NEW POTENTIAL ANTITUMORAL AGENTS OF POLYPHENOLIC NATURE OBTAINED FROM *HELLEBORUS PURPURASCENS* BY MEMBRANARY MICRO- AND ULTRAFILTRATION TECHNIQUES

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Abstract: The *in vitro* action of some total aqueous and hydroalcoholic extracts and of their concentrate or permeate fractions, separated by membranary micro- and ultrafiltrations of the primary homogenates, obtained from *Helleborus purpurascens* ethnomedicinal plant, upon the cell protein biosynthesis, proliferation, viability and development of the HeLa cancerous cells cultures was investigated. The significant proteinsynthesis alteration, protein dynamics modification, decrease of total cell number, cell viability diminution, inhibitory impact upon the cell cultures development, during studied evolution period, suggest the behaviour of these polyphenolic hellebore extracts as *in vitro* active cytostatic and cytotoxic agents. Our preliminary characterization of these vegetal biopreparations as protein, mitotic, growth inhibitors offers the informational background for further investigations, on many other cancerous and normal cell lines and adequate experimental models to *in vitro* prescreening, as well as for their introduction in the in *vivo* antitumoral screening program on different experimental tumoral systems.

INTRODUCTION

From the ancient times, using plants as a source of remedies to treat many diseases has captured the attention of the people. With the progress in chemistry, systematic studies have been conducted to identify bioactive compounds in plant extracts and to evaluate their biological activity. Polyphenols, as bioactive constituents, show numerous pharmacological effects (anti-inflammatory, antioxidant, antibacterial, anti-estrogenic, immunomodulatory, neurogenic etc.).

The genus *Helleborus* (Ranunculaceae) includes approximately 20 species of herbaceous, perennial rhizomatous plants, widely spreaded in Europe and Asia (Ardelean and Mohan, 2008; Watanabe et al., 2003). Hellebore extracts have been extensively investigated for their biological activities (Rosselli et al., 2009), conferred by the biologically active constituents (bufadienolides, saponosides; ecdysteroids, protoanemonin) (Stanescu et al., 2004) which allow their use in medicine, in the treatment of urologic and rheumatic diseases, several tumor types and hematological malignancies (Horstmann et al., 2008; Lupu et al., 2009; Yang et al., 2010). An original medicine utilizing purified *Helleborus* extracts, prepared in Romania and registered as BOICIL in USA (Kerek, 1981, US Patent) proved antalgic and antirrheumatic activity, and MCS-18 compound, isolated from *H. purpurascens*, has beneficial effects in inflammatory and autoimmune disorders by attenuating antibody production (Kerek et al., 2008; Littmann et al., 2008). The antiinflammatory action is attributed to steroidal constituents (Lacaille-Dubois and Wagner, 2000).

Although continuous progresses are registered in cancer prophylaxis, diagnosis and treatment, the neoplastic disease still holds pride of place in contemporary pathology. The antineoplastic chemotherapy is still characterized, by a relatively low effectiveness (De Vita, 2004; Stroescu, 1998), fact which explains the major significance given to the oncobiologic research, oriented towards optimizing its efficiency, by discovery of new oncochemotherapeutic medicines with preferential action upon cancerous cells and lower impact upon health ones (Miron, 2000; Owens, 2001). In recent years a lot of studies are focused on anticancer, immunomodulating, cytotoxic and antioxidant activities of *Helleborus* extracts (Rosselli et al., 2009; Paun-Roman, 2010), but there are some contradictions and debates relative to this issue.

An attractive source of new cytostatic agents is *H. purpurascens*, which have a high content of biologically active polyphenolic compounds. Therefore, we have considered opportune to obtain some total polyphenolic extracts or their fractions from *H. purpurascens*, as well as to investigate their antineoplastic capacity. The aim of present paper is to emphasize on the modern technology’s applications for the *H. purpurascens* extracts processing by a multi-stages membranary micro- and ultrafiltrations cascade, to obtain some natural biopreparations and to investigate, in an *in vitro* preliminary screening on the HeLa neoplastic cells, their impact upon the cell proteinsynthesis, proliferation, viability and development degree of cell cultures, in view of highlighting and evaluating their cytostatic and cytotoxic properties.

MATERIALS AND METHODS

16 natural polyphenolic biopreparations of vegetal origin were performed from diverse anatomical components (roots and rhizomes) of *Helleborus purpurascens* medicinal plant. The roots and rhizomes were dried, homogenized and grounded to a fine powder, using the GRINDOMIX GM200 mill; the extracts were prepared by maceration in cold distillate water or in aqueous: alcoholic (ethanol) mixtures 50% v/v , as solvents. The contact time between the plant and the solvent was maintained of 24 h for aqueous extracts and 7 days for hydro-alcoholic extracts, extracts have been sporadic, mechanically stirring, working temperature (20^{o}C). The herbal's mass concentration in the solvent was of 6% (w/v) for aqueous and hydroalcoholic extracts. After filtering the initial extracts through Isolab quantitative filter paper "medium" for removing the coarse suspensions and sterilization, each filtrate was processed by microfiltration (MF), using Millipore membranes with 0.45 μm pores, to remove the impurities. Microfiltration (MF) process is meant to perform feed clarification and sterilization. These steps were followed by a four-stages ultrafiltration cascade, using four ultrafiltration plane membrane types from regenerated cellulose (Millipore) with cut-off 30,000 MWCO (UF1), 10,000 MWCO (UF2), 3,000 MWCO (UF3) and 1,000 MWCO (UF4). The permeate obtained from UF1 was introduced into the cross-flow circuits for UF2 and then the resulted permeate from UF2 or UF3 was eventually introduced into the cross-flow circuits for UF3 or UF4. A KMS Laboratory Cell CF-1 installation purchased from Koch Membrane (Germany) firm was used for both MF and UF.

The concentration ratio in ultrafiltration processes (expressed as permeate and concentrate volume ratio) were of 2:1. All ultrafiltration experiments – meant for concentration of rejected solutes and fractionation of solutes – were carried out at room temperature (cca. 23 C).

The total polyphenols (PT) were assessed in the hydrous and alcoholic total vegetal extracts or in the separated concentrate and permeate fractions through the Singleton and Rossi' s spectrophotomerical method (36, 40), the concentrations being expressed in equivalents galic acid/ L (GAE/L) compared to the standard curve with galic acid.

Cell oncobiology experimental protocol.

In vitro testing of the cytostatic and cytotoxic actions, on cancerous cell cultures, has included a series of total and fractionated, hydrous and hydroalcoholic polyphenolic extracts: **HphE** (*H. purpurascens* total hydrous extract); **0.45 μm MF-HphE (**microfiltrate of **HphE**); **30,000Da UF1C-HpE** (concentrate of first ultrafiltrate); **30,000Da UF1P-HphE** (permeate of first ultrafiltrate); **10,000Da UF2C-HphE** (concentrate of second ultrafiltrate); **10,000Da UF2P-HphE** (permeate of second ultrafiltrate); **3,000Da UF3C- HphE** (concentrate of third ultrafiltrate); **3,000Da UF3P-HphE** (permeate of third ultrafiltrate); **1,000DaUF4C- HphE** (concentrate of fourth ultrafiltrate); **1,000Da UF4P-HphE** (permeate of fourth ultrafiltrate); **HphaE** (*H. purpurascens* total hydroalcoholic extract); **0.45 μm MF-HphaE (**microfiltrate of **HphaE**); **30.000Da UF1C-HphaE** (concentrate of first ultrafiltrate); **30,000Da UF1P-HphaE** (permeate of first ultrafiltrate); **3,000Da UF2C-HphaE** (concentrate of second ultrafiltrate); 3**,000Da UF2P-HphaE** (permeate of second ultrafiltrate).

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% CO2 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 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) or with one containing the hydrous or hydroalcoholic polyphenolic extracts, in a dose of 5 μg/mL (treated cultures).

After 24 and 48 h of *in vitro* treatment, the medium was discarded from the test tubes, the layer of cells was washed with PBS (salin phosphate buffer) and then subjected to the analysis methods for: the evaluation of the total protein content using Lowry method modified by Oyama (Oyama et al., 1956) and tracing of the protein dynamics; the cytometrical assessment of the total cell number with Türk haemocytometer on the basis of the formula: N = n x d . 104, 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 et al., 1998); the mathematical estimation of the % cytotoxicity = Ncmt / Nctt x 100,where: Ncmt = living cells number of the treated sample; Nctt = total cells number of the treated sample (Doyle et al., 1998); mathematical evaluation of cell cultures degree after the action of the hellebore extracts, the inhibition of this last process representing their cytostatic effect upon cell protein biosynthesis and cell mitosis, as well as their cytotoxic action upon cell viability.

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 (Cann, 2002).

RESULTS AND DISCUSSIONS

The *in vitro* action of some total aqueous and hydroalcoholic polyphenolic extracts and of their concentrate or permeate fractions, separated by membranary micro- and ultrafiltrations of the primary homogenates, obtained from *Helleborus purpurascens* ethnomedicinal plant, upon the cell protein biosynthesis, proliferation, viability and development of the HeLa cancerous cells was investigated in the present work.

Thus, in a first experimental model, the reactivity profile of the cell proteinsynthesis process of the HeLa tumoral cells cultures was outlined by the registered total protein concentrations and dynamics during their evolution period in the presence of the tested biopreparations

It can be seen, in figure 1, that the control group presents a 50% progressive augmentation of the total protein content in untreated HeLa neoplastic cells cultures, from 24 hours age up to 72 hours age. The protein dynamics of the untreated tumoral cell cultures has presented an ascendant route with increasing amplitude These characteristics of the untreated cell cultures are the expression of an inherent proteinsynthesis enhancement, conditioned by the cell proliferation process, which assures a double number of functional cells and, respectively, the normal development of the control cultures, appreciated by us as reference percentage value (100%).

In all ten variants, the cultures incubated with the total hydrous polyphenolic extract or with its derivated fractions have presented lower protein concentrations than control values, which attended the statistical and cytostatical significance both at 48 hours and at 72 hours, after 24 and 48 hours treatments with the bioactive preparations. Therefore, we assist at decline of the cell proteinbiosynthesis, induced by the tested agents. The proteinsynthesis inhibitory impact of the diverse bioproducts is characterized by different manifestation degrees, its intensity depending both on the separation mode (micro- or ultrafiltration) of samples and the fractionated type (concentrate or permeate).

The UF1P-HphE polyphenolic fraction has determined the biggest reductions of the total protein concentrations (19.79 and 10.91 μg protein/culture, respectively) to the 48 and 72 hours old HeLa cultures, treated 24 and 48 hours with this agent. The impact of the others polyphenolic samples (HphE, MF-HphE, UF1C- HphE, UF2C- HphE, UF2P- HphE, UF3 C and P- HphE, UF4 C and P- HphE) upon the protein biogenesis of the HeLa cultures was more attenuated, but still significant. The successive graphical transposition of the total proteins value, obtained at different time intervals of treated cell cultures evolution, traces their proteinsynthesis dynamics. Contrary to the controls, in the case of the treated cancerous cultures, it is observed that the protein dynamics is characterized by a descendent route and by a decrease amplitude, the most visible modifications being cronologically registered in the case of UF1P-HphE, UF2C- HphE, UF2P, MF-HphE, UF4 C and P- HphE, UF3 C and P- HphE and UF1C- HphE biopreparations.

The negative changes of the cell protein contents and dynamics, have demonstrated the induction of a very significant proteinsynthesis inhibitory impact, which confirms the cytostatic property of these bioproducts. The total protein content in the HeLa cells incubated with total hydrous extract (HphE variant) was similar both after 24 and 48 hours treatment indicating a blocking of protein biosynthesis process in a short period after incubation. This result is consistent with data from literature according to which 2 hours of contact between cell cultures and polyphenols, such as gallic acid, are sufficient to induce apoptosis (Inoue et al., 1994).

Significant alterations of the protein biosynthesis were also registered during the 48 and 72

hours evolution of the HeLa cultures treated either with a total hydroalcoholic extract or with its permeate and concentrate derivated fractions. These modifications are suggested by the negative quantitative variations, which have different degrees of expression, the amplitude of the cytostatic potential being dependently on the bioproduct type used in cell treatments.

During the entire investigated time interval (72 h), the cell cultures treated with different hydroalcoholic polyphenolic preparations, as compared to the control values, were characterized by significantly smaller protein contents (μg protein/culture), confirming the perturbation of the tumoral cells protein biogenesis.

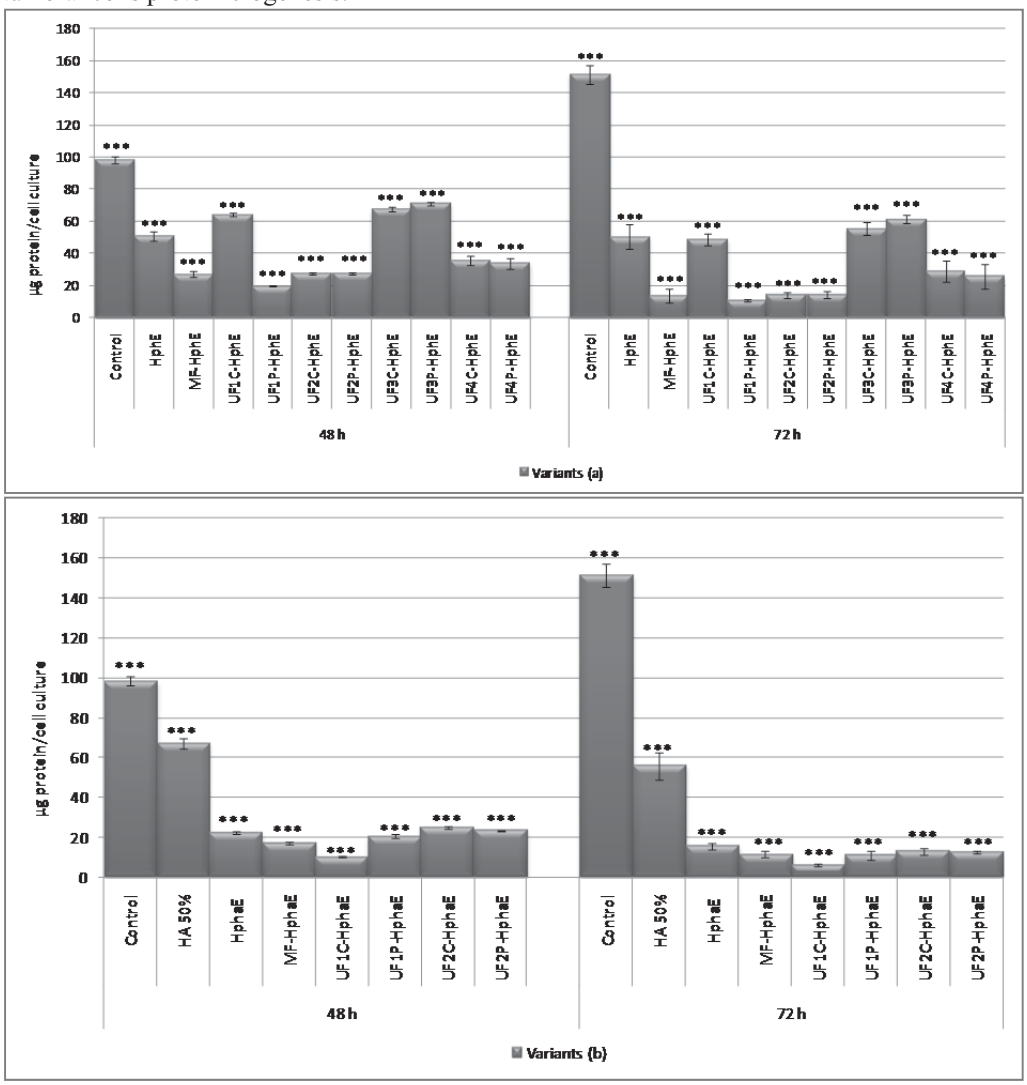

Fig. 1. The total protein content (μg/culture) and proteinsynthesis dynamics of the HeLa cancerous cell cultures treated with total hydrous (a) and hydroalcoholic (b) polyphenolic extracts from *Helleborus purpurascens* and their concentrate or permeate fractions (15μg/mL), during a 72 hours of evolution. Significantly different from control: *p<0.05, **p<0.01, ***p<0.001

This profound negative impact of hydroalcoholic bioproducts upon intracellular protein biogenesis, with maximum expression at UF1C-HphaE extract – and not UF1P-HphE – is also outlined by the descendent route and very low amplitude of the proteinsynthesis dynamics. The significant cytostatic potential of all hydroalcoholic polyphenolic products seems to be somewhat higher than that of hydrous samples, this difference being attributed to the extractive agent (HA 50%), which has himself a cytotoxic impact.

In a new series of tests, we have investigated the reactivity of HeLa cells' proliferation process, expressed by the total cell number evolution during the development time of treated cultures, which has revealed the sense and extent of the bioactive extracts' interference with this cellular process. The mean values of the evaluation index of the cell mitosis, registered at different ages of the control and treated cell cultures, are included in Table 1.

In the case of control HeLa 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. Instead, the 48 hours old HeLa cell cultures, treated 24 hours with the hydrous hellebore extracts, are characterized by a decrease of the total cells number, comparatively with the corresponding control value. This numerical decline signals a regression of the cellular proliferation rate – due to the hellebore extracts – which expresses the mitoinhibitory property of these bioproducts, their potential being variable from one preparation to another. Although all treated HeLa cultures, with different hydrous extracts, have presented statistic and cytostatic significant decreases of total cells number, six extracts (MF- HphE, UF1P-HphE, UF2C-HphE, UF2P-HphE, UF4C-HphE and UF4P-HphE) have been characterized by an antiproliferiative effect of 61.05 - 76.29%, these values being superior to the 50% minimum reference level imposed by the American prescreening program for the appreciation of a new drug as potential antitumoral agent.

Table 1. The total cells number variation and cellular proliferation reactivity of the HeLa neoplastic cells incubated with the hydrous and hydroalcoholic biopreparations (in a dose of 15 μg/mL), obtained from *Helleborus purpurascens*. In brackets is indicated the number of experimental cultures for each type

Variant	24 h	48 h		% Proliferation rate	% Antiproliferative degree
	X●10^3 ± ES	X●10^3 ± ES	p		
Hydrous extracts					
Control	152.34±2.3(5)	358.98±11.23 (5)	–	100	-
HphE	152.34±2.35 (5)	194.25±10.10 (5)	<0.001	54.11	45.89
MF-HphE	152.34±2.35 (5)	117.51±5.6 (5)	<0.001	32.73	67.27
UF1C-HphE	152.34±2.35 (5)	240.22±4.12 (5)	<0.001	66.92	33.08
UF1P-HphE	152.34±2.35 (5)	85.10±5.10 (5)	<0.001	23.71	76.29
UF2C-HphE	152.34±2.35 (5)	118.92±6.03 (5)	<0.001	33.13	66.87
UF2P-HphE	152.34±2.35 (5)	120.04±4.12 (5)	<0.001	33.44	66.56
UF3C-HphE	152.34±2.35 (5)	251.95±4.32 (5)	<0.001	70.18	29.82
UF3P-HphE	152.34±2.35 (5)	263.83±6.23 (5)	<0.001	73.49	26.51
UF4C-HphE	152.34±2.35 (5)	139.83±5.00 (5)	<0.001	38.95	61.05
UF4P-HphE	152.34±2.35 (5)	133.78±6.00 (5)	<0.001	37.27	62.73
Hydroalcoholic extracts					
Control	152.34±2.35 (5)	358.98±11.23 (5)	–	100	-
HA 50%	152.34±2.35 (5)	250.02±7.89 (5)	<0.001	69.65	30.35
HphaE	152.34±2.35 (5)	93.95±3.69 (5)	<0.001	26.17	73.83
MF-HphaE	152.34±2.35 (5)	75.44±4.36 (5)	<0.001	21.02	78.98
UF1C-HphaE	152.34±2.35 (5)	51.58±2.14 (5)	<0.001	14.37	85.63

Variant	24 h	48 h		% Proliferation rate	% Antiproliferative degree
	X●10^3 ± ES	X●10^3 ± ES	p		
UF1P-HphaE	152.34±2.35 (5)	87.83±4.21 (5)	<0.001	24.47	75.53
UF2C-HphaE	152.34±2.35 (5)	103.74±3.89 (5)	<0.001	28.90	71.10
UF2P-HphaE	152.34±2.35 (5)	97.91±6.21 (5)	<0.001	27.27	72.73

The total number of the tumoral HeLa cells, from the cultures' componence incubated with the hydroalcoholic bioproducts, is much lower than control, demonstrating a strong mitoinhibitory potential of these extracts. The mitoinhibitory impact of the 50% HA was of about 30%. In the light of this reference value, the antiproliferative level of the total and fractionated hydroalcoholic products was ranged between 51% (UF2C-HphaE) and 79.37% (UF1C-HphaE), both limits being superior to the 50% minimum standard level, imposed by the American prescreening program.

Some experimental studies have highlighted that the mitoinhibitory effect of various total polyphenolic extracts, upon cancerous cell lines, could be induced by polyphenolic glycosides of bufadienolide type, which were also isolated from underground vegetal organs of the *Helleborus* sp (Bassarello et al., 2008; Kemertelidze, 2008; Muzashvili et al., 2006).

In an another *in vitro* experimental model, was followed the action of the total polyphenolic hellebore extracts and of their filtrated fractions upon cell viability, expressed by numerical differences between alive and dead cells. Using this parameter we assessed the cytotoxic potential of all investigated biopreparations upon HeLa cell cultures.

As illustrated in the below Figure 2, the control cultures have been characterized by a major number of alive cells and very few dead ones, their cell viability being in around of 98.23%. Contrary, the treated cultures have presented preponderantly death cells, the significant descreases of cell viability ranging from one type of hellebore extract to another.

Thus, the total hydrous polyphenolic extracts as well as the concentrate and permeate derived fractions have induced either a nonsignificant low cytotoxic impact – in the case of HphE (10.2%), UF1C-HphE (2.23%), UF3C-HphE (13.71%) and UF3P-HphE (9.64%) extracts– or a significant strong cytotoxic effect, which was reported in the case of MF- HphE (54.14), UF1P-HphE (65.40%), UF2C- HphE (63.89) and UF2P- HphE (50.95%) extracts, between these limits enrolling UF4C and P – HphE samples, which generated a moderate declin of the cell viability (43.15 and 45.61%).

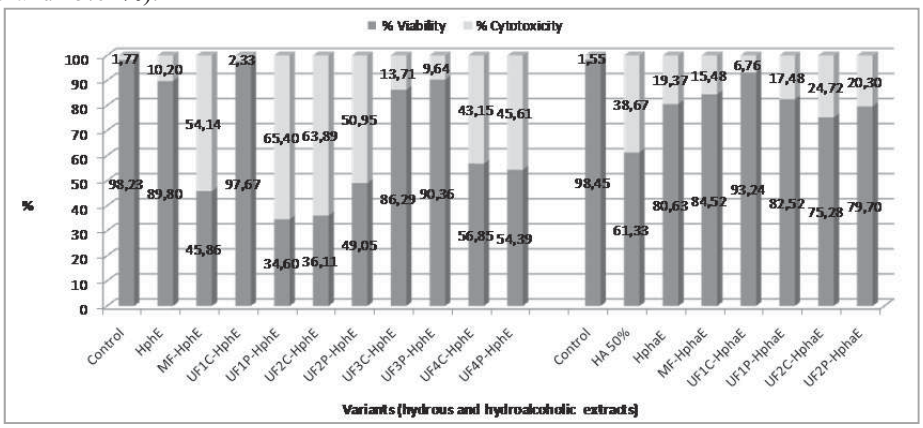

Fig. 2. HeLa cancerous cell cultures viability and the cytotoxic impact of the total and fractionated, hydrous and hydroalcoholic hellebore biopreparations (15 µg/mL).

The level of the viability regression of the HeLa cell cultures subjected to the hydroalcoholic hellebore treatment has reached values ranging between 6.76% (UF1C-HphaE) - 24.72% (UF2C-HphaE), all revealing a moderate cytotoxic effect. In the light of the cytotoxic impact of the HA 50% vehiculating agent (38,67%), we can suppose that the normal cytotoxic potential of the compounds is attenuated by the presence of diluted ethanol, which was used as extractant solvent.

Like in the case of the proliferation process, it can assume similar action patterns of the studied agents. In a same context, Watanabe et al., 2003 revealed that some glucosidal bufadienolides were cytotoxic both upon the several neoplastic cell lines and on healty cell cultures, while the rhamnosidal bufadienolides had higher specificity on cancerous cells than on normal ones. On the other hand, in vitro administration of the aqueous extracts from Helleborus niger has been correlated with induction of sister chromatid exchanges, which is the molecular mechanism of their property to destabilize the DNA and to cause apoptotic cell death in various cancerous cell lines (Büssing. and Schweitzer, 1988, Jesse et al., 2009).

A last *in vitro* experimental model was used for investigation of the consequences of hellebore’s interaction with the HeLa neoplastic cells upon the cell cultures development degree, the percentage values of this index being included in Figures 3 and 4.

The neoplastic cell cultures incubated with the total or fractionated hydrous hellebore extracts have reached various levels of cultures development process. In comparison with the development degree of control cell cultures, considered as the 100% reference value, we observed significantly diminished percentage values of this process, which allowed highlighting and evaluation of an inhibitory impact of the polyphenolic samples upon cell cultures development In relation to their inhibitory potentials on HeLa cell cultures development, the ten extracts can be grouped as follows:

- the biopreparations which exert a very strong cytostatic effect – over 90% (MF-HphE, UF1P- HphE, UF2C-HphE and UF2P-HphE), with very low limits of variation (from 90.57% - UF2P-HphE, to 90.81% - MF-HphE);
- the concentrate and permeate fractions, obtained after the fourth ultrafiltration, which have an significant inhibitory rate of 80.91% (U43C HphE) and 82.96% (UF4P- HphE), respectively;
- the biopreparations which induce an inhibition of tumor cell development level smaller than 70%: HphE, UF1P- HphE, UF3C HphE and UF3P- HphE, the limits of variation ranging from 59.46% (UF3P- HphE) to 67.80% (UF1P- HphE).

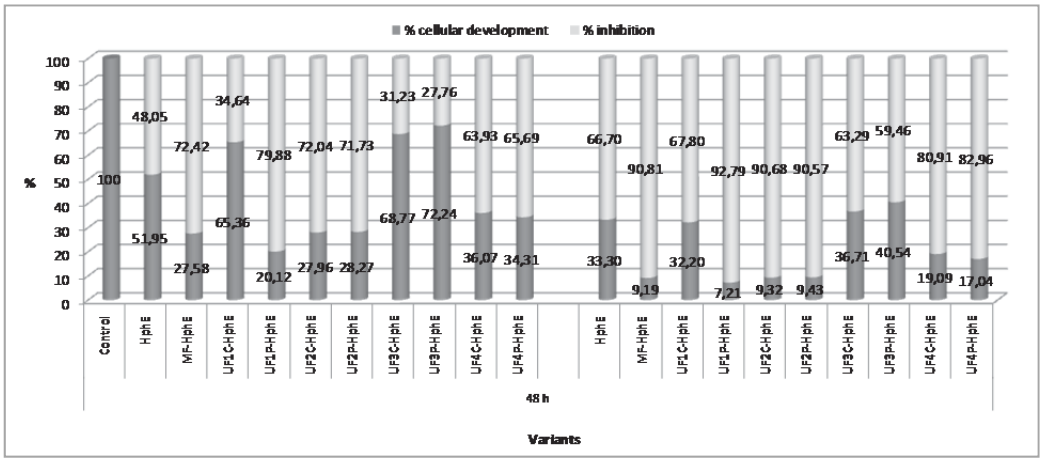

Fig. 3. The development degree of HeLa tumor cell cultures during their evolution in presence of *Helleborus purpurascens* total and fractionated hydrous extracts (in a dose of 15 μg/mL) and their corresponding inhibitory impact, comparatively to the control ones

Therefore, indifferently of the used hydrous extract, we have emphasized the capacity of all the tested hydrous polyphenolic extracts to inhibit the development of HeLa cell cultures, their action levels being statistically and cytostatically significant.

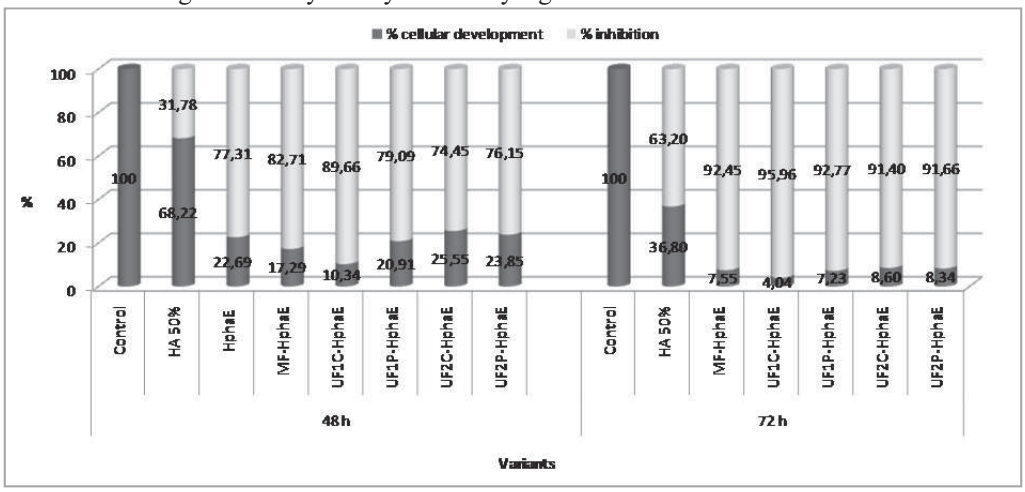

Fig. 4. Variations of the Hela cell cultures development level and the corresponding inhibition degrees, after the polyphenolic treatment with 15 μg/mL from total and fractionated hydroalcoholic extracts, as compared with untreated tumoral cultures

In vitro treatment of HeLa cancerous cell cultures, with the total and fractionated hydroalcoholic Helleborus extracts, has conditioned a more pronounced decrease of cell cultures development after 24 and 48 hours incubation, this potentiation of effect being, probably, due to the presence of HA 50% in the componence of the samples, himself inducing a perturbation of cell cultures development of 31.78 and 63.2%. Thus, the maximum inhibition rates, above 90%,

were registered at the 72 hours old treated cultures (Fig. 4) and have characterized MF- HphaE, UF1C- HphaE, UF1P- HphaE, UF2C- HphaE and UF2P- HphaE.

The inhibitory impact upon HeLa cancerous cell cultures development – this last aspect expressing *in vitro* antitumoral effect, which remember us the *in vivo* tumoral regression – which has characterized both total hydrous or hydroalcoholic hellebore extracts and their permeate or concentrate fractions, is consequence of cell proteinosynthesis and proliferation alterations, as well as of aggression upon cellular viability induced by the tested polyphenolic samples.

The inhibitory potential of the bioactive extracts upon HeLa cultures development is dependent on the used extract - the most active being UF1P- HphE and UF1C- HphaE– this can be probably amplified by the prolongation of the *in vitro* treatment period.

The cytostatic potentials of the total hydrous or hydroalcoholic hellebore extracts are inferiorly that ones of microfiltrates and some ultrafiltrates (UF 1, 2, 4- permeates). These observations confirm the opportunity of utilization of the membranary micro- and ultrafiltration techniques of the total extracts, which assure a specific repartition of the bioactive polyphenolic compounds in the different fractions and an *in vitro* various cytostatic effectiveness.

The analysis of our experimental results has highlighted the indubitable interference of the hydrous and hydroalcoholic, total and fractionated polyphenolic biopreparations, extracted from *Helleborus purpurascens*, with cell proteinbiosynthesis, mitosis processes and cell viability of the HeLa neoplastic cells. The inhibitory impact upon cell proteinsynthesis, the antiproliferative effect, the decrease of cell viability have conditioned the regression of the cell cultures development, this *in vitro* antitumoral action expressing the *in vitro* strong cytostatic and moderate cytotoxic properties of these bioactive extracts.

The action pattern of the bioactive preparations is similarly, but the *amplitudes of the in vitro* cytostatic and cytotoxic potential are different from a sample to another, the polyphenolic agents being characterized by a superior cytostatic impact rather than a cytotoxic one. The complementarity of these actions suggested that the vegetal polyphenolic extracts had a major *in vitro* antitumoral effectiveness. This conclusion is not hazardous if we take into account that *in vitro* American prescreening program imposes the induction of a minimum inhibitory impact upon the cell cultures developmentof at least 50%.

Our results, corroborating with the specialty literature (Cunha-Filho et al., 2010), have argued that the autochthonous total or fractionated, hydroalcoholic or hydrous polyphenolic hellebore extracts may be included in the category of cytostatic and cytotoxic agents, which, finally, could enrich the oncochemotherapeutic arsenal.

CONCLUSIONS

In vitro investigation of the interaction of some total and fractionated polyphenolic preparations, extracted from roots and rhizomes of *Helleborus purpurascens* perennial medicinal plant with HeLa cancerous cells has highlighted a decrease of cell proteic content, a regression of total cell number during evolution of the cultures, a moderate increase of dead cells number, a negative perturbation of cell cultures development.

All these observations have suggested a cytostatic and cytotoxic impact expressed by the inhibition of cellular proteinsynthesis, by alterations of protein dynamics, by mitostatic effect, by cell viability decrease and by the cell cultures regression.

The negative repercussions upon some cytophysiological processes of HeLa neoplastic cells allow us to characterize some polyphenolic biopreparations as potential cytostatic and cytotoxic agents.

Preliminary characterization of our total or fractionated polyphenolic samples as potential cytostatic agents imposes the extending and thoroughgoing of the *in vitro* research, on other human malignant and normal cell lines, in order to: prove their cytostatic effect reproducibility and the dependence of cytostatic effectiveness on the treatment doses; highlight another cell, subcell and molecular effects of these natural biopreparations and their probably action mechanism; evaluate the reactivity degree of the normal cells to the action of these new cytostatic products.

The elucidation of cytostatic property of some autochthonous polyphenolic biopreparations will impose their introduction in the *in vivo* screening circuit, on tumor-bearing animals, in order to qualitative and quantitative pharmacological evaluation of their antineoplastic pharmacodynamic effect. The final pharmacological characterization of a polyphenolic bioproduct as antineoplastic agent with possible clinical significance will provide a new pathway of superior capitalization of *Helleborus purpurascens* medicinal plant.

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