THE CYTOSTATIC POTENTIAL CONFIRMATION OF SOME FUNGAL AUTOCHTHONOUS BIOPREPARATIONS UPON HeLa NEOPLASTIC CELLS CULTURES

PINCU ROTINBERG[1*], COSMIN TEODOR MIHAI[1], DANIELA GHERGHEL[1], GABRIELA CAPRARU[1], DORINA IUREA[1], CRĂIȚA MARIA ROȘU[1], STEFANIA SURDU[1]

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Abstract: Some biopreparations of alkaloid-ergolinic nature have been extracted from hyphal and supernatant components, which were centrifugally separated from the submerged culture media of three strains of *Claviceps purpurea* (T1-3, T2-1 and T13-1), these being in different ontogenetic development stages (4, 6, 8, 10, and 12 days, respectively). *In vitro* testing of their interaction with cellular protein synthesis process of HeLa neoplastic cells cultures, has highlighted the cellular protein biosynthesis alteration, modifications of the protein dynamics sense and amplitude, as well as the cell cultures development inhibition. The protein synthesis inhibitory impact has confirmed the cytostatic action of these natural bioproducts, their cytostatic effectiveness being dependent of the *Claviceps purpurea* (T1-3, T2-1 and T13-1) strains specificity, the strain ontogenetic age, the biochemical nature of the intracellularly synthesized, stocked and extracellularly discharged substratum, as well as of their obtaining sources.

INTRODUCTION

At present, the antineoplastic chemotherapy still holds a priority in the fight against cancer disease, a real scourge of contemporary times. Although there has been continuous progress in cancer diagnosis and treatment as a result of recent discoveries in cellular, subcellular and molecular oncobiology, antineoplastic chemotherapy is still of little effectiveness, a fact explained especially by its negative impact on the normal cells of the organism under neoplasm aggression, and by the development of resistance phenomenon of the malignant cells to the cytostatic drugs action (DeVita et al, 1991; Klein et al, 1985; Stroescu, 1995; Weinhouse, 1980).

The augmentation of the oncochemotherapy effectiveness represents a major biomedical desideratum, which imposes the broadening of the chemopharmaceutical research~~s~~. The main directions of investigations are: the discovery and design of new oncolytic agents that should specifically target the tumoral cells; the identification of new therapeutic ways of action upon carcinogenesis process; the conceiving of new strategies and programs of anticancerous chemotherapy; the use of different drug monitored delivery and transport systems and the discovery of agents which can potentiate the antitumoral effect of the oncochemotherapeutic drugs (Adams, 2002; Anderson et al, 2002; Valeriote et al, 1984; Weinhouse, 1980; Calabresi and Parks, 1985; Chiricuta, 1988; Davey and Tudhope, 1983; Edwards, 1975; Klein and Klein, 1985; Seethala and Prabhavathi, 2001).

These major objectives can be performed only on the basis of the last information from molecular and cellular biology, structural and functional genomics, proteomics, and respectively, metabolomics, as well as pharmacogenomics and toxicogenomics (Anderson and Chiplin, 2002; Cook, 2002; Simson, 2001; Wong, 2002; Workman and Kaye, 2002;Adams, 2002; Cruce, 1999; Habeck, 2002; Karp, 1996; Lyden et al, 2001; Miron, 2000; Roobol et al, 1984; Stroescu, 1995).

Some natural ergolinic alkaloid structures and their semisynthetic derivatives have been mentioned as agents with numerous, diverse and important pharmacological properties, which are the expression of their interaction with the membranary and intracellularly receptors, as well as of their interference with cellular and molecular processes (Bruneton, 1995; Ciulei et al, 1993; Kren and Martinkova, 2001).

We have assumed that the alkaloid-ergolinic principles can be implied in the cytophysiological alteration of the tumoral cells, from where a new possibility of biomedical capitalization as possible antitumoral drugs will rise. On this hypothesis, foreseen a cytostatic action of these biosynthetic products. Previous *in vitro* research~~es~~ allowed the selection of some new autochthonous cytostatic agents, of alkaloid-ergolinic nature, obtained from either hybrided or modified parental strains of *Claviceps purpurea* (T1-3, T2-1 and T13-1) (Gherghel et al., 2004; Rotinberg et al., 2004).

In this context, our interest has been oriented towards two main new chemopharmaceutic investigation directions for the establishment of the optimal ontogenetic moment to capitalize the mycelium and/or culture medium of *Claviceps purpurea* microfungus for obtaining some biopreparations with superior cytostatic potential, as well as for specifying the molecular support from their biochemical composition responsible for the cytostatic property. Thus, the present paper includes the results of our investigations concerning the *in vitro* reactivity of HeLa cancerous cells proteinsynthesis and

development to the action of some biopreparations extracted and separated from hyphal and supernatant materials of different submerged strains of *Claviceps purpurea* (T1-3, T2-1, and T13-1) at different ontogenetic stages of development (4, 6, 8, 10, and respectively 12 days).

MATERIALS AND METHODS

In vitro testing of the cytostatic action on HeLa cell cultures included a series of coded hydrosoluble alkaloid-ergolinic extracts. The biopreparations were obtained from mycelium (MT1-3:1 days,MT1-3: 6 days, MT1-3:8 days, MT1-3:10days and MT1-3: 12 days: MT2-1:4 days, MT2-1:6 days, MT2-1:8 days, MT2-1:10 days and MT2-1:12 days; MT13-1:4 days, MT13-1: 6 days, MT13-1:8 days, MT13-1: 10 days and MT 13-1:12 days) and supernatants (ST1-3:4 days, ST1-3: 6 days, ST1-3:8 days, ST1-3:10 days and ST1-3: 12 days: ST2-1:4 days, ST2-1:6 days, ST2-1:8 days, ST2-1:10 days and ST2-1:12 days; ST13-1:4 days, ST13-1: 6 days, ST13-1:8 days, ST13-1: 10 days and ST13-1:12 days) separated from three strains (T1-3, T2-1 and T13-1) of *Claviceps purpurea* microfungus in different ontogenetic stages (4-12 days old).

The biological material used in the *in vitro* experiments, was represented by mycoplasm-negative negroid human cervix epitheloid carcinoma HeLa cells, which were cultured in DMEM medium (Dulbeco's Modified Essential Medium, Biochrom AG, Germany) supplemented with 10% fetal bovine serum, (Sigma, Germany), 100 µg/mL streptomycin (Biochrom AG, Germany), 100 IU/mL penicillin (Biochrom AG, Germany) and 50 µg/mL amphotericin B (Biochrom AG, Germany), at a density of 5 x 10^5 cells in 75 cm^2 flasks, in a humidified 5% CO_2 atmosphere at 37^o C.

When the cells reached confluence, they were detached from the flask with 0.25% trypsin + 0.02% EDTA (ethylenediaminetetraacetic acid, Biochrom AG, Germany) in the normal medium and then centrifuged at 1800 rpm for 2 min. The cells, at a density of 1 x 10^5 cells/mL, were seeded in the experimental tubes containing growth DMEM medium and were introduced at 37^o C. The culture medium of the 24 h cell cultures was changed either with a normal one (control cultures) or with one containing the alkaloid-ergolinic bioproducts, in a dose of 5 µg/mL (treated cultures).

After 24 and 48 hours of *in vitro* treatment, the medium was discarded from the test tubes, the cells monolayer was washed with PBS (saline phosphate buffer) and then subjected to the analysis methods for the evaluation of the total protein content (Lowry method modified by Oyama) and tracing of the protein dynamics (Oyama and Eagle, 1956). Also, the cellular cultures development degree after the action of the alkaloid-ergolinic bioproducts was evaluated, the inhibition of this last process expressing their cytostatic effect due to the inhibitory impact upon cell protein biosynthesis.

The cytostatic property signification of the studied biopreparations was appreciated on the basis of the American prescreening program, which imposed a minimum induced inhibitory impact of 50% for the *in vitro* selection of the potential antitumoral agents (Leiter et al, 1965). For each culture type and time interval, five culture tubes were used and the results were evaluated statistically by Student's test (Snedecor, 1968).

RESULTS AND DISCUSSIONS

In an adequate experimental model we have investigated the *in vitro* cytophysiological behavior of the tumoral cells cultures during their evolution up to 24, 48 and respectively 72 hours after the inoculation of the test tubes with HeLa type cells. The evaluation indices of the parental and daughter cell cultures reactivity to the chemical treatment were: the proteinsynthesis intensity; protein dynamics and the cell cultures development degree.

In a first series of tests, we have investigated the proteinsynthesis process of the HeLa cells cultures, expressed by the total proteins concentration and by the protein dynamics, the aim of the research being the highlighting and appraising of the proteinsynthesis inhibitory impact induced by the alkaloid-ergolinic extracts.

Generally speaking, we have observed at the untreated, control, HeLa neoplastic cells cultures, a progressive augmentation – with about 50 %– of the total protein content from 24 hours age up to 72 hours age. The successive graphical transposition of the protein values, obtained at different time intervals during the cultures evolution, traces the proteinosynthesis dynamics, which in the case of the control cell cultures, is characterized by an ascendant route with progressive increased amplitude. These characteristics of the untreated cell cultures are the expression of an inherent protenisynthesis enhancement, conditioned by the cell proliferation

process, which assures the normal development of the control cultures, which we consider as reference percentage value (100%).

The cultures incubated with the alkaloid-ergolinic extracts, as compared to the control values, were characterized by a progressive decrease of the protein concentrations, which sometimes attended the statistical significance both at 48 hours and at 72 hours after an incubation with the extracts for 24 and 48 hours.

Thus, it can be seen from Fig. 1, that the HeLa cell cultures evolution, incubated 24 and 48 h with MT1-3: 4 days and MT1-3: 6 days mycelian biopreparations, is characterized by significant reductions of the total protein concentrations, by negative modifications of sense and amplitude of protein dynamics route, these variations arguing the induction of a very significant proteinsynthesis inhibitory impact, which confirms the cytostatic property of these two bioproducts. Moderate alterations of the protein biosynthesis were registered during the 72 hours evolution of the HeLa cultures treated with MT1-3: 8, 10 and 12 days extracts, the quantitative and qualitative regressions of the protein content and protein dynamics highlighting a nonsignificant cytostatic effect of the latter.

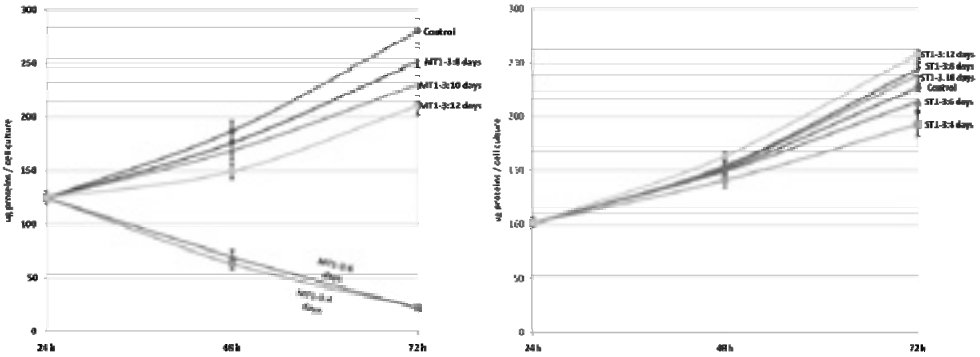

Fig. 1. The total protein contents (µg/culture) and proteinsynthesis dynamics of the HeLa cancerous cell cultures treated with the MT1-3 or ST1-3 biopreparations (in a dose of 5µg/mL), extracted from mycelia and supernatants of the different ages T1-3 *Claviceps purpurea* strain.

From the same figure, contrary to the MT1-3: 4 days and MT1-3: 6 days mycelian biopreparations, in the case of bioproducts extracted from the supernatants, separated by centrifugal removal of strain mycelium, a significant negative impact upon intracellular protein content of the HeLa tumor cell cultures can’t be distinguished, as compared to the control cultures. The MT1-3: 8, 10 and 12 days extracts, as well as all ST1-3 products are not characterized by a significant cytostatic property.

Therefore, we assist to an inhibition of the protein biosynthesis induced by some bioactive extracts. The amplitude of the proteinsynthesis inhibitory potential is dependent on alkaloid-ergolinic extract type. Thus, the MT1-3: 4 days and MT1-3: 6 days have caused a very profound diminution of the protein concentrations, while the impact of the others extracts (MT1-3: 8, 10 and 12 days extracts, as well as all ST1-3 products) upon the protein biogenesis of the HeLa cultures was much more attenuated.

The protein dynamics, in the case of the treated tumoral cell cultures evolution, is characterized by a descendent route and by a more or less significant decrease in amplitude, the most cytostatic active biopreparatins being cronologically: MT1-3: 4 days and MT1-3: 6 days.

In a second series of tests, we have investigated the proteinsynthesis process of the HeLa cells cultures, in the conditions of their *in vitro* treatment with others alkaloid –ergolinic biopreparations, which were obtained from hyphal and supernatant materials of different T2-1 strain ontogenetic ages (4, 6, 8, 10, and 12 days, respectively).

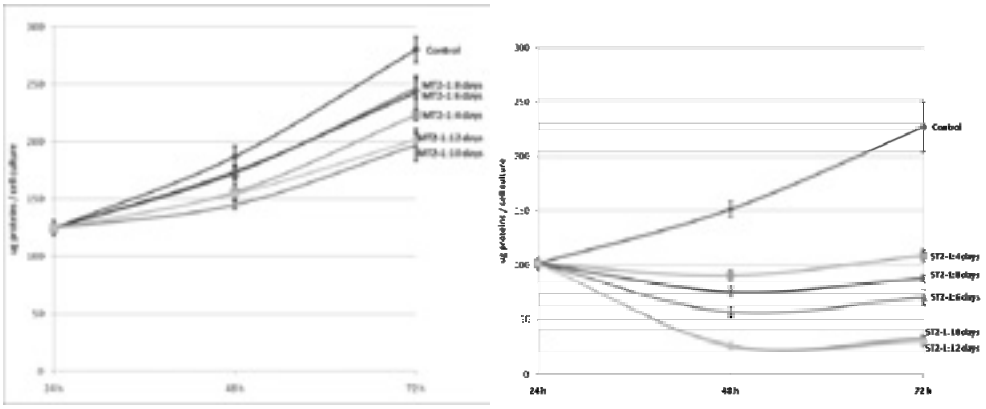

Fig.2. The interference of the MT2-1 and ST2-1 biopreparations (5µg/mL), obtained from hyphal and supernatant materials of different T2-1 strain ontogenetic ages (4, 6, 8, 10, and 12 days, respectively), with the cellular proteinsynthesis process of HeLa cells cultures.

In comparison with the control HeLa cellular cultures, the evolution of the treated cultures is characterized – at 48 and 72 hours ages – by lower total protein concentrations. These negative quantitative variations have different degrees, which are dependent on the product type used in cell treatments. Thus, it is observed from Fig.2, that the 24 and 48 hours treatments of the HeLa cellular cultures with the biopreparations obtained from the MT2-1 strain hyphal material of different ontogenetic ages have induced small amplitude modifications of the protein contents and of protein dynamics, these being non relevant in the frame of extracts evaluation as cytostatic agents, because their proteic perturbing impact (about 25%) is inferior to the minimum one imposed by the American prescreening program (50%).

The entire evolution of HeLa cultures, treated with the ST2-1 bioproducts – extracted from T2-1 strain postcentrifugal supernatants corresponding to the various ontogenetic development stages (4, 6, 8, 10, and 12 days, respectively) has highlighted statistic and cytostatic significant decreases of protein contents at all studied time intervals. This profound negative impact of bioproducts, with maximum expression at ST2-1: 10 days and ST2-1:12 days extracts, upon intracellular protein biogenesis, is also emphasized by the descendent route and very reduced amplitude of the proteinsynthesis dynamics (Fig. 2).

Finally, a last group of in vitro tested biopreparations has included some extracts obtained from hyphal and supernatant materials, which were centrifugally separated from T 13-1 *Claviceps purpurea* strain submerged cultures of different ontogenetic ages.

It is observed from Fig. 3, that the 24 and 48 hours treatments of the HeLa cellular cultures with the MT 13-1 and ST 13-1 biopreparations – corresponding to the various

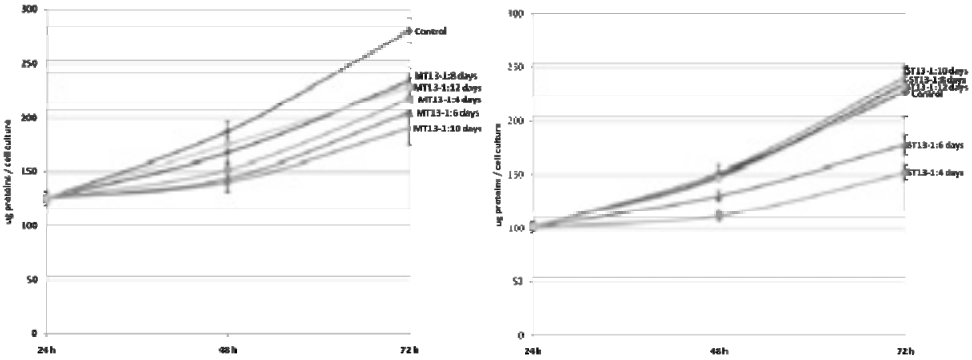

Fig.3. The modulation of HeLa neoplastic cells proteinsynthesis, expressed by cultures protein contents and protein dynamics, in the experimental conditions of the action of the MT13-1 and ST13-1 bioproducts (in a dose of 5µg/mL), extracted from the hyphal and supernatant materials of different old T13-1 *Clavicesps purpurea* strain.

ontogenetic development stages (4, 6, 8, 10, and 12 days, respectively) – have induced small amplitude modifications both for protein contents and for protein dynamics at all studied time intervals, these not being significant from cytostatic point of view, because their proteic perturbating impact (about 25%) is inferior to the minimum one imposed by the American prescreening program (50%) for the appreciation of a new drug as potential antitumoral agent.

Our bulk of results, registered on an adequate *in vitro* experimental model to the selection of new possible cytostatics, has highlighted that from the thirty tested extracts only 7 have confirmed the cytostatic property of the *Claviceps purpurea* ergolinic-alkaloids, that we previously characterized as potential antineoplastic agents (Gherghel et al., 2004; Rotinberg et al., 2004). These bioactive extracts were obtained from 4 and 6 days old T1-3 strain hyphal material, (MT1-3: 4 days and MT1-3: 6 days), as well as from 4, 6, 8,10 and 12 days, respectively, old T2-1 strain supernatants (ST2-1: 4 days, ST2-1: 6 days, ST2-1: 8 days, ST2-1: 10 days and ST2-1: 12 days), they profoundly perturbing the cellular proteinsynthesis process and probably, the cell proliferation.

The normal ongoing of both cellular processes consequently assures the cell cultures development. In this context, we performed another experimental model, which proposed to follow the impact of the selected cytostatic active extracts upon the HeLa cell cultures development level, the percentage values of this index being included in the next figure.

Thus, from Fig.4 it can be seen, that in comparison with the development degree of control cell cultures, which we considered as the 100% reference, the neoplastic cell cultures incubated with the MT1-3:4 days and MT1-3:6 days, as well as with ST2-1:4 days, ST2-1:6 days, ST2-1:8 days, ST2-1:10 days and ST2-1:12 days biopreparations, have revealed different degrees of development, the following maximum levels, at 72 hours of evolution, were registered: 7.9%, 7.8%, 47.8%, 31.1%, 38.6%, 14.4% and 13.4%, respectively. As compared to the 100% reference development value of the control

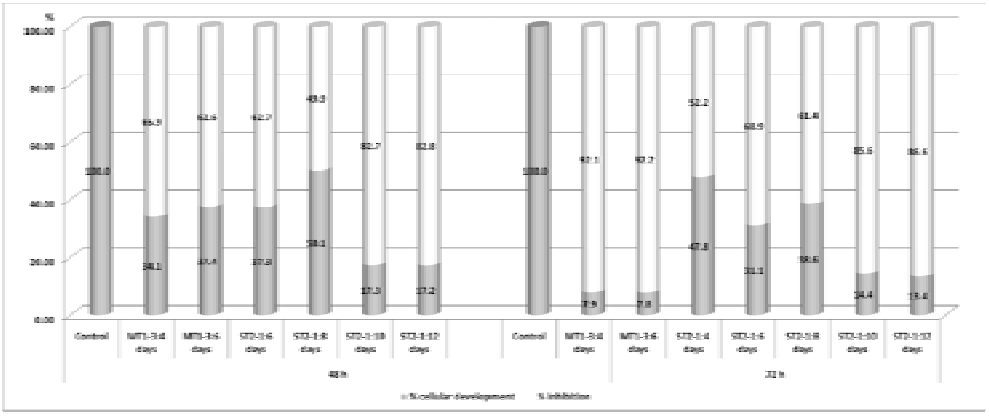

Fig. 4. The development degree of HeLa tumor cell cultures during their evolution, in the conditions of their incubation in the presence of the bioproducts, obtained from the T1-3 strain mycelium and T2-2 strain supernatant, by different ontogenetic ages, compared with the control cultures..

cultures, an inhibitory impact of the bioactive extracts upon the HeLa cultures development can be evidenced and assessed, its intensity being in consequent order: 92.1%, 92.2%, 52.2%, 68.9%, 61.4%, 85.6% and 86.6%, respectively.

Our *in vitro* testing of the effect of some bioactive fungal preparations upon the HeLa tumoral cells has highlighted a more or less pronounced negative effect induced by these bioactive products upon cell cultures development This effect of some extracts can be due to the inhibitory action upon cell proteinsynthesis process and probably upon cell mitosis.

According to the alteration degree of the cells protein biosynthesis and of cellular cultures development we can classify the seven extracts in two categories:

– the former group, including the mycelian preparations MT1-3: 4 days and MT1-3:6 days, was characterized by a very strong cytostatic potential (over 90%), which has surpassed the minimum 50% imposed level and

– the latter group, that includes the supernatant products ST2-1: 4, 6, 8, 10 and 12 days, respectively, which has conditioned a significant cytostatic effectiveness, in ascendent order from 52.2% to 86.6%, these values being also superior to the 50% minimum reference level imposed by the American prescreening program .

The noticeable differences between the cytostatic potential of all mentioned ergolinic-alkaloid biopreparations – extracted from the hyphal and supernatant material of those three *Claviceps pur*purea submerged strains – could be determined, on one hand, by a qualitative and/or quantitative compositional heterogeneity of these extracts, and on the other hand, by a different out-turn of alkaloidic bioproduction during *Claviceps purpurea* strains ontogenesis .Thus, micelian bioproducts isolated from young T1-3 strain (of 4 and 6 days) are similar to the supernatant ones separated from old T2-1 strain (of 10 and 12 days). Therefore, it seems that corresponding hyphal and supernatant biopreparations of the two strains resemble from point of view of their chemical composition, because both categories of extracts are characterized by a very high cytostatic effectiveness. In the first case, the molecular substratum responsible for this pharmacodynamic property still has (at 4 and 6 days ages of the T1-3 mycelia) intracellular location and in the second one, the biochemical molecules responsible for the bioactive effect have been extracellularly expelled (at 10 and 12 days ages of the T2-1 supernatants). These data

suggest that the bioactive molecules are synthesized in different moments of the ontogenetic evolution of those two alkaloids producers strains.

The analysis of the experimental results, obtained in the present study, highlights the perturbation of the neoplastic HeLa cells proteinsynthesis by the alkaloid-ergolinic biopreparations, obtained from mycelium or postcentrifugal culture medium of some strains of *Claviceps purpurea*. The proteinsynthesis inhibitory impact, induced by these bioactive extracts, consequently determines the inhibition of the cell culture development, which confirm their *in vitro* cytostatic property. The most pronounced cytostatic activity have shown the bioproducts resulted from T1-3 strain mycelium of 4 and 6 days – its cells being probably characterized, in early ontogenetic stages, by an intense cell biosynthesis of glucanic exopolysaccharides and ergolinic alkaloids precursor biomolecules, as well as the biopreparations obtained from T2-1 strain supernatant (especially ST2-1:10 days and ST2-1:12 days), they corresponding to a cell functional state characterized by a great alkaloid bioproduction and extracellular discharge, in the late ontogenetic stages.

The expression of the cytostatic action of some alkaloid-ergolinic bioproducts upon HeLa neoplastic cells, completes the data from the specialty literature, which signals a multitude of cellular, subcellular and molecular effects of these bioagents. They assure the *in vivo* global manifestation of their diverse pharmacodynamic actions (Aruoma, 2003, Jhon, 2003), our results being a scientific start point, wich supports a new possible medical capitalization of the ergolinic-alkaloid, from some *Claviceps purpurea* strains, as potential anticancerous drugs.

The cytostatic effectiveness of the MT1-3: 4 and 6 days or of the ST2-1 10 and 12 days alkaloidic biopreparations, extracted from *Claviceps purpurea* T1-3 andT2-1strains hyphal and supernatant materials, is closely dependent on the strains specificity, the strain ontogenetic age, the biochemical nature of the intracellularly synthetized , stocked and extracellularly discharged substratum, as well as on their obtaining sources. These explanations must be rigorously respected for obtaining a new oncochemotherapeutic agent which could interest the clinical trial.

CONCLUSIONS

In vitro investigation of the interaction of some alkaloid-ergolinic products, extracted from mycelium or supernatant of some *Claviceps purpurea* submerged strains, with HeLa cancerous cells cultures has highlighted, quantified and confirmed the cytostatic impact of the MT1-3:4 and 6 days or of the ST2-1 10 and 12 days alkaloidic biopreparations, obtained from *Claviceps purpurea* T1-3 andT2-1strains, expressed by the inhibition of proteinsynthesis and cell cultures development.

The amplitude of the cytostatic potential of these biopreparations is a dependent variable of: the strain specificity; its ontogenetic age; chemical structures responsible for the biologic active effect; source of their extraction.

The expression of the cytostatic impact of these four products imposes the testing of their action upon cell viability, the cytotoxicity being a component of the global pharmacodynamic antineoplastic effect.

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[1] Biological Research Institute, Bd. Carol I, 20A, 700505, Iaşi, Romania

[*] Corresponding Author: Rotinberg Pincu, pincu.rotinberg@uaic.ro