CONTRIBUTIONS TO THE COMPLEX STUDY ON ANTITUMOR ACTIVITY OF USNEA BARBATA (L.) F.H.WIGG.

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ABSTRACT

Usnea barbata (L.)F.H.Wigg. - known as "old man's beard", "tree moss", "songluo" is a lichen in the family *Parmeliaceae*, genus *Usnea*. *Usnea* species have recorded history of therapeutic use dating back over three thousand years in Chinese medicine. The lichen secondary metabolites have shown an impressive range of biological proprieties, including antibiotic, antifungal, antiviral, anti-inflammatory, or anticancer activities.

In this study, the antitumor activity of *Usnea barbata* extract was evaluated by observing the morphological changes on squamous cells carcinoma cell-line CAL 27 (ATCC® CRL-2095 ™) in contact with different concentrations of extract, ranged between 12.5–400 µg/mL. The results obtained were quantified by the intensity of morphological changes of the tumor cells after 24 hours of contact. The most significant activity were recorded for 400 µg/mL extract.

This study shows that *Usnea barbata* (L.)F.H.Wigg. extract has antitumor activity. The analysis of the obtained results showed that the cytotoxicity of lichen extract on CAL 27 tumor cells is directly related to the concentration of the applied solution.

Keywords: *Usnea barbata (L.) F.H.Wigg extract, antitumor action, CAL 27 cell line, cell morphology*

INTRODUCTION

Usnea barbata (L.)F.H.Wigg. - known as "old man's beard", "tree moss", "songluo" is a lichen in the family *Parmeliaceae,* genus *Usnea. Usnea* species has a recorded history of therapeutic use dating back over three thousand years in

Chinese medicine [1], [2]. This genus name – *Usnea,* may come from the time of the Arabian school of medicine and pharmacy. *Usnea* species are used today in traditional Chinese medicine, contemporary homeopathic and naturopathic medicines and in various systems of traditional medicine worldwide [3]. The numerous therapeutic applications are due to their phytochemicals contained and which represent almost 60 organic compounds with various structures: polyphenols, dibenzofurans, depsidones, depside, depsones, fatty acids, lactones, quinones, polysaccharides [4]. These secondary metabolites have shown a high range of biologicalactivities including antibiotic, antifungal, antiviral, anti-inflammatory, anticancer proprieties [5], [6].

In this study, the antitumor activity of *Usnea barbata* (L.) F.H.Wigg. was evaluated by observing the morphological changes on squamous cells carcinoma cell-line CAL 27 (ATCC® CRL-2095 ™) after 24 hours of contact with various concentration of dry lichen extract.

MATERIAL AND METHOD

1. Harvesting and identification of lichen species

The harvesting of the vegetal material was made from Călimani mountains (900 m), Suceava county, and the identification of this lichen species was performed at the Department of Pharmaceutical Botany of the Faculty of Pharmacy within the Ovidius University of Constanta [7].

2. Plant material and lichen extract preparation

The freshly harvested lichen was cleaned of impurities and dried at a constant temperature, below 25^{o}C, in an airy room, sheltered from the sun's rays [7].

To obtain *Usnea barbata* extract (UBE), the dry lichen was ground in powder form and kept for 8 hours with acetone, at 70^{o} C, in a continuous reflux on Soxhlet. After refluxing, the evaporation of the solvent was performed on a rotary evaporator and the dry extract was transferred to a glass vessel with a sealed lid and stored in the freezer at a temperature below -20 ° C until further processing [7].

3. Evaluation of the cytotoxic action of UBE on squamous cells tongue carcinoma cell line, CAL 27 (ATCC® CRL-2095 ™)

The tumor cell line CAL 27 (ATCC® CRL-2095 ™) is widely used to obtain oral squamous cell carcinoma (OSCC) for *in vitro* and *in vivo* studies, being considered representative for the study of this cancer type.

Working hypothesis: UBE has antitumor action on tongue squamous cell carcinoma.

Aim of the study: evaluation of the antitumor action according to the concentration of the extract and the exposure time.

Preparation of cell line material:

The CAL 27 cell line was supplied by the American Type Culture Collection (ATCC) and purchased from an authorized LGC Standards GmbH - Germany distributor. CAL 27 human tumor cell line (ATCC® CRL-2095 ™) are squamous epithelial cells isolated from lingual carcinoma; were grown in a special medium – jue modified Dulbecco (DMEM, Biochrom AG, Germany containing 10% fetal bovine serum (Sigma, Germany), 100 µg/mL streptomycin (Biochrom AG, Germany), 100 IU/mL penicillin (Biochrom AG, Germany). For optimal cell growth, the flasks were maintained in a Binder incubator, in a particular microclimate, at 37 degrees Celsius, in humidity (to reduce evaporation), in presence of 5% CO_2 to maintain the pH of the culture medium [8].

After 24 hours of incubation, the formed cell monolayer was detached with a trypsin-EDTA solution. After cells counting and establishing of the viability by trypan blue exclusion test using Cellometer Mini – Nexcelom Bioscience, the cells from the initial flasks were split into 96-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) at a density of $8x10^3$ cells/well, and then incubated under the same temperature and humidity conditions in the Binder incubator. The following final UBE concentrations in dimethyl sulfoxide (DMSO) as solvent (µg/mL) were tested: 12.5, 25, 50, 100, 200 and 400 µg/mL.

After applying of the six concentrations of UBE the cells were thermostated for 24 hours. Apart from untreated control, the 0.2% DMSO variant was performed, in order to evaluate the interaction of the cells with the solvent.

Determination of the morphological changes

The morphological changes of CAL 27 cells, after the contact with six different concentrations of UBE (12.5-400µg/mL), were detected with the 10x objective under the inverted light microscope (NIKON Eclipse TS100); the images have been taken with MShot MS60-2 digital camera.

RESULTS

The changes on morphology of CAL 27 cells are directly proportional with the concentration of UBE used.

As shown in Figure 1, at the moment of the effective realization of the experimental groups, the cells have a globular appearance and do not adhere to the substrate, being in Brownian motion in the culture medium.

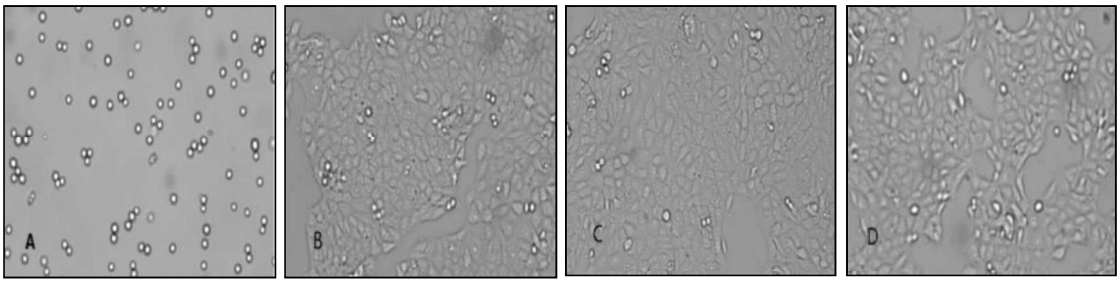

Figure 1. *Morphology of CAL 27 cells in the initial inoculation phase (A), after 24 hours of contact with 0.2% DMSO (B), after the contact with UBE tested solutions: 12.25 µg/mL (C); 25 µg/mL (D)*

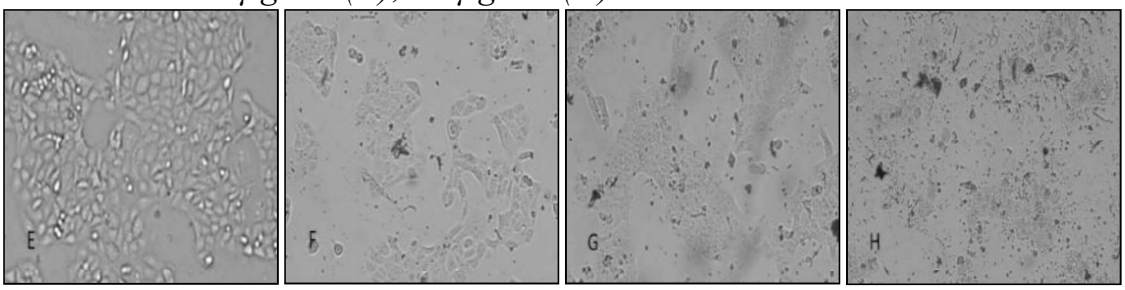

Figure 2. *Morphology of CAL 27 cells, after the contact with UBE tested solutions: 50 µg/mL (E); 100 µg/mL (F); 200 µg/mL (G); 400 µg/mL (H)*

The study on control cells, and also in DMSO 0.2%, indicated that, after 24 h of treatment, these cells have a normal morphology and are adherent to the substrate (Figure 2). The morphological study on the tumor cells exposed to UBE showed that CAL27 cells had different degrees of morphological changes, as follows:

- loss of cell adhesion,
- membrane contraction,
- formation of abnormal cell folds,
- cell fragmentation,
- reduction of the density of living cells (figures: 1., C-D and 2., E-H)

These changes are most significant at the maximum concentration of UBE in the tested solutions.

DISCUSSIONS

By analysis of the figures 1 and 2, it is found, compared to the control, the minimal interference of EUB with cell viability, in the range of concentrations 12.5–50 µg/mL inclusive, registering a minimal cytotoxic effect.

Slight decreases in cell viability are observed at concentrations of 100 and 200 µg/mL, which correspond to insignificant cytotoxic effects. The non-cytotoxic effect of the solvent (0.2% DMSO) on CAL 27 cells was noted; on this

observation is based the conclusion that the induced cytotoxicity is generated exclusively by UBE action.

The antiproliferative effect on some tumor cell lines has been attributed to usnic acid, as shown by other studies presented in the literature [9], [10].

In the accessed scientific data it is highlighted that the cytotoxic effect of classical chemotherapeutics and plant extracts differs, depending to the type of tumor cells on which the studies are performed [11], [12].

CONCLUSIONS

The extract of *Usnea barbata* (L.) F.H.Wigg. has cytotoxic activity on CAL 27 cells, its intensity being directly proportional to the concentration of UBE.

The main morphological cell changes include loss of adhesion, membrane contraction, abnormal cell folds, cell fragmentation and, consequently, the reduction of the living cells density as an expression of the UBE antitumor effect.

The results of our preliminary study provide a valuable basis for future studies in order to suppose the mechanisms of UBE tumor cell death induction, and to determine the effective doses of this extract without toxic effects on normal cells.

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