

THE BIOCHEMICAL INVESTIGATIONS OF SOME *CLAVICEPS PURPUREA* BIOPRODUCTS AND THEIR *IN VITRO* CITOSTATIC POTENTIAL

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Abstract

The sclerotia of the pyrenomicete *Claviceps purpurea* (Fr.) Tul. (*Hypocreaceae*) contains numerous active compounds of which the most known are ergoline alkaloids. In this paper, the mycelian extracts and the corresponding concentrated supernatants, separated from cultures of nine *Claviceps purpurea* strains, have been biochemically investigated by determination of alkaloids, glucans and proteins. The cytostatic potential of extracts and concentrated supernatants has been also tested on HeLa tumor cells cultures in the presence of bioactive samples. Generally, the cytostatic potential of these bioactive agents was inversely correlated with the intra - or extracellular ergoline alkaloids content, which seemed not to be the exclusive biochemical substratum for this *in vitro* pharmacodynamic effect.

Rezumat

Scleroții ciupercii *Claviceps purpurea* (Fr.) Tul. (*Hypocreaceae*) conțin numeroși compuși activi, cei mai cunoscuți fiind alcaloizii de tip ergolinic, structuri cu un spectru larg de utilizări terapeutice. În lucrarea de față, extracte miceliene și din supernatantul culturii a 9 tulpini de *Claviceps purpurea* au fost caracterizate din punct de vedere al conținutului în alcaloizi, glucani și proteine, precum și al efectului citostatic. Proprietățile citostatice ale biopreparatelor obținute au fost demonstrate prin efectul inhibitor asupra biosintezei proteice celulare, prin testarea pe culturi celulare tumorale HeLa. În general, creșterea procentului de inhibiție a biosintezei proteice se corelează cu diminuarea concentrației în alcaloizi ergolinici a extractului testat. În aceste condiții, aceștia nu sunt singurii compuși bioactivi responsabili de efectul farmacodinamic, *in vitro*.

Keywords: *Claviceps purpurea* extracts, biochemical investigations, cytostatic potential

Introduction

Despite major scientific and technological progress in pharmaceutical chemistry, antineoplastic agents derived from natural

products still make an enormous contribution to drug discovery today. The pyrenomycete *Claviceps purpurea* (Fr.) Tul. (*Hypocreaceae*) is probably the best known species of the *Claviceps* genus. The fungus produces different ergot alkaloids (ergoline and clavine type) with a wide range of biological activities. The broad spectrum of ergot alkaloids effects are mostly based on their interactions with adrenergic, serotonergic or dopaminergic receptors, as well as on their interference with some cellular and molecular processes. The ergoline alkaloids are used in the treatment of uterine atonia, postpartum bleeding, migraine, orthostatic hypotension, cerebral insufficiency, hyperprolactinemia or Parkinson disease. The antitumoral effect observed in the case of some clavine-type alkaloids reinforced the interest for *Claviceps purpurea* as a possible source of new oncotherapeutical agents [4,8].

This paper presents the results of our investigations concerning the cytostatic potential and the biochemical features of some *Claviceps purpurea* submerged hybride strains' extracts, the biological material being obtained by somatic hybridization at the Institute of Biological Research from Iasi, Romania.

Materials and Methods

Biological Material. Nine strains of *Claviceps purpurea* (T2-5, T3-2, T11-2, T12-2, T14-2, T15-1, T7-1, T10-1 T11-1), were analyzed according to the procedure:

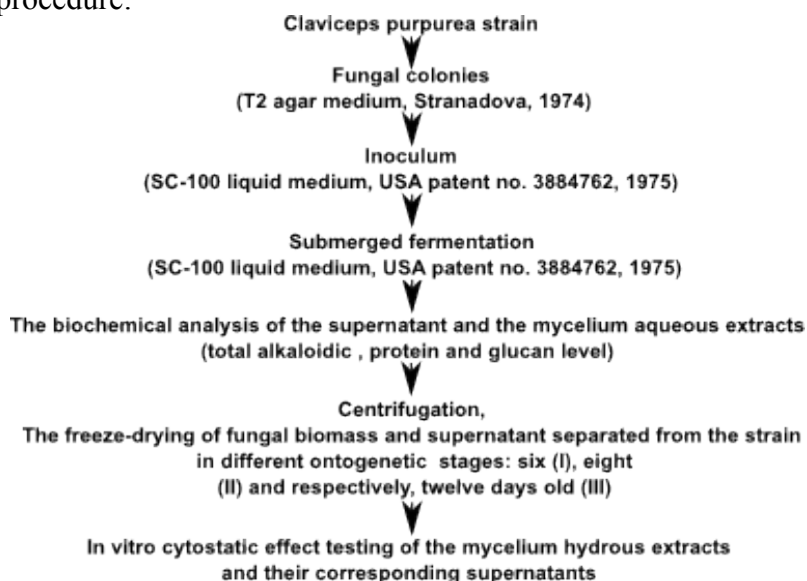


Figure 1

The experimental procedure for obtaining the studied biological material

Two types of samples (obtained according to figure 1) were taken into study:

- *mycelium aqueous* extracts
- the concentrated supernatants (the culture liquid) obtained after centrifugation.

Samples were collected after 6, 8 and 12 days of fungal fermentation and were prepared according to the above steps (Figure 1), biochemically analyzed and tested for their citostatic activity.

The biochemical analysis

Total alkaloid content (CAT) was measured using van Urk color reagent (sulfuric acid 70%, p-dimethylaminobenzaldehyde 0.125 g, 1:1 FeCl₃ solution, 0.3 mL, per 100 mL of color reagent). The calibration curve was generated using 0.01% ergotamine tartrate in 1% tartaric acid [7]. Protein concentration in fungal extracts was determined by Bradford method [1]. Glucan level was gravimetrically quantified, after supernatant precipitation with ethanol (1:1 v/v).

Evaluation of cytostatic properties of fungal extracts

In vitro cytostatic activity of fungal extracts was evaluated using HeLa cancer epithelial cell cultures. The cells were maintained on 75 cm² culture flasks, containing DMEM growth medium (Dulbecco's Modified Essential Medium, Biochrom AG, Germany) supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin (Biochrom AG, Germany), 100 UI/mL penicillin (Biochrom AG, Germany) and 50 µg/mL amphotericin B (Biochrom AG, Germany). The flasks with the tumoral cells at a density of 5×10^5 were incubated at 37°C, in a humidified atmosphere with 5% CO₂ [2]. After the initial cell cultures reached the monolayer stage through confluence of HeLa cells, they were detached from the lower plate with a solution of 0.25% trypsin and 0.02% EDTA (ethylenediaminetetraacetic acid, Biochrom AG, Germany), then centrifuged at 1800 rpm for 2 minutes and re-suspended in normal medium. Two mL of cell suspension, with a density of 1×10^5 cells/mL, were inoculated in test tubes and kept in an incubator at the same ambient conditions, for different time intervals. After 24-hours incubation, when the daughter cells reached the monolayer stage, the growth medium was decanted and replaced either with fresh growth medium (control culture) or with fresh growth medium containing 5 µg/mL ergoline extracts (treated culture). After 48 hours (corresponding to 72-hours culture age), the growth medium was removed, the cell layer washed with PBS (phosphate buffer solution) and then subjected to the analysis method for the evaluation of the total protein content according to Lowry's method, modified by Oyama [6]. The registered protein values were used

for mathematical evaluation of cellular cultures development degree after the action of the ergoline alkaloid biopreparations, the inhibition of this last process representing their cytostatic effect.

The cytostatic significance of the studied biopreparations was evaluated on the basis of the American prescreening program criteria, which imposed, among other, the induction of a minimum inhibitory impact of 50 of the % for *in vitro* preliminary testing and selection of new potential antitumoral agents [3]. For each culture type and time interval five culture tubes were used and the results were evaluated statistically by Student's "t" test [2] at a statistical significance level fixed at $p \leq 0.05$.

Results and Discussion

The fungal extracts were characterized by a content of total alkaloid ranging from 1.25 to 11.10 mg/mL for the mycelium extract, and from 6.14 to 16.40 mg/mL for the supernatant (Figure 2).

The glucan concentrations were 5.20 - 19.40 $\mu\text{g/mL}$ in the mycelium extract and 7.25 $\mu\text{g/mL}$ - 18.52 $\mu\text{g/mL}$ in the concentrated supernatants (Figure 3).

Protein content ranged from 0.70 mg/mL to 3.93 mg/mL in the mycelium extract, while in the supernatant was 0.36 - 1.41 mg/mL (Figure 4).

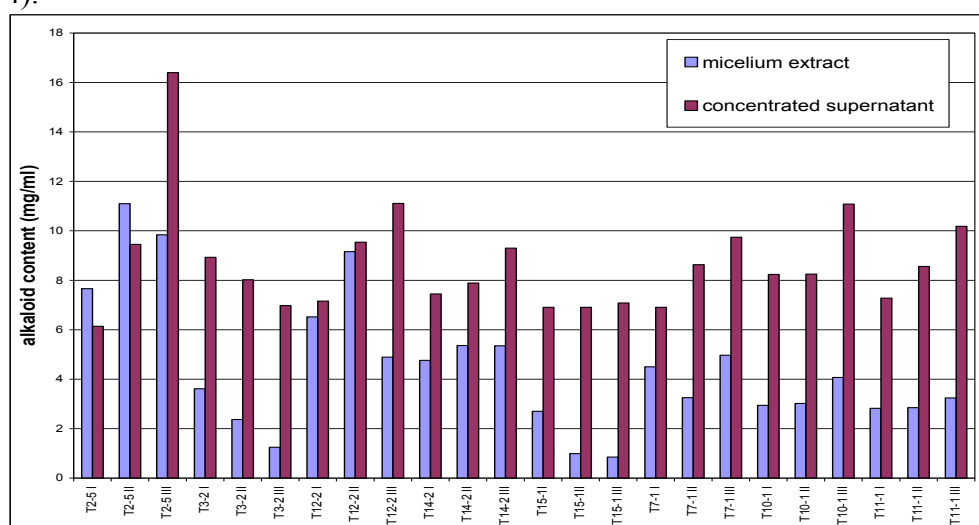
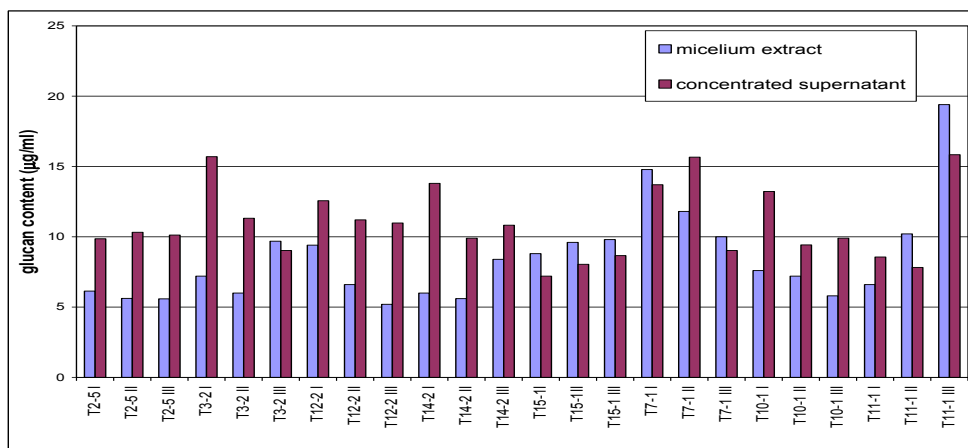


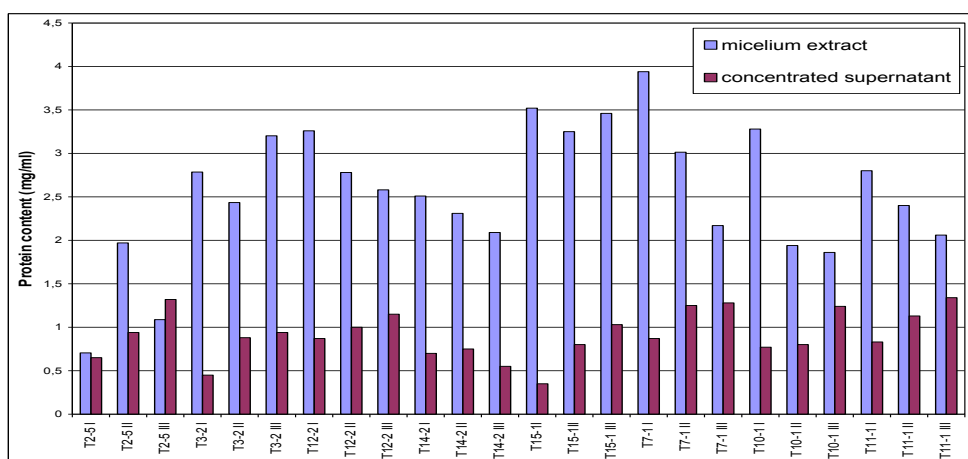
Figure 2

Total alkaloid content (mg/mL) of fungal aqueous extracts obtained from different strains of *Claviceps purpurea*

**Figure 3**

Glucan content (µg/mL) of fungal aqueous extracts obtained from different strains of *Claviceps purpurea*

In the initial step of the *in vitro* prescreening, on HeLa neoplastic cells cultures, we tested the reactivity of the cell protein biosynthesis, of the culture development process to the action of a large number (54) of autochthonous bioproducts derived from mycelium and supernatant of nine *Claviceps purpurea* hybride submerged cultivated strains.

**Figure 4**

Protein content (mg/mL) of fungal aqueous extracts obtained from different strains of *Claviceps purpurea*

The mycelium extracts and the concentrated supernatants of the T2-5, T11-2, T12-2, T14-2, T15-1, T7-1 strains, showed no cytostatic activity. They were also statistically non-significant ($p > 0.05$) in regard to the

development and protein biosynthesis alteration. Therefore we have excluded them from our sphere of interest. Thus, we selected only three hybride submerged strains (T 3-2, T 10-1 and T11-1) of *Claviceps purpurea* for study and we presented the experimental results regarding the interaction of their hyphal extracts and concentrated supernatants with some processes of the HeLa tumoral cells.

In vitro cytostatic potential of mycelium extracts (A) or concentrated supernatants (B), obtained from the T3-2, T10-1 and T11-1 strains of *Claviceps purpurea* at different ontogenetical stages (I - 6 days, II - 8 days, III - 12 days), was expressed as inhibition of the development of HeLa cell cultures, in comparison with the control cultures (Figure 5). High cytostatic activity was observed for the concentrated mycelian extracts of 12-days old T3-2 strain (of 72%) and T11-1 strain (64.2%). In the case of the T11-1 strain the magnitude of the negative impact on the cell cultures development was proportional with the age of the strains. After 6 days the inhibitory effect was reduced while after 12 days, it was registered the most significant inhibitory effect. In the case of the T3-2 strain, the similar amplitudes of the inhibitory effects suggested a constant level of extracellular cytostatic active compounds within the studied ontogenetic interval (6-12 days), which remained stationary because of their simultaneous synthesis and download in and from hyphal cells. The cell growth inhibition achieved with supernatants from three of the strains of *Claviceps purpurea* (T3-2, T10 and T11) was complementary to that obtained with the mycelium extracts.

The most active bioproducts were obtained from T3-2 strain mycelium in an ontogenetic time interval between 6 and 12 days, proving that the peak of maximum cellular synthesis of cytostatic biomolecules characterised the age of 12 days.

It can be concluded that the inhibitory effectiveness of our autochthonous fungal bioproducts upon development of the HeLa cancer cells cultures – evaluated in this paper only on the basis of the induced protein decline – was correlated with their source (*Claviceps purpurea* submerged hybride strain), its ontogenetic age, mycelian or supernatant origin of bioproduct, the strains biochemical and bioproductive particularities. Generally, we observed an inverse proportional relationship between the total ergoline alkaloids level and the effect intensity induced by the bioproducts separated from diverse *Claviceps purpurea* hybrid strains, cultivated in submerged conditions.

This fact suggests that the biochemical mechanism of pharmacodynamic effect of our fungal products is not exclusively of alkaloid-ergolinic nature, but it can be represented by other cell

biomolecules (glucans, proteins, etc.) which are present in the chemical composition of the tested mycelium extracts and supernatants.

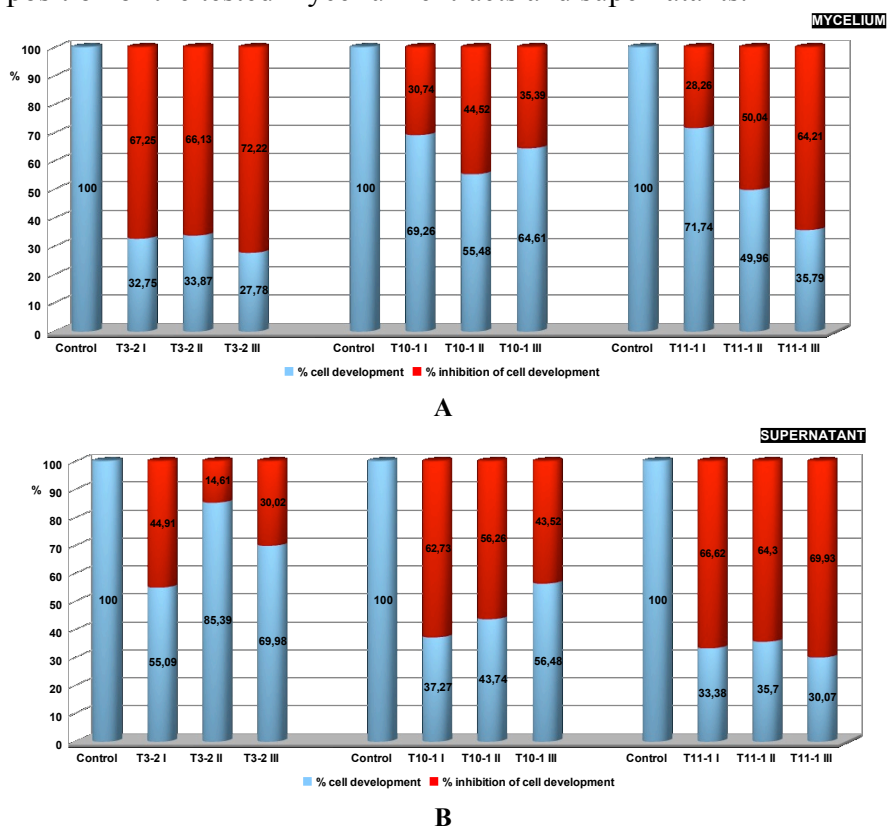


Figure 5

HeLa tumoral cell cultures development under *in vitro* treatment with 5 µg/mL ergoline compounds from mycelium extracts (A) and concentrated supernatants (B), obtained from *Claviceps purpurea* strains, and their corresponding cytostatic effect

Conclusions

The maximum cytostatic potential characterized the extract obtained from T3-2 III submerged strain mycelium and respectively T11-1 III supernatant, prelevated in the 12th day of *Claviceps purpurea* culture and from the 6 days old T10-1 strain supernatant. It is difficult, in this phase of the study, to indicate the exact biochemical mechanism responsible for the registered cytostatic effect of our fungal bioproducts, because both the mycelium extracts and supernatants contained ergoline alkaloids, glucans and proteins with potential cytostatic activity.

Acknowledgements

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