic or hematogenous, loco-regional or distant, to other tissues and organs; it has an estimated global incidence of 6 million cases per year, being the second leading cause of death after cardiovascular disease (Einarsdóttir *et al.*, 2010).

Oro-maxillofacial malignancies are generally characterized by: infiltrative-destructive tumor growth with local invasion and loco-regional and distant metastatic dissemination (with the formation of cervical metastases, or in more distant organs) (Nuñez-Aguilar *et al.*, 2018).

The National Institute for the Study of Cancer shows that the overall survival rate in this type of neoplasms is 63% and that its value varies depending on the area of localization in the oral cavity and the time of initiation of treatment; thus, if treated at the onset of the disease, oral cancer has a survival rate of 84%, compared to: 65%, if it spread to the lymph nodes of the neck and 39%, if it spread at a distance (Dhanuthai *et al.*, 2018).

It is known that most synthetic chemotherapeutics currently used in antitumor therapy develop resistance over time and have non-selective toxicity against normal cells; these side effects, combined with side effects and multiple drug interactions, are major disadvantages of chemotherapies (Yin *et al.*, 2019).

Therefore, obtaining new antibacterial and antitumor drugs remains a major clinical challenge. In this context, plants are a valuable source of biologically active natural compounds, and research is aimed at their use in anti-infective and anti-cancer therapy. Isolated constituents may be useful as

alternative therapeutic agents or as core nuclei for new synthetic products with increased activity and / or reduced toxicity (Zitvogel *et al.*, 2013).

Lichens are also found in the category of plants that have remarkable antitumor, antibacterial and antioxidant properties (Shrestha and St. Clair, 2013).

They are a unique group in the plant world and, at the same time, are the most widespread symbiotic organisms in nature, inhabiting more than 8% of the earth's surface (Ranković *et al.*, 2012).

An important representative of lichens is the genus Usnea Dill. ex. Adans, with over 350 species spread over the entire surface of the globe (Prateeksha *et al.*, 2016). It is clear from the literature that many species belonging to this genus have been used in traditional medicine for thousands of years, in the treatment of various diseases (Shanmugam *et al.*, 2017). Representatives of the genus Usnea are also found in Romania; thus, starting from all the considerations mentioned above, the idea of carrying out our studies, which aimed to highlight the antitumor action of Usnea barbata (L.) F.H. Wigg., harvested from the Călimani mountains (900 m), Suceava county.

2. Theoretical background

By the UHPLC-ESI-OT-MS-MS technique, in Usnea barbata (L.) F.H.Wigg. the following groups of secondary metabolites have been isolated:

- Depside: barbatolic acid, thamnolic acid, haemathamnolic acid, lecanoric acid, gyrophoric acid, diffractaic acid, methyl-8-hydroxy-4-O-divaricatic acid, barbatic acid (figure 10), sekikaic acid, 8-hydroxybarbatic acid, atranorin and chloroatranorin;
- Depsidone: salazinic acid, connorstictic acid, siphulellic acid, galbanic acid, norstictic acid, α-acetylconstictic acid, stictic acid, lobaric acid;
- Lipids: tetrahydroxyyeicosanoic acid, tetrahydroxydocosanoic acid tetrahydroxytricosanoic acid, tetrahydroxydocosanoic acid, tetrahydroxyhexacosanoic acid, nonahydroxyoctacosanoic acid heptahydroxytricosatrienoic acid;
- Diphenyl ethers: β-alectoronic acid;
- Dibenzofurans: usnic acid (Salgado *et al.*, 2018).

These secondary metabolites are responsible for many biological actions, including the antineoplastic effect of lichen extracts; In numerous studies in the accessed literature, this activity has been evaluated, and the results obtained so far are promising (Molnár and Farkas, 2010).

Although extracts of Usnea barbata (L.) F.H.Wigg. have been used for thousands of years in traditional medicine, there are currently few scientific studies on their antitumor potential. In the scientific literature various anticancer mechanisms if lichens are mentioned: cell cycle arrest, Inhibition of growth factor signalling and DNA repair, disabling replicative immortality by inhibiting telomerase activity, inhibition of invasion and metastasis , supression of genome instability, apoptotic cell death, necrosis, autophagy, ROS-Dependent Mitochondria Molecular Mechanisms (Tram *et al.*, 2020).

These aspects were the basis of our researches; I believe that the evaluation of the antitumor mechanisms of Usnea barbata (L) F.H.Wigg. could lead to the optimization of treatment regimens for oncological pathology, especially in oral cancer (Popovici *et al.*, 2020).

Our previous studies have been shown that the death of tumor cells is real and also tried to elucidate the mechanisms by which this phenomenon occurs.(Popovici *et al.*, 2020).

Given the fact that proteins are fundamental elements for maintaining life, it is estimated that the decrease in the amount of total protein leads to cell death (Ettinger, Ganry and Fernandes, 2019). Based on these considerations, the working hypothesis of our study is that the application of different concentrations of Usnea barbata (L.) F.H.Wigg extract generates the death of tumor cells through this mechanism (Deo, Bijlani and Deo, 1979).

3. Research methodology

✓ Obtaining the dry lichen

The heels of Usnea barbata (L.) F.H.Wigg. were picked manually, directly from the branches of conifers, in march 2020. The freshly harvested plant material was cleaned of impurities and dried at a constant temperature, below 25oC, in an airy room, sheltered from the sun's rays. After drying, the vegetable product obtained was kept for a long time under the same conditions, for use in subsequent studies. The identification of the lichen species was realized at the Department of Pharmaceutical Botany of the Faculty of Pharmacy, Ovidius University, Constanta.

✓ Obtaining the dry lichen extract

To obtain the dry extract, the lichen was ground in powder form and kept for 8 hours with acetone, at 70o C, in a continuous reflux on Soxhlet. After the completed reflux the TURBOVAP 500 Caliper rotary evaporator was used to evaporate the solvent; the dry extract (UBE) was transferred to a glass vessel with a sealed lid and stored in the freezer at a temperature below -20 ° C until further processing (Popovici *et al.*, 2019).

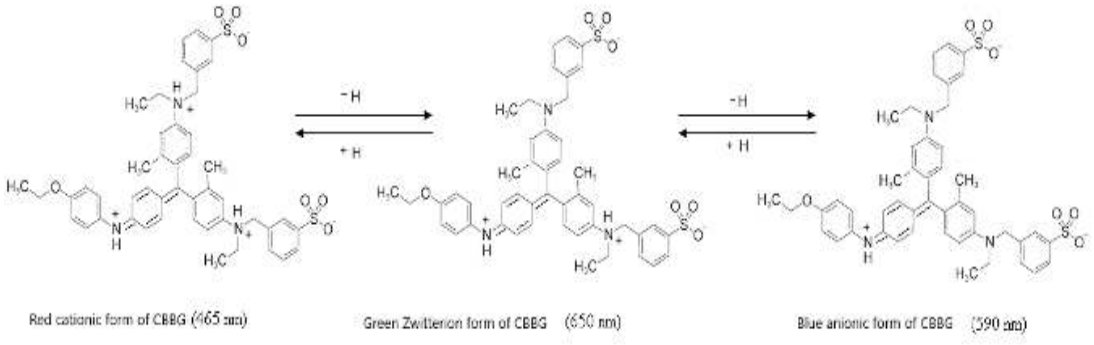
✓ Protein dosing by the Bradford method

The Bradford method is a sensitive analysis; it can dose proteins with a concentration between 0.1 - 2 mg / mL.(Aminian *et al.*, 2013)

Principle of the method

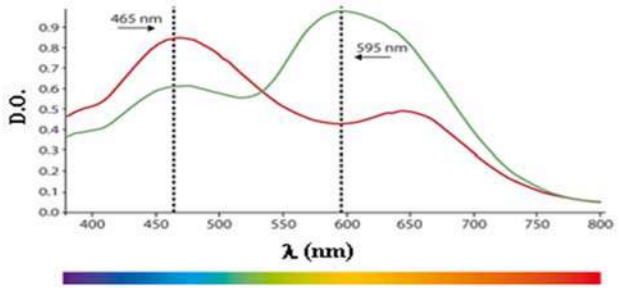
In a strongly acidic medium, Coomassie Brilliant Blue G (CBBG) is predominantly red, with a maximum absorbance of around 465 nm, and a green form with a maximum absorbance at 650 nm and to a lesser extent in blue anionic form, with a maximum absorbance at 590 nm (*Figure no. 1, 2.*). Of the ionic forms of CBBG, only the anionic form binds to proteins at the level of positively charged lysine and arginine residues, forming a blue complex. Simultaneously with the formation of the blue complex there is a change in the maximum absorption capacity from 465 nm to 595 nm *(Figure no. 2*). The color intensity measured at 595 nm is directly proportional to the amount of protein in the sample to be analyzed (Aminian *et al.*, 2013).

Figure no. 1. Ionic structures of CBBG



Source: (Aminian *et al.*, 2013)

Figure no. 2. Changing the maximum absorption range of CBBG in the presence of proteins, from 465 nm to 595 nm



Source: (Aminian *et al.*, 2013)

Reagents

- Standard solution of bovine serum albumin (BSA) 2 mg / ml prepared extemporaneously in the same medium in which the proteins were extracted;
- Bradford reagent: 100 mg of CBBG is dissolved in 50 ml of 95% ethanol. The alcoholic solution is mixed with 100 ml of 85% phosphoric acid and made up to 1 liter with distilled water;
- Total protein extract (EPT);
- Extraction medium (Kamizake *et al.*, 2003)

Phases of the working technique

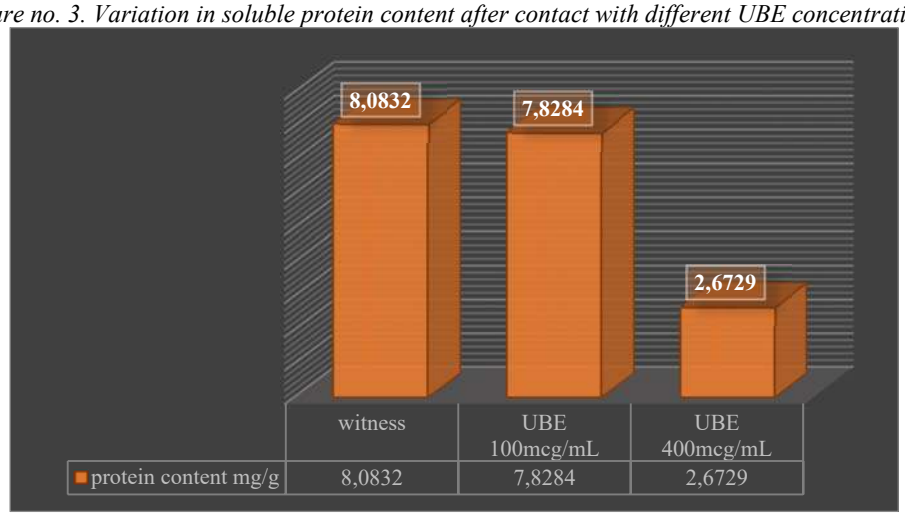
- dilutions of EPT are made whose concentration in proteins we want to determine;
- few dilutions are made from the stock solution of 2 mg / ml BSA
- in a 96-well plate, pipette 5 µl of each sample (consisting of EPT dilutions) and each standard (dilutions of the BSA stock solution from tubes 1 - 7) into separate wells and add 250 µl. Bradford reagent;
- the plate is shaken for 30 s and left to stand for 15 minutes at room temperature;
- an automatic plate reader reads the optical densities (extinctions) of both EPT-containing samples and BSA standards at a wavelength between 590 - 690 nm;
- a standard curve is drawn noting on the abscissa the protein concentration in mg / ml and on the ordinate the values corresponding to the BSA standards;
- using the standard curve, the units of D.O.595 obtained for EPT dilutions are transformed into concentration values (mg / ml) and multiplied by the corresponding dilution factor (Martina and Vojtech, 2015).

4. Findings

Analysis of the obtained results showed that at the UBE concentration of 100 µg / mL the amount of soluble proteins is similar to the value recorded in the control.

In contrast, at the maximum tested dose (400 µg/mL), the protein content decreased significantly, being almost 3 times lower compared to the control, thus proving the cytotoxic effect of the lichen extract *(Figure no. 3.*).

Figure no. 3. Variation in soluble protein content after contact with different UBE concentrations



Source: Authors' contribution

Discussions

In previous cytotoxicity studies on CAL27 tumor cells line, we demonstrated that the maximum cytotoxicity at 24 hours was recorded at the value of the UBE concentration of 400 µg / mL (2 times higher than at 100 µg / mL (Popovici *et al.*, 2020); in the present study, at this value of the UBE concentration, there was a marked decrease in soluble proteins (3 times higher than at 100 µg / mL).

One possible mechanism by which significant decreased of total proteins content could cause tumor cell death at the same concentration of UBE would be intense intracellular induced oxidative stress. Reactive oxygen species (ROS) production and thiol redoxstate imbalance are induced immediately upon protein deprivation and represent important mediators of autophagy, and, finally, for tumor cells death (Filomeni, De Zio and Cecconi, 2015).

Neoplastic cells have a higher specific aminoacids (glutamine, arginine, asparagine) demand versus normal cells; by total protein decreasing, these aminoacids become unavailable. For example, the tumor cells are considered as glutamine-addict, such that glutamine deficiency may induce apoptosis (Kwong Lam Fung and Chi-Fung Chan, 2017).

5. Conclusions

The results of this study show the possibility of correlation between the significant decrease of total proteins content and the high cytotoxic effect on CAL27 tumor cells, both recorded at the same concentration of UBE.

Its paves the way for discovery of the possible molecular mechanisms that could explain the antitumor effect of Usnea barbata (L.) F.H.Wigg., which could be triggered by protein/aminoacids deficiency.

6. Acknoledgement

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