CELL PROLIFERATION AND VIABILITY OF HELA AND HEP-2P NEOPLASTIC CULTURES *IN VITRO* TREATED WITH NEW POLYPHENOLIC OR POLYSACCHARIDIC BIOPREPARATIONS OF VEGETABLE AND FUNGAL ORIGIN

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Abstract: The *in vitro* action of some new autochthonous polysaccharidic and polyphenolic biopreparations, specifically extracted from fungal and vegetable sources, upon the proliferation and viability of the HeLa and Hep-2p cancerous cells was investigated. The significant perturbation of the mitosis process of the tumoral cells, as well as the profound diminution of the cell viability of the neoplastic cell cultures argue the behaviour of some polysaccharidic and polyphenolic extracts as *in vitro* active cytostatic and cytotoxic agents. This primary characterization of some natural fungal or vegetable extracts as cytostatic and/or cytotoxic agents offers the informational background for the introduction of those biopreparations in the *in vivo* antitumoral screening program on different experimental tumoral systems.

INTRODUCTION

The morphological, structural, physiological, genetical, biochemical, biophysical and antigenic features of the tumoral cells – although assure yet their relative invulnerability – provide the numerous targets for chemotherapy, immunotherapy, genic therapy and biochemical therapy of the malignant diseases (Alison, 2003; Bast *et al.*, 2000; Miron, 2000; Owens, 2001; Ruddon, 2007).

Despite the fact that there has been continuous progress in cancer diagnosis and treatment as a result of recent discoveries in cellular and molecular oncobiology, structural and functional genomics, pharmacogenomics and toxicogenomics, proteomics and metabolomics, antineoplastic therapy is still of little effectiveness (Abrams, 2003; Bronchud, 2000; Lodish *et al.*, 2003; Lyden et al., 2001; Weinstein, 2001; Adams, 2002; Anderson et al., 2002; Habeck, 2002).

One of the most significant objectives of contemporary studies in pathology consists in improving the efficacy of means to control the carcinogenesis. In the fight against cancerous diseases, chemotherapy holds pride of place, but it is still of small effectiveness, a fact explained especially by its negative impact on the normal cells of the organism under neoplasm aggression and by the development of a resistance phenomenon of the tumoral cells to the cytostatic drugs action. Consequently, for the improvement of the oncochemotherapy there are necessary the extending and thoroughgoing researches for: 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 monithorized delivery and transport systems; the discovery of agents which can potentiate the antitumoral effect of the oncochemoterapeutic drugs (Devita *et al.*, 2001; Leiter et al., 1965; Stroescu, 1998; Weinstein, 2001).

Identification of some new substances with cytostatic action, preferentially towards the malignant cells and less upon the host's normal cells, represents, at present, a major concern in the general effort to struggle against the cancerous disease (Grunberger et al., 1988; Bradley, 2001; Lyden, 2001; Adams, 2002; Anderson&Chiplin, 2002; Habeck, 2002).

The identification of a new antitumoral agent and its introduction in clinical practice – the main purpose of the screening chemotherapeutic programs – are the result of some complex preclinical and clinical pharmacological investigations according to appropriate experimental patterns, which use various testing biological systems having different degrees of reactivity (Figg &McLeod, 2004; Leiter et al., 1965; Jungstandt et al., 1971; Boyd, 1989; Bissery and Chabot, 1991; Phillips et al., 1991; Seethala et al., 2001; Donger et al, 2002).

The multitude of biosynthesis, semisynthesis and synthesis substances with supposed antitumor action imposed in the last 20 years the introduction and intensive utilization of an *"in vitro"* test on neoplasic cell cultures of human origin as part of the screening programms meant for a preliminary selection of the potential cytostatic and/or cytotoxic drugs (Dold, 1988; Boyd, 1989; Hrushesky, 1990; Skehan et al., 1990).

In a previous research, we have evaluated the *"in vitro"* cytostatic action of some polysaccharidic or polyphenolic biopreparations, specifically extracted from *Pleurotus sp., Agaricus sp., Rosa canina, Hippophae rhamnoides* and *Vitis vinifera*, through the comparative analysis of the total protein levels, proteinsynthesis dynamics and

cell cultures development degree during the 24, 48 and 72 hours evolution of control and treated cell cultures. In comparison with the control cultures, the evolution of the treated ones was characterized, in the case of polyphenolic biopreparations, or not, in the case of the polysaccharidic extracts, by lower total protein concentrations, associated with alteration of the protein dynamics and with a significant inhibitory impact upon the HeLa cell culture development (Mihai et al., 2007).

In the present paper, we proposed to investigate the *in vitro* impact of these vegetable and fungal biopreparations of poliphenolic or polysaccharidic nature upon cellular proliferation and viability of HeLa and HEp-2p tumoral cells cultures, in order to enlarge the experimental data basis whis is necessary for their appreciation as cytostatic and/or cytotoxic agents.

MATERIAL AND METHODS

In vitro testing of the cytostatic and cytotoxic actions, on HeLa and HEp-2p cell cultures, included a series of hydrosoluble polysaccharidic and polyphenolic extracts, coded EPZArPlsp., EPZAcPlsp, EPZArAsp, EPZAcAsp, $EPFHA_{30}{}^{o}Rc$, $EPFHA_{30}{}^{o}Hr$, $EPFHA_{30}{}^{o}Vv$, $EPFHA_{50}{}^{o}Rc$, $EPFHA_{50}{}^{o}Hr$, $EPFHA_{50}{}^{o}Vv$.

The polysaccharidic agents were obtained by aqueous extraction, with cold (r) or hot (c) water, from the fungal species of *Pleurotus sp.* (EPZArPlsp., EPZAcPlsp) and *Agaricus sp.* (EPZArAsp, EPZAcAsp), meanwhile the polyphenolic biopreparations were specifically extracted from the bark's shrubs, through a hydroalcoholic extraction with 30^{o} or 50^{o} alcohol, of *Rosa canina* ($EPFHA_{30}{}^{o}Rc$, $EPFHA_{50}{}^{o}Rc$), *Hippophae rhamnoides* ($EPFHA_{30}{}^{o}Hr$, $EPFHA_{50}{}^{o}Hr$) and *Vitis vinifera* ($EPFHA_{30}{}^{o}Vv$, $EPFHA_{50}{}^{o}Vv$). The entire process of extraction and purification of the studied agents was performed at SC "PLANTAVOREL" SA, Piatra–Neamt.

The biological material used in the *in vitro* experiments, was represented by mycoplasm-negative negroid human cervix epitheliod carcinoma HeLa and by human laryngeal carcinoma HEp-2p cells, which were cultured in DMEM 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 $5x10^5$ cells in 75 cm^2 flasks, in a humidified 5% CO_2 atmosphere at 37ºC.

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 minutes. The cells were seeded at a density of $1x10^5$ cells / ml in the experimental tubes containing 2 ml of DMEM medium. The medium of the 24 hours cell cultures was changed either with a normal one (control cultures) or with one containing either the polysaccharidic agents or the polyphenolic extracts (treated cultures), in a dose of 1.5 mg/ml (Leiter et al., 1965; Doyle and Griffiths, 1998; Seethala et al., 2001).

After 48 hours of *in vitro* treatment, the medium was discarded from the test tubes, the layer of cells was washed with PBS and then subjected to the analysis methods for: the cytometrical assessment of the total cellular number with Türk haemocytometer after the formula: $N = n \times d \times 10^4$, where, N= total cellular number; n = number cells from a square of 1/25; d = dilution of 2; the mathematical estimation of the cell proliferation inhibition (% mitoinhibitory impact = Nt / Nm x 100, where: Nt = treated sample cells number; Nm = control sample cells number); the cytometrical assessment of the alive and dead cells number by trypan blue exclusion test (Doyle and Griffiths, 1998); the mathematical estimation of the cytotoxical degree (% cytotoxicity = Nctt – Ncvt / Nctm x 100, where: Nctt = treated cell total number; Ncvt = treated alive cells number; Nctm = control cells total number).

The cytostatic property of the studied biopreparations was considered on the basis of the American prescreening program, which imposed an induced minimum inhibitory impact of 50% for the *in vitro* selection of the potential antitumoral agents.

For each culture type and time interval five culture tubes were used and the results were evaluated statistically by Student's "t" test (Motulsk, 1995).

RESULTS AND DISCUSSIONS

In a first series of tests, we have investigated the proliferation process of the HEp-2p and HeLa cells cultures, expressed by the total cell number, the aim of the researches being of highlighting and appraisal of the mitoinhibitory impact induced by the polysaccharidic and polyphenolic extracts.

The mean values of the evaluation index of the cellular proliferation reactivity, registered at different ages of the control and treated cell cultures, are included in Table I.

In the case of control HeLa or HEp-2p neoplastic cell cultures, it can be observed a progressive augmentation of the total cell number from 24 hours age up to 48 hours age, which assures the normal development of this culture type. The cultures incubated with the

polysaccharidic or polyphenolic extracts, as compared to the control values, were characterized by a progressive decrease of the total cell number, which sometimes attended the statistical significance at 48 hours age, after a 24 hours incubation with extracts. Therefore, we assist at an inhibition of the cell proliferation induced by some bioactive extracts.

The amplitude of the mitoinhibitory potential is correlated to the used polysaccharidic or polyphenolic extracts and to the used cell cultures. Thus, upon the HeLa cell cultures, the polysaccharidic bioproducts have caused a diminution of the proliferation only with 26.7% (EPZArAsp), 2.1% (EPZAcAsp), 5.99% (EPZAcPlsp), while the EPZArPlsp conducted to a stimulation of the proliferative process with 5%. Instead, the reactivity of the HEp-2p cell cultures to the action of the polysaccharidic extracts is different, the antiproliferative impact being higher. Thus, EPZArAsp has determined a diminution of proliferation rate of 77.0%, EPZAcAsp of 77.9%, EPZArPlsp of 82.0% and EPZAcPlsp of 75.0%, these values being significantly cytostatic and statistical.

Table I The antiproliferative effect of the polysaccharidic and polyphenolic agents induced upon the treated HEp-2p and HeLa cells cultures and expressed by the total cell number (n x 10^5) decreases. Figures in brackets indicate the number of experimental cultures for each type.

Experimental group	**HeLa**				**HEp-2p**			
	X ± ES	p	% Proliferation rate	% antiproliferative degree	**X ± ES**	p	% Proliferation rate	% Antiproliferative degree
Control 24 h	0,1630 ± 0,02 (5)	–	–	–	0,2240 ± 0,03 (5)	–	–	–
Control 48 h	0,3330 ± 0,03 (5)	–	100,00	0,00	0,3180 ±0,02 (5)	–	100,00	0,00
EPZArAsp	0,2440 ± 0,02 (5)	<0,05	73,27	26,73	0,0731 ± 0,05 (5)	<0,001	22,99	77,01
EPZAcAsp	0,3260 ± 0,03 (5)	NS	97,90	2,10	0,0700 ± 0,02 (5)	<0,001	22,01	77,99
Control 24 h	0,1481 ± 0,02 (5)	–	–	–	0,1450 ± 0,003 (5)	–	–	–
Control 48 h	0,3020 ± 0,04 (5)	–	100,00	0,00	0,2530 ± 0,002 (5)	–	100,00	0,00
EPZArPlsp	0,3171 ± 0,02 (5)	NS	105,00	0,00	0,0455 ± 0,004 (5)	<0,001	17,98	82,02
EPZAcPlsp	0,2839 ± 0,01 (5)	NS	94,01	5,99	0,0632 ± 0,007 (5)	<0,001	24,98	75,02
Control 24 h	0,1586 ± 0,04 (5)	–	–	–	0,1648 ± 0,01 (5)	–	–	–
Control 48 h	0,3185 ± 0,03 (5)	–	100,00	0,00	0,2558 ± 0,04 (5)	–	100,00	0,00
EPFHA30ºRc	0,0480 ± 0,02 (5)	<0,001	15,07	84,93	0,0357 ± 0,07 (5)	<0,02	13,96	86,04
EPFHA30ºHr	0,0733 ± 0,004 (5)	<0,001	23,01	76,99	0,0741 ± 0,03 (5)	<0,002	28,97	71,03
EPFHA30ºVv	0,1369 ± 0,01 (5)	<0,001	42,98	57,02	0,1202 ± 0,01 (5)	<0,01	46,99	53,01
Control 24 h	0,1686 ± 0,005 (5)	–	–	–	0,1718 ± 0,02 (5)	–	–	–
Control 48 h	0,3295 ± 0,002 (5)	–	100,00	0,00	0,2628 ± 0,02 (5)	–	100,00	0,00
EPFHA50ºRc	0,0560 ± 0,001 (5)	<0,001	17,00	83,00	0,0288 ± 0,03 (5)	<0,001	10,96	89,04
EPFHA50ºHr	0,1251 ± 0,002 (5)	<0,001	37,97	62,03	0,063 ± 0,003 (5)	<0,001	23,97	76,03
EPFHA50ºVv	0,1977 ± 0,003 (5)	<0,001	60,00	40,00	0,1130 ± 0,01 (5)	<0,001	43,00	57,00

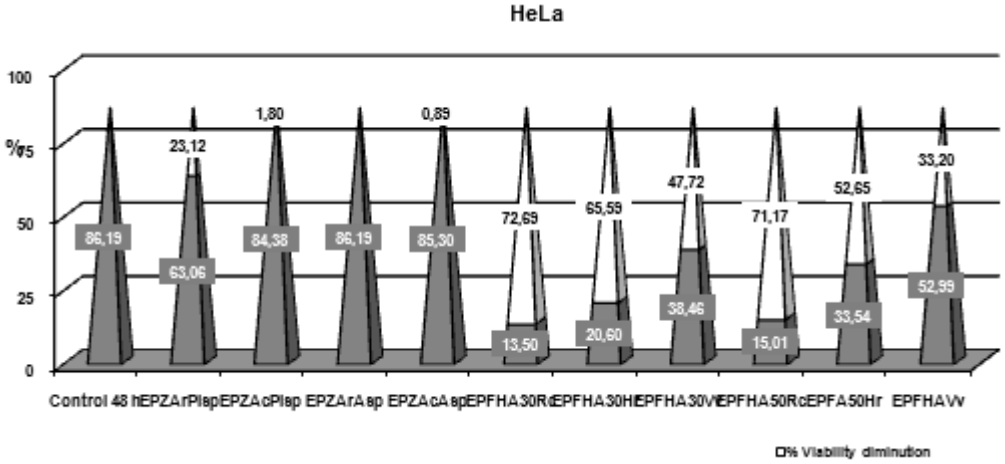
The impact of the polyphenolic extracts upon the Hela or HEp-2p cell cultures is characterized by a very profound diminution of the total cell number, as compared to the cell number of the control cultures. Between that two lines of cell cultures are not evidenced significant differences in reactivity. In the case of the polyphenolic extracts, obtained by extraction with hydroalcohol 30º, the mitoinhibitory impact upon HeLa cell

cultures was of 84.9%, for EPFHA30^{o}Rc, of 76.9%, for EPFHA30^{o}Hr and of 57.0%, for EPFHA30^{o}Vv, while the antiproliferative impact upon HEp-2p cells was about of 86.0%, 71.0% and 53.0%. The polyphenolic agents, extracted with hydroalcohol 50^{o}, have determined a reduction of the proliferative process with 83.0% (EPFHA50^{o}Rc), 62.0% (EPFHA50^{o}Hr) and 40.0% (EPFHA50^{o}Vv), in the case of HeLa cultures, and with 89.0% (EPFHA50^{o}Rc), 76.0% (EPFHA50^{o}Hr) and 57.0% (EPFHA50^{o}Vv), in the case of HEp-2p cells. Unlike the polysaccharidic extracts, the polyphenolic ones have induced similar effects upon the cell cultures used, the amplitude of the mitoinhibitory impact being slight different from a bioproduct to other. Also, the extraction method, with alcohol of 30^{o} or 50^{o}, didn't significantly influence the amplitude of the antiproliferative impact.

In the next *in vitro* experimental model, was followed the action of the fungal polysaccharidic agents and of the vegetable polyphenolic extracts upon cell viability, expressed by numerical differences between alive and dead cells, them visualization being performed with trypan blue stain. The obtained results are summarized in Figure 1. The comparative analysis of the alive and dead cells number from the HeLa cell cultures, control and respectively treated for 24 hours with polysaccharidic or polyphenolic extracts, in a dose of 1.5 mg/ml, reveals a different behavior. Thus, the control cultures are characterized by a greater number of alive cells than dead ones, while the treated cultures have presented a bigger number of dead cells than alive ones. Like in the case of the proliferation process, we can observe the same action patterns of the studied agents.

So, the polysaccharidic extracts have differently action upon the two cell cultures:

- upon the HeLa cells the cytotoxic effect was very weak (23.1%, 1.8% or 0.9% in the case of the EPZArPlsp, EPZAcPlsp or EPZAcAsp) or null (EPZArAsp);



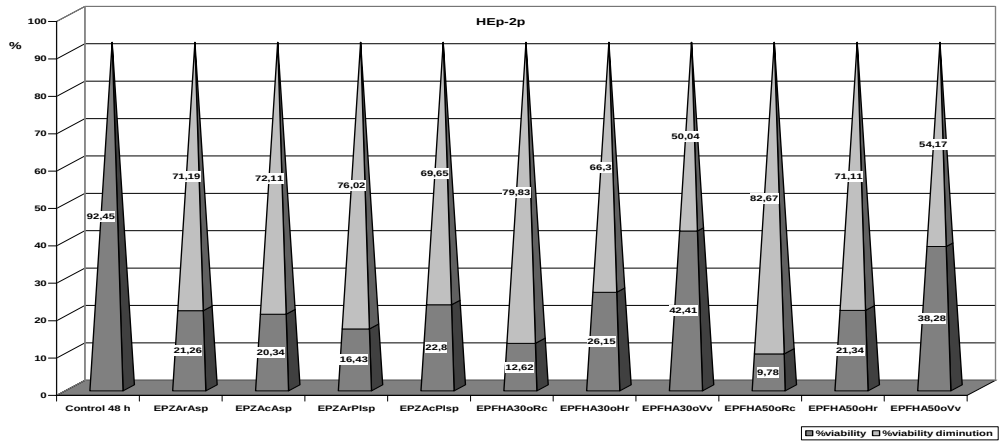


Figure 1 The tumoral cell cultures viability and the cytotoxic impact of the polysaccharides or polyphenols extracts.

- upon HEp-2p cells the polysaccharidic agents have a pronounced cytotoxic effect, the degree of viability diminution reaching procentual values of 71.2% (EPZArPlsp), 72.1% (EPZAcPlsp), 76.0% (EPZArAsp) and 69.6% (EPZAcAsp).

The level of the viability regression of the tumoral cells submitted to the polyphenolic treatment has attended values of 72.7%, 65.6% and respectively 47.7% or of 79.8%, 66.3% and respectively 50.0% in the case of the biopreparations obtained, with hydroalcohol 30^o, from the *Rosa canina*, *Hypophae rhamnoides* and respectively *Vitis vinifera* and used upon HeLa or HEp-2p cell cultures. In the case of the HeLa or HEp-2p cell cultures treatment with the same polyphenolic agents, but obtained after utilization of the hydroalcohol 50^o, the values of the cytotoxic impact were: 71.2% or 82.7% (EPFHA50^{o}Rc), 52.7% or 71.1% (EPFHA50^{o}Hr) and 33.2% or 54.2% (EPFHA50^{o}Vv), values which were similar with those obtained in the first case.

The present paper, through the proposed targets, the *in vitro* used experimental models and the registered results aims to identify new chemical agents with cytostatic and cytotoxic action, which, finally, will enrich the oncochemotherapeutic arsenal.

Our focus on the polysaccharidic and polyphenolic compounds, obtained from fungal or vegetable sources, is justified by the abundance of informations about their cellular, subcellular and molecular actions, which are materialized by bactericide, virulicide, fungicide, antiseptic, antibiotic, antianemic, antiinflamatory, cardiovascular, immunomodulatory and even antitumoral pharmacological effects of these natural biomolecules.

Conferring the attribute of antitumoral agent to a new chemical product is the result of a complex, multidirectional and multistage process of investigation on various and adequate experimental models, represented by testing biological systems with different organizational levels and specific reactive capacities (Jungstand et al., 1971; Cook, 2002; Crouch&Slater, 2001; Hrushesky, 1990; Philips et al., 1990; Abrams, 2003; Bronchud, 2000; Devita *et al.*, 2001; DiPiro *et al.*, 2005; Figg &McLeod, 2004).

The methodology and experimental protocol applied in our study correspond to the chemotherapeutical prescreening program on cancer cells cultures, elaborated by the National Institute for Cancer Chemotherapy of USA for the selection of some possible active

cancerostatic agents. For a first step of investigation, the reference program imposes the induction of a minimum mitoinhibitory impact or a minimum cell viability decrease, upon the cancerous cell cultures, by the tested product of at least 50%, for it to be considered a potential cytostatic or cytotoxic agent (Leiter et al., 1965; Riddell et al., 1986; Dold, 1988; Boyd, 1989; Philips et al., 1990; Figg &McLeod, 2004).

Our *in vitro* testing of the effect of some new fungal polysaccharidic or vegetable polyphenolic preparations upon the HeLa and HEp-2p tumoral cells has highlighted a more or less pronounced negative impact induced by these bioactive natural products.

Thus, the polysaccharidic agents have showed a dual pattern of action, dependent by the used tumoral cell line. In the case of the HeLa cells, the mitoinhibitory and the cytotoxic impact were insignificantly from cytostatic and statistic point of view. Not the same situation was registered when the polysaccharidic treatment was applied to the HEp-2p cell cultures, the diminution of the total cell number and of the cell viability being very pronounced and, also, significant cytostatically and statistically. The specific reactivity of these two cancerous cell lines may be due to the different origins of cells and to their membranary permeability. Probably, the membrane of the HeLa cells is not very permeable for the polysaccharidic macromolecules or it is deprived by specific receptors for this type of biomolecules.

The negative impact of the polyphenolic extracts upon the HeLa and HEp-2p tumoral cells proliferation and viability is characterized by similarity, in both cases the effects being significantly cytostatic and statistical. We can, also, perform a hierarchy of the polyphenolic biopreparations from the point of view of their mitoinhibitory and cytotoxicity effectiveness: on the first position is the extract obtained from *Rosa canina*; on the second position is the biopreparation from *Hipophae rhamnoides* and on the last place is the extract from *Vitis vinifera*. In relation to the cytostatic and cytotoxic potential of the mentioned polyphenolic biopreparations, the signaled differences could be explained, on one hand, by their qualitative and/or quantitative compositional heterogeneity and on the other hand, by the different outturn of vegetable species in the polyphenols bioproduction.

The analysis of our experimental results, obtained in the present study, highlights the perturbation of the HeLa and HEp-2p neoplastic cells proliferation and viability by the biopreparations, extracted from fungal and vegetal sources.

CONCLUSIONS

The expression of the mitoinhibitory effect and cell viability decrease impact after the in vitro treatment of the tumoral cells – together with the profound perturbation of proteinbiosynthesis and of cell culture development - allow us to characterize the original, autochthonous polysaccharidic and polyphenolic biopreparations as potential cytostatic and cytotoxic agents.

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