Synthesis of Some Total Polyphenolic Extracts from the *Vitis vinifera* Seeds and the Study of Their Cytostatic and Cytotoxic Activities

COSTICĂ SAVIN[1], PINCU ROTINBERG[2], COSMIN MIHAI[2], ALINA MANTĂLUTA[1], ANCUTA VASILE[1], RODICA PASA[1], DOINA DAMIAN[1], DUMITRU COJOCARU*[3]

[1]Iaşi Research-Development Station for Viticulture and Vinification, 48 Aleea Mihail Sadoveanu, 700489, Iaşi, Romania

[2]The Institute of Biological Researches Iaşi, 47 Lascăr Catargiu Str., 700107 Iaşi, Romania

[3]Alexandru Ioan Cuza University Iaşi, Faculty of Biology, 22 Carol I Blv., 700505, Iaşi Romania

Four total, hydrosoluble polyphenolic biopreparations from the seed of Vitis vinifera have been obtained and physicochemical characterized. Their in vitro testing, on HeLa neoplastic cell cultures, has highlighted the cell proteinsynthesis alteration; protein dynamics modification; decrease of total cell number; cell viability diminution; inhibitory impact upon the cell cultures development. It has been concluded that the polyphenolic extracts are behaving as cytostatic and cytotoxic agents.

Keywords: polyphenols, polyphenolic biopreparations, cellular cultures, cytostatic activity, cytotoxic activity

Despite the fact that there has been continuous progress in cancer prophylaxis, diagnosis and treatment, the neoplastic disease still holds pride of place in contemporary pathology. The antineoplastic chemotherapy – a main way in cancer treatment – is still characterized, by a relatively low effectiveness [1, 2], fact which explains the major significance given to the oncobiologic research, oriented towards optimizing its efficiency, by discovery of new oncochemotherapeutic agents with preferential action upon cancerous cells and lower upon health ones. [3, 4].

A great part of the biologically active compounds are polyphenols, which represent a class of over 8000 compounds, the majority being identified in different anatomic segments of the plants [5, 6]. The interest for this group of metabolites is justified because of the biological properties manifested through their anti-inflammatory [7], anti-oxidant [8, 9], antiestrogenic [10], antibacterial [11], immunomodulatory [12] and antitumoral [13-15] action etc, part of the pharmaco-dynamic effects of some polyphenolic products being biomedically capitalized in curative and preventive, single or associated treatments. Among the flavonoids we mention the galic acid and the flavan monomers 3-ol represented by the cafeic acid, genistein, siringic acid, catechine, epicatechine 3-o galat, myrecitin, cumaric acid, quercitin, dimers, trimers and polymers of procyanidins. [15, 17, 18]. An attractive source of polyphenols is represented by *Vitis vinifera* seeds, which have the highest content (5-8 %) of these bioactive compounds [19].

In the light of the things presented above, and from the perspective of our preoccupations of superior exploitation of seeds waste resulted from the vinification process, we considered opportune and important to obtain some total polyphenolic extracts and to investigate their pharmacological properties.

In a first stage of the chemico-pharmaceutical researches, we have proposed, in the present paper, to obtain and characterize from the physico-chemical point of view the total polyphenolic, alcoholic and concentrated extracts, to investigate *in vitro* the impact of these biopreparations of vegetal nature on the proteinsynthesis, proliferation, viability and on the development degree of HeLa cell cultures, in view of highlighting and appreciating their cytostatic and cytotoxic properties.

Experimental part

The total polyphenolic extracts studied in this paper were obtained from the *Vitis vinifera* grape seeds according to the varieties, in the following manner: EPF_1 (Cabernet Sauvignon), EPF_2 (black Băbească), EPF_3 (black Feteasca) and EPF_4 (Merlot). For ensuring the reproductibility of experimental results, the vegetal materials were processed so that the humidity reach the value of 0.5 %, the physical purity be of 99.5%, and the genetic purity of 100%. After the crumbling, at dimensions of 1-2 mm, the vegetal materials were degreased with ethyl ether.

The process of extracting the total polyphenols was achieved in continuous system in the Soxhlet apparatus using as solvent the ethylic alcohol in ratio 1/10 (vegetal material (g) solvent (mL). The four total polyphenolic alcohol extracts were concentrated in the rotary evaporator at the temperature of 30°C, to eliminate the ethylic alcohol, for the testing of the cytostatic and/or cytotoxic activities.

Materials and methods

The total polyphenols (PT) were determined in the vegetal alcohol and concentrated extracts through the spectrophotometer method Folin-Ciocâlteu [20], the concentrations being expressed in equivalents galic acid/ L (GAE/L) compared to the standard curve with galic acid.

The colour index (IC) was determined spectro-phometrically by cumulating the values determined at 420 nm, 520 nm and 620 nm, according to STAS 6182/35-75.

The tanoid matter index (IM*T)* was determined by spectrophometrically determining the absorbance at 280 nm of vegetal extracts diluted with ethanol 50% [21].

* *Tel.: (+40)0232 201363*

Table 1
THE PHYSICO-CHEMICAL CHARACTERIZATION OF ALCOHOLIC POLYPHENOLIC EXTRACTS

The physico-chemical parameters	Total polyphenolic alcoholic extracts from *Vitis vinifera seeds*			
	EPF_1	EPF_2	EPF_3	EPF_4
Color index (IC)	0,0336	0,0310	0,0158	0,0302
Tanoid matter index (IMT)	396,4	690,4	463,56	336,00
Total polyphenols (GAE/L)	0,966	1,076	1,0368	0,880
Anthocyanins (A) (mg/mL)	16,80	6,47	14,35	6,30

The anthocyanic compounds were determined through the method of discolouring with sodium bisulphate ($NaHSO_4$) - the R. Gayon-Sonestreet method [22]. At *p*H 3.5 the anthocyanic compounds are in totality under the form of pseudobases, and at *p*H 0.6 under the form of intensely colored cations. The difference of absorbance is directly proportional with the quantity of anthocyanins from the sample to analyze. The values obtained reported to the standard curve, achieved with pure anthocyanins allow establish to the concentration of anthocyanins (mg/mL).

The dry substance (s.u.) and ash determination was achieved gravimetrically, in the platinum crucibles.

The biological material used in the in vitro experiments, was represented by mycoplasm-negative negroid human cervix epitheliod 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 $5 \cdot 10^5$ cells in 75 cm^2 flasks, in a humidified 5% CO_2 atmosphere at 37°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, 2 mL at a density of $1 \cdot 10^5$ cells/mL, were seeded in the experimental tubes containing DMEM medium and introduced at 37°C. The medium of the 24 h cell cultures was changed either with a normal one (control cultures) or with one containing the polyphenolic extracts (treated cultures), in a dose of 1,5 mg/mL [23, 24].

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 (Lowry method modified by Oyama) [25] and tracing of the protein dynamics; the cytometrical assessment of the total cellular number with Türk haemocytometer on the basis of the formula: $N = n \cdot d \cdot 10^4$, 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 . 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 [24]; the mathematical estimation of the cytotoxical degree % cytotoxicity = Ntct – Ncat / Ntcc . 100, where: Ntct = treated cells total number; Ncat = treated alive cells number; Ntcc = control cells total number [24]; mathematical evaluation of cellular cultures degree after the action of the polyphenolic biopreparations, 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 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 [23]. For each culture type and time interval, five culture tubes were used and the results were evaluated statistically by Student's "t" test [26].

Results and discussions

In the alcohol phase the four polyphenolic extracts obtained in the extraction process were characterized from the point of view of the color index (IC), the tanoid matter index (IMT), of the contents in total polyphenols (PT), and anthocyanins (A). The data obtained are mentioned in table 1.

From the values obtained, we notice a variability of the chemical constants according to the taxonomic appurtenance of the vegetal material subjected to the extraction process. We notice, however, the total polyphenolic extracts EPF2, and EPF3 obtained from the seeds of the black Băbească and Merlot varieties, which have high values for IMT and PF compared to EPF1 and EPF4 polyphenolic extracts.

After the concentration of the four alcoholic polyphenolic extracts in the rotary evaporator and the rerun of the residue in 25 mL distilled water, we determined the total polyphenols, dry substance and ash. The data obtained are mentioned in table 2.

From the data presented, we ascertain, as we expected, higher values in total polyphenols in the EPF2 and EPF3 extracts. Determining the dry substance, we ascertain different values between 82.10 and 534.60 mg/mL that suggests the structural complex nature of the vegetal

Table 2
THE CHEMICAL CHARACTERISTICS OF
TOTAL POLYPHENOLIC CONCENTRATED EXTRACTS

The physico-chemical parameters	Total polyphenolic concentrated extracts			
	EPF_1	EPF_2	EPF_3	EPF_4
Total polyphenols (GAE/L)	36,7	37,7	36,6	37,5
Dry substance (mg/mL)	534,6	82,10	234,6	242,8
Ash (g/L)	1,30	0,50	1,80	1,6
pH	5,5	5,6	5,5	5,5

Table 3
THE INFLUENCE OF THE POLYPHENOLIC BIOPREPARATIONS, IN A DOSE OF 1.5 mg/ml, UPON HeLa TUMORAL CELLS PROTEINSYNTHESIS

Experimental group	24 hours	48 hours (24 hours incubated with EPF)			72 hours (48 hours incubated with EPF)		
	X±ES	X±ES	t	p	X±ES	t	p
Control	168.5±9.8 (5)	224.3±13.4 (5)	–	–	261.2±18.9 (5)	–	–
EPF_1	168.5±9.8 (5)	54.1 ± 4.5 (5)	12.0	<0.001	34.9 ± 4.2 (5)	11.7	<0.001
EPF_2	168.5±9.8 (5)	58.9 ± 6.4 (5)	12.1	<0.001	56.8 ± 6.5 (5)	9.5	<0.001
EPF_3	168.5±9.8 (5)	25.8 ± 4.1 (5)	10.1	<0.001	10.7 ± 3.0 (5)	12.3	<0.001
EPF_4	168.5±9.8 (5)	68.9 ± 4.4 (5)	11.3	<0.001	64.5 ± 8.4 (5)	7.4	<0.001

*Figures in brackets indicate the number of experimental cultures for each type.

polyphenolic extracts obtained. Thus, in appreciating the cytostatic and/or cytotoxic action we considered correct to establish the concentration field reporting ourselves to the dry substance, constant parameter, which comprises in totality the range of active compounds present in the concentrated extracts and not in the total polyphenol concentration, determined compared to the curve with galic acid, which is selective.

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 the proteinsynthesis inhibitory impact induced by the polyphenolic extracts. The mean values of the evaluation index of the cell protein biosynthesis reactivity, registered at different ages of the control and treated cell cultures, are included in table 3.

The cell cultures, treated for 24 and respectively 48 h with the polyphenolic preparations, as compared to the control values, were characterized, during the entire investigated time interval, by significantly smaller protein concentrations (μg protein/culture), suggesting the perturbation of the tumoral cells protein biogenesis. Thus, if the untreated tumoral cellular cultures protein dynamics presents an ascendant route with progressive increased amplitude, the protein dynamics of the treated cell cultures reveals a descendent route of decreased amplitude. The above presented experimental data, significantly emphasize the proteinsynthesis inhibitory impact induced by the polyphenolic extracts, the intensity of the effect depending on *Vitis vinifera* variety. The maximum inhibitory potential characterizes only the biopreparation obtained from seeds of black Feteasca variety, followed by the Cabernet Sauvignon, black Babeasca and Merlot extracts.

Another highlighting and evaluating parameter of the natural polyphenolic biopreparations cytostatic effect, was the total cellular number, which expresses the sense and extent of the bioactive agents interference with the cancerous cells proliferation process. The registered results in the conditions of their mitoinhibitory action testing are presented in table 4. The treated HeLa cell cultures, of 48 or 72 h old, are characterized by a progressive and significant decrease of the total cells number, comparatively with the corresponding control cultures. Thus, a regression of the cellular proliferation process to the polyphenolic action is highlighted, leading to the expression of a mitoinhibitory effect with a slight variable amplitude according to the vegetable extract type.

In the next *in vitro* experimental model, the action of the vegetable polyphenolic extracts upon cell viability has been followed and expressed by numerical differences between alive and dead cells their visualization being performed with trypan blue stain.The obtained results are presented in table 5.

The comparative analysis of the alive and dead cells number from the HeLa cell cultures, control and respectively treated for 48 h with polyphenolic extracts, in a dose of 1.5 mg/mL, reveals a different behavior of those two cell culture types. Thus, the control cultures are characterized by a greater number of alive cells than the dead ones, while the treated cultures have presented a bigger number of dead cells than alive cells. Therefore, we have stated that vegetable extracts of polyphenolic nature diminished the neoplastic cells viability; them being characterized by a moderate cytotoxic potential.

The final experiment of this study has investigated the consequences of polyphenolic extracts interaction with the HeLa tumoral cells upon the cell cultures development degree, the percentage values of this parameter being included in table 6.

In comparison with the development degree of control cell cultures, considered by us as 100%, the neoplastic cell cultures incubated with the tested vegetable bio-preparations have presented significantly diminished procentual values.The inhibitory potential of the bioactive extracts upon HeLa cultures development is dependant on the used extract - the most active being EPF3 – and it can be amplified by the prolongation of the *in vitro* treatment period. Thus, EPF3 induces an inhibitory effect upon cell cultures development of 88.5%, at 48 h and 95.9%, at 72 h.

Table 4
THE MITOSIS INHIBITION, EXPRESSED BY THE TOTAL CELL NUMBER (n . 10^5) DECREASES OF THE TREATED HeLa CELLS CULTURES

Cell culture	48 hours				72 hours			
	X±ES	p	proliferation rate %	antiproliferative degree %	X±ES	p	proliferation rate %	antiproliferative degree %
Control	0.229±0.022 (5)	–	–	–	0.286±0.027 (5)	–	–	–
EPF_1	0.143±0.018 (5)	<0.05	62.4	37.6	0.147±0.020 (5)	<0.001	51.5	48.5
EPF_2	0.134±0.008 (5)	<0.002	58.5	41.5	0.135±0.010 (5)	<0.001	47.2	52.8
EPF_3	0.139±0.015 (5)	<0.01	60.8	39.2	0.155±0.018 (5)	<0.001	54.1	45.9
EPF_4	0.116±0.008 (5)	<0.001	50.6	49.4	0.101±0.011 (5)	<0.001	35.3	64.7

*Figures in brackets indicate the number of experimental cultures for each type.

Table 5
THE TUMORAL CELL CULTURES VIABILITY AND THE CYTOTOXIC IMPACT OF THE POLYPHENOLS

Cell culture	Alive cells number x 10^5 X±ES	p	% Viability	Dead cells number x 10^5 X±ES	p	% Cytotoxicity
Control	0.278±0.024 (5)	–	97.2	0.008±0.001 (5)	–	2.8
EPF_1	0.060±0.004 (5)	<0.001	69.6	0.087±0.005 (5)	<0.001	30.4
EPF_2	0.058±0.003 (5)	<0.001	73.1	0.077±0.004 (5)	<0.001	26.9
EPF_3	0.055±0.002 (5)	<0.001	65.0	0.100±0.011 (5)	<0.001	35.0
EPF_4	0.027±0.001 (5)	<0.001	74.2	0.074±0.002 (5)	<0.001	25.8

*Figures in brackets indicate the number of experimental cultures for each type.

Table 6
THE HeLa CELLS CULTURES DEVELOPMENT AND ITS INHIBITION DEGREE IN THE PRESENCE OF POLYPHENOLIC BIOPREPARATIONS (1.5 mg / mL)

Cell culture	24 hours	48 hours		72 hours	
	cell cultures development (%)	cell cultures development (%)	development inhibition (%)	cell cultures development (%)	development inhibition (%)
Control	100	100	0	100	0
EPF_1	100	24.1	75.9	13.4	86.6
EPF_2	100	26.2	73.8	21.7	78.3
EPF_3	100	11.5	88.5	4.1	95.9
EPF_4	100	30.7	69.3	24.7	75.3

The present paper, through the proposed objective, the experimental models used *in vitro*, the results registered, regard the identification of the new natural polyphenolic agents with cytostatic and cytotoxic action, which finally enriche the oncochemotherapeutic arsenal, which does not include until now an antineoplastic medicine of polyphenolic nature despite of the clinic trials, which are performed today with polyphenolic commercial preparations [15, 16, 27, 28].

The analysis of the experimental results, obtained in the present study, highlights the perturbation of the neoplastic HeLa cells proteinsynthesis, proliferation and viability processes by the polyphenolic biopreparations, obtained from different *Vitis vinifera* varieties seeds. The proteinsynthesis and mitosis inhibitory impact, the cell viability decrease conditions the inhibition of the cell culture development induced by these bioactive extracts.

The *in vitro* cytostatic and cytotoxic actions intensities are different, the polyphenolic biopreparations being characterized by a superior cytostatic potential (75.3 - 95.9%) rather than **a** cytotoxic one (25.8 - 30.4%). The complementarity of the actions suggests that the vegetable extracts have 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 culture development by the tested product of at least 50%.

The slight difference between *in vitro* cytostatic and cytotoxic efficiency of various polyphenolic biopreparations, obtained from seeds of Cabernet Sauvignon, black Băbească, black Fetească şi Merlot varieties, could be the result of the qualitative and quantitative heterogeneity of the polyphenolic structures from the obtained extracts.

The expression of the cytostatic and cytotoxic actions of the polyphenolic extracts upon HeLa neoplastic cells confirms the data from the specialty literature, which signals *in vitro*, on different cancerous cells lines, a multitude of cells, subcells and molecular effects, which concur and assure the *in vivo* global pharmacological manifestation, on experimental and clinical models, of anticarcinogenic action of some polyphenolic biopreparations [15, 17].

Conclusions

In vitro investigation of the interaction of some global polyphenolic preparations, extracted from the Vitis vinifera varieties seeds, with HeLa cancerous cells has highlighted and has quantified their cytostatic and cytotoxic impact, expressed by the inhibition of proteinsynthesis and cellular proliferation, by cell viability decrease and by the inhibition of cell cultures development.

The manifestation of these biological properties imposes the introduction of the autochthonous polyphenolic biopreparations in the *in vivo* screening circuit, on tumor-bearing animals, in order to qualitative and quantitative pharmacological evaluation of their antineoplastic pharmacodynamic effects.

References

1.DE VITA, V.T. JR., Cancer: Principles and Practice of Oncology, 7, Philadelphia, Lippincott, De Vita Jr. et al (eds.), 2004, p. 276
2.STROESCU V., Pharmacological basis of medical practice, Ed. Medicală, Bucureşti, 1998, p. 48-63, p. 207-225, p. 1050
3.MIRON, L., Oncologie generală¸ Ed. Egal, Bacău, 2000, p. 9
4.OWENS, J.,. Drug Discovery Today, **6,** 2001, p. 1203
5.GEORGESCU, C., BRATU, I., TAMAS, M., Rev. Chim. (Bucureşti), **56**, nr. **7**, 2005, p. 779
6.DEHELEAN, C. A., VLASE, L., PEEV, C., ANTAL, D., VOICA, C., Rev. Chim. (Bucureşti), **57**, nr. **8,** 2006, p. 862
7.MIDDLETON, E., KANDASWAMI, C., Biochem. Pharmacol., **43,** 1992, p. 1167
8.ARUOMA, O., Mutat. Res, **523/524**, 2003, p. 9
9.IVANOVA, D., GEROVA, D., CHERVENKOV, T., YANKOVA, T., J. Ethnopharmacol., **96**, nr. 1-2, 2005, p. 145
10.BHAT, K.P., LANTVIT, D., CHRISTOV, K., METHA, R.G., MOON, R.C., PEZZUTO, J.M., Cancer Res, **61**, 2001, p. 7456
11.DOUGHARI, J.H., ELMAHMOOD, A.M., MANZARA, S., Afr. J. Microbiol. Res., 2007, p. 37
12.JUE, T., HONG-XIANG, S., YI-PING, Y., 2008, J. Ethnopharmacol., **119**, nr. 2, p. 266

13.MUKHTAR, H., AHMAD, N., Toxicol. Sci., **52**, 1999, p. 111
14.FERGUSON, L.R., Mutat. Res., **475,** 2001, p. 89
15.LAMBERT, J.D., HONG, J., YANG, G.Y., LIAO, J., YANG, C.S., 2005, Am. J. Clin. Nutr.; **81** (1 Suppl), 2005 Jan, p. 284S
16.TUZZ-YING, S., HUNG-CHI, L., NAE-CHERNG, Y., MIAO-LIN, H., 2007, J. Ethnopharmacol., **115**, p. 50
17.GRUNBERGER, D, BANERJEE, R, EISINGER, K, OLTZ, E. M., EFROS L, CALDWELL, M, ESTEVEZ, V, NAKANISHI, K., 1988, Experientia, **44**, nr. 3, p. 230-2
18.JHON S., J. Med. Food, **6,** nr. 4, 2003, p. 291
19.BIŢĂ, M. G., PREDA M., Rev. Chim. (Bucureşti), **58**, nr. 6, 2007, p. 494
20.SINGLETON, VL., ORTHOFER R., Methods Enzymol, **299,** 1999, p. 152
21.KONTEK, A., Anale I.C.V.V., **10,** 1983, p. 321
22.RIBEREAU – GAYON P., Bull. Soc. Chim., Paris 1965, p. 260
23.LEITER, J., ABOTT, D.J., SCHEPARTZ, S.A., Cancer Res., **25**, 1965, p. 20
24.DOYLE, A., GRIFFITHS, J.B., Cell and Tissue Culture: Laboratory Procedures in Biotechnology, John Wiley & Sons Ltd, West Sussex, 1998, p. 57
25.OYAMA, V., EAGLE, H., Proc. Soc. Exp. Biol. Med**., 91**, 1956, p. 305
26.SNEDECOR G.M., Metode statistice aplicate în agricultură şi biologie, Ed. Did. Ped., Bucuresti, 1968
27.Zhao, C., Giusti, M.M., Malik, M., Moyer, M.P., Magnuson, B.A., 2004, J Agric Food Chem., **52**, nr. 20, p. 6122-8
28.HEBER, D., 2008, Cancer Lett., **269**, nr. 2, p. 262

Manuscript received: 10.04.2008