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Study of Cytostatic and Cytotoxic Activity of Several Polyphenolic Extracts Obtained from *Vitis vinifera*

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Abstract

The study of polyphenolic extracts from skins, seeds, grape pomace and lees is justified by the huge amount of information from specialized literature, drawing attention to the many pharmacological effects of these biomolecules. The concentrated vegetal extracts obtained from seeds, skins, grape pomace and lees (yeast deposit after fermentation) were characterized according to their content of total polyphenols, anthocyanins, dry matter, ash and pH. The biological material used in the *in vitro* testing experiments in order to study cytostatic and cytotoxic effects were stabilized cultures of HeLa cancer cells, uncontaminated with mycoplasma and derived from a human uterine cervix carcinoma. Another action of the cytostatic substances could be exerted upon the cell proliferation process. The cell division process of the HeLa cell cultures treated with seeds by-product showed remarkable quantitative changes. The study has also evidenced a great number of dead cells in the composition of the treated HeLa cell cultures, their existence pointing out that the bioactive agent induced a major decrease of the cells viability. The obtained results in the context of the complex evaluation of the *in vitro* antitumoral property of the extracts, obtained from seeds, skins, grape pomace and wine lees from the 'Arcaș' grape variety, on HeLa cells cultures, have demonstrated the significant cytostatic and cytotoxic potential of the seeds polyphenolic biopreparate. The results obtained show that polyphenolic extracts from *Vitis vinifera* seeds act as cytostatic and cytotoxic agents.

Keywords: apoptosis, by-products of wine making, protein synthesis, tumor cells

Introduction

In Romania, as well as in other grapevine growing countries, grapes are used mostly for wine production. Grapes contain complexes of polyphenolic compounds, of which only one third is found in must (Țârdea *et al.,* 2010), whereas the rest is easily accessible and relatively easy to extract from skins, seed and pulp. Therefore, it was decided that a systematic study is necessary in order to characterize their properties.

The capacity of red grapevine varieties to accumulate different classes of polyphenols, which used to be a rather narrow segment of research, is now becoming a wider and wider field of interest. The study of polyphenolic extracts from skins, seeds, grape pomace and lees is justified by the huge amount of information from specialized literature, drawing attention to the many pharmacological effects of these biomolecules. From these polyphenolic products, bactericide (Baydar *et al.*, 2004), anti-anemic and anti-inflamatory (Joi *et al*., 2010; Youdim *et al.*, 2002), anti-oxidant (Katalinic *et al.*, 2009) cardio-vascular (Cheng *et al.,* 2007) and even oncolitic (Kaur *et al.*, 2006; Paun Roman *et al*., 2008) effects are reported. Their comprehensive influence at the level of the animal or human organism is part of complex interactions between phenolic bio-molecules and cellular, sub-cellular and molecular structures (membranes, organelles, enzymes, metabolites, nucleic acids etc.) that create possibilities of intervention as non-enzymatic protectors, as acceptors and donors of electrons, as metabolic and membranotrophic modulators (Hosu *et al.*, 2011).

The main aim of this study is to identify new anti-tumor preparations made from plant material high in polyphenols and based on a preliminary *in vitro* screening on neoplasic cells cultures.

Material and methods

In the *in vitro* screening test cycle four alcoholic extracts were used from skins, seeds, grape pomace and lees from the 'Arcaș' grape variety. The extraction was done in a Soxhlet apparatus with an extraction ratio of 1:10 (g plant material/mL alcohol). The four alcoholic extracts were concentrated in a rotary evaporator at a temperature of 30°C, from 200 mL to 20 mL.

Total polyphenols were determined spectrophotometrically according to Folin-Ciocâlteu (Singleton *et al*., 1999); anthocyanin compounds according to Ribereau Gayon-Sonestreet (Ribereau-Gayon, 1965) and dry matter gravimetrically. The reference substance was gallic acid.

The biological material used in the *in vitro* testing experiments in order to study cytostatic and cytotoxic effects were stabilized cultures of HeLa cancer cells, uncontaminated with mycoplasma and derived from a human uterine cervix carcinoma. Growth was maintained in DMEM medium (Dulbeco's Modified Essential Medium, Biochrom AG, Germany) supplemented with 10% fetal 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 a humidified 5% CO_2 atmosphere at 37°C (Doyle *et al*., 1998; Leiter *et al*., 1965).

When the cells confluenced and reached the monolayer stage of culture, they were detached by means of 0.25% trypsin + 0.02% EDTA (ethylenediaminetetraacetic acid, Biochrom AG, Germany), re-suspended in normal medium in order to obtain the necessary cellular mass for the *in vitro* investigations.

Preparation

The cells were centrifuged at 1800 rpm for 2 minutes. Two mL of cell suspension, with a density of 1 x 10^5 cells/mL DMEM medium, were seeded in the test-tubes and incubated at 37°C for 24, 48, and 72 hrs. The medium of the 24-hour cell cultures was changed either with a normal one (control cultures) or with one containing the bioactive polyphenolic samples in a dose of 30 μg/mL).

After another 24 and respectively 48 hours of *in vitro* development, the growth medium was decanted from the test cultures, the cells were washed with TFS (saline phosphate buffer) and analyzed for total protein content (using the Lowry method modified by Oyama) (Lowry *et al*., 1951; Oyama *et al*., 1956)

In order to evaluate cellular proliferation, the cytometric method in a continuous flow system was used (flow-cytometer Beckman Cell Lab Quanta SC) with carboxyfluorescein succinimidyl ester (CFSE). The specific emission of fluorescein (FITC) was registered with the blue laser at 488 nm. The process of cellular apoptosis was observed by the same method, using fluorochromes 7-AAD (7-Aminoactinomycin D, connecting to the nuclear DNA) and annexine-5 FITC (bound to the phosphatidylserine expressed on the internal surface of the apoptotic cellular membranes (Coder, 2003; Lyons *et al.,* 2003; Macey, 2007).

The significance of the cytostatic effects (inhibition of protein synthesis, mito-inhibitory effects perturbing cellular viability and apoptosis) as well as the cytotoxic action (ratio of live vs. dead cells) were calculated according to a comparative analysis between results obtained from the present study and standard values stipulated in International American and American programs of *in vitro* prescreening for the selection of new potential anti-neoplasic agents (Leiter *et al*., 1965; Takimoto, 2003).

All tests were done with 5 replicates. Statistical significances were tested according to the t-test (Cann, 2002).

Results and discussion

The concentrated vegetal extracts obtained from seeds, skins, grape pomace and lees (yeast deposit after fermentation) were characterized according to their content of total polyphenols, anthocyanins, dry matter, ash and pH (Tab. 1). Total polyphenolic extracts from seeds have a concentration of 25.3 g gallic acid/L, while the anthocyanins in skins reached 2293.7 acid gallic/L.

The dry matter content of seed extracts is 48.8 mg/L, contrary to skin extracts of 138.6 mg/L. The extracts from the fermentation by-products pomace and lees have both low contents of polyphenols and anthocyanins.

In view of the complex nature of polyphenolic and anthocyanic plant extracts and their cytostatic and/or cytotoxic effects, is it necessary to refer the results to a dry matter basis (mg/mL). Therefore, in the present experi-

Tab. 1. Physico-chemical characteristics of concentrated polyphenolic extracts obtained from the 'Arcaş' grape variety

Parameters	U.M	Seed	Skin	Pomace	Lees
Total polyphenols	g galic acid/L	25.3	11.6	4.4	1.8
Anthocyans	g galic acid/L	-	2293.7	17.5	17.5
Dry substance	mg/L	48.8	138.6	41.6	29.0
pH		5.5	5.6	5.5	5.6

Tab. 2. Protein concentration (μg protein/culture) of HeLa tumor culture cells of different ages, and their growing conditions in the presence of tested 30 μg/mL polyphenolic extracts

Experimental sample	24 hours	48 hours (24 hours of treatment)			72 hours (48 hours of treatment)		
	X±ES	X±ES	t	p<	X±ES	t	p<
Control sample	148.5±9.0 (5)	212.3±10.4 (5)	-	-	298.5±16.0(5)	-	-
Seeds extract	148.5±9.0 (5)	125.5 ± 9.0(5)	6.33	0.001	84.5± 12.1(5)	10.70	0,001
Skins extract	148.5±9.0 (5)	205. 8 ± 9.5(5)	0.46	N.S.	314.3±13.0(5)	0.77	N.S.
Grape pomace extract	148.5±9.0 (5)	190.5 ± 11.0(5)	1.44	N.S.	263.3± 5.0(5)	2.11	N.S.
Lees extract	148.5±9.0 (5)	200.6 ± 12.5(5)	0.72	N.S.	284.5± 13.82	0.67	N.S.

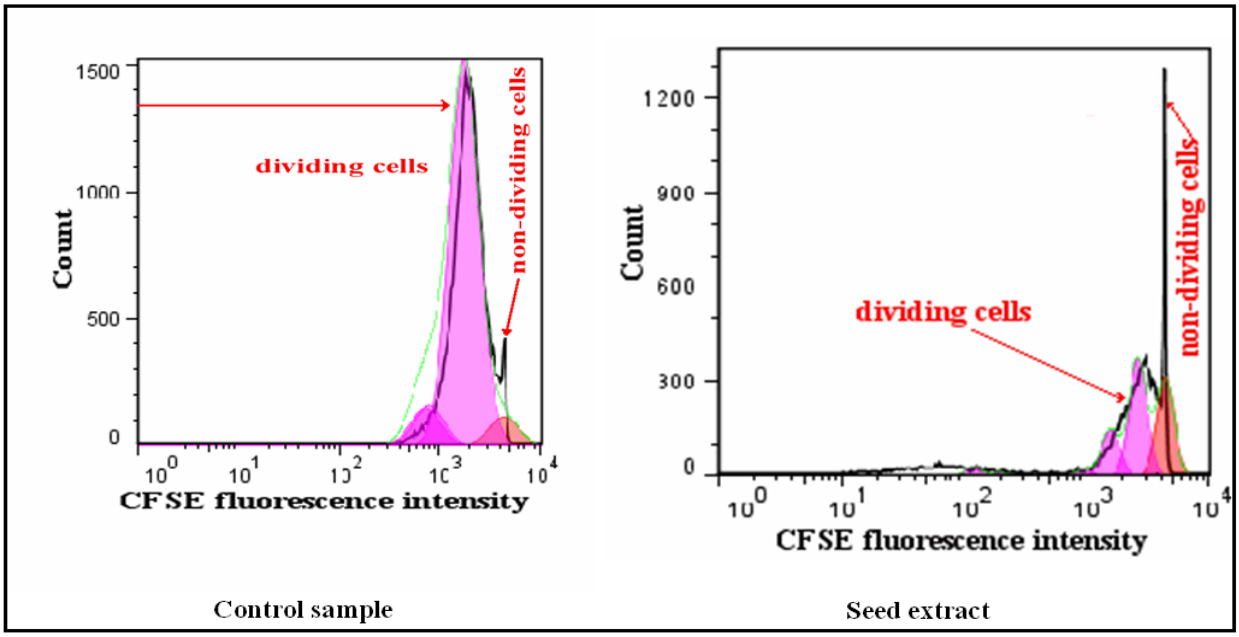
Note: Figures in brackets indicate the number of replicates

ment, concentrations were calculated according to this parameter.

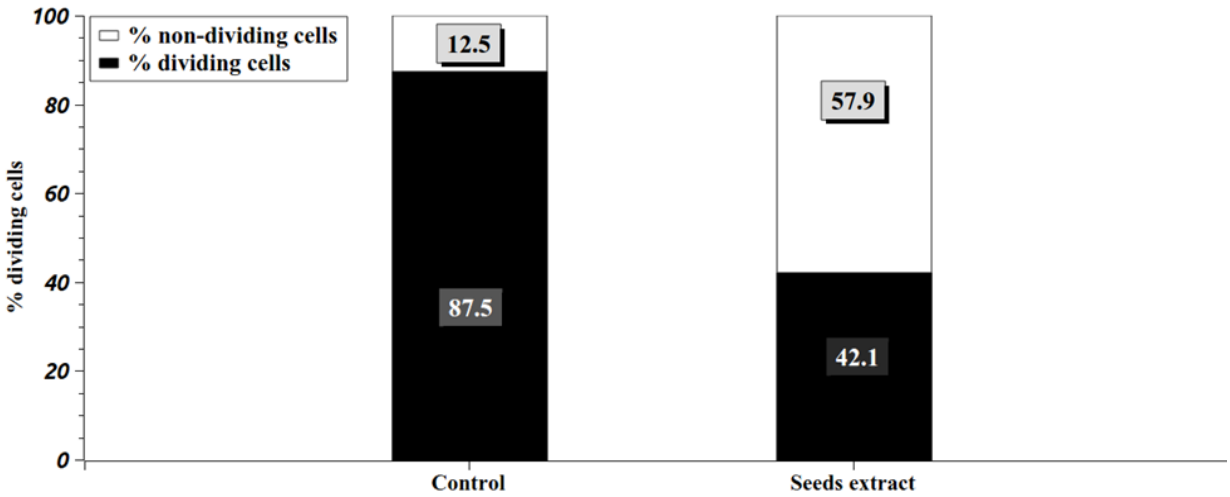
The results of the *in vitro* reactivity of human nature HeLa cancerous cells to concentrated polyphenolic extracts from the 'Arcaş' variety will be presented in what follows. The *in vitro* research focused on the interference of the bio-active compounds with the protein synthesis, mitosis and cellular apoptosis and on their impact on cellular viability.

In a first trial, the *in vitro* interaction between polyphenolic and anthocyanic structures of the extracts was analyzed at 30.0 mg/mL on protein biosynthesis of HeLa malign cells and their impact on total cellular protein concentration. Protein synthesis determined during culture evolution and culture development, as well as HeLa cells reactivity with certain polyphenolic compounds in comparison to the control sample, were also analyzed. The average protein contents observed at different stages, are presented in Tab. 2. The number in brackets indicates the number of replicates.

Total protein concentration increases in the control sample during the time course from 24 to 72 hrs. This progressive growth indicates the intensification of cellular protein-synthesis, together with the development of the



A.



B.

Fig. 1. Characteristics of cell proliferation process of HeLa human cancer cells incubated with seeds extract in the active cytostatic dose of 30 µg/mL (A= amplitude and fluorescence of the nondividing or dividing cells; B= percentual quantification of the cell sub-populations)

Tab. 3. Degree of HeLa cells cultures development in the presence of polyphenolic extracts (30 µg/mL)

Experimental variant	24 hours	48 hours	72 hours
	Development of cellular cultures (%)	Development of cellular cultures (%)	Development of cellular cultures (%)
Control sample	100	100	100
Seeds extract	100	59.11	28.31
Skins extract	100	96.94	105.30
Grape pomace extract	100	89.73	88.21
Lees extract	100	94.49	95.31

cellular multiplication rate, showing a normal development of control cultures.

HeLa cultures, treated with seed extract, had lower protein contents after 72 hours, compared to the control sample. This indicates that seed extracts may be a powerful inhibitor of protein synthesis in HeLa cultures *in vivo*. Grape pomace and wine lees in a similar dose showed nearly no effect in the *in vitro* test. The reduction in protein content is not significant. Skin extract seems to stimulate protein synthesis, but the effect is not significant.

Another aspect is presented in Tab. 3, illustrating the interaction between polyphenolic extract and the degree of growth inhibition of HeLa cells.

In comparison to the control cultures, seed, skin, pomace and lees extracts reduced more or less the development degree of HeLa cells cultures after 48 and respectively 72 hours treatments. Thus, it can be observed that the seed extract induced a significant regression of cultures during its entire period of evolution, while the other ones had a minimum and insignificant inhibitory impact. The inhibiting potential of the extracts on the development of HeLa cells cultures is dependent on the origin of the extract. The most active is the seed extract, which reduced the development with 40.89% (after a treatment of 24 hours, at a 48 hours age) and 71.69% (after a treatment of 48 hours, at 72 hours age). The skin, pomace and respectively, lees extracts have perturbed the cultures development only by 3.1% (in the first case), 10.3% or 11.8% (in the second case) and respectively, by 5.5% or 5.0% (in the third case).

Because of the low reactivity of the HeLa cells protein synthesis process and of cell cultures development to the action of our skin, pomace and lees extracts, it has been considered opportune their exclusion from the ulterior cell oncobiology investigations for evaluation of the cytostatic property of some new *Vitis vinifera* polyphenolic by-products.

Another action of the cytostatic substances could be exerted upon the cell proliferation process. Thus, it has been followed the interaction of the bioactive seeds polyphenolic biopreparation with the HeLa human neoplastic cells mitosis. The cell division process of the HeLa cell cultures treated with seeds by-product showed remarkable quantitative changes (Fig.1 A and B).

It can be observed from the flowcytometrical highlighting and quantification of the cell proliferation-expressed by inverse proportion relationship between the daughter cells number (increased) and their fluorescence intensity

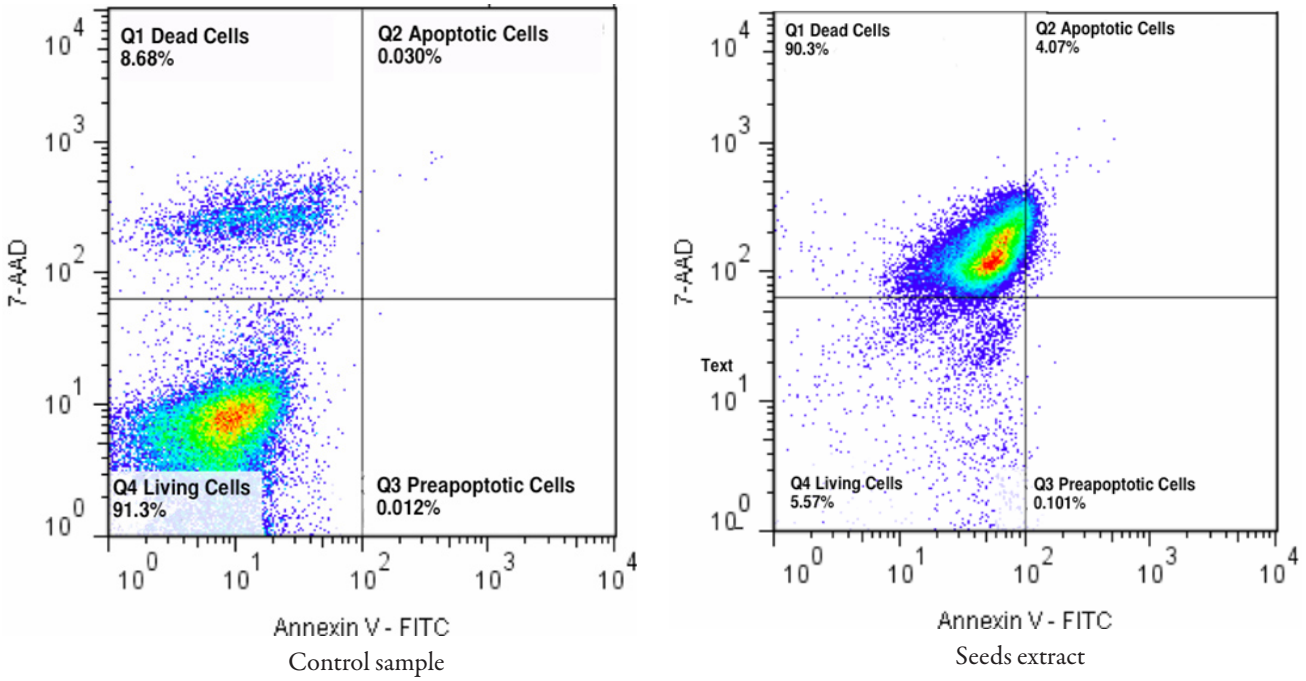


Fig. 2. Bivariate analysis Annexin V/7-AAD of the effect, induced by seed extract (30 µg/mL), on the percentual distribution of preapoptotic, apoptotic, dead and alive cells sub-populations from control and treated HeLa tumor cells cultures

(decreased), which is evaluated by cell proliferation analysis module of the FlowJo software-that:

• a great number of dividing cells and an attenuated fluorescence of the daughter cells have characterized the untreated, control HeLa cells cultures;

• a small number of dividing cells and an intense fluorescence, have characterized the daughter cells of the treated HeLa cells cultures.

These findings argue the inducing of a significant mitoinhibitory effect upon HeLa cells proliferation process by *Vitis vinifera* seeds polyphenolic extract. The interaction of the bioactive agent, with the cellular structures implied in the cell division, blocks cells in the interphase before entering cell mitosis.

Our results are partially confirmed by *in vitro* and *in vivo* recent studies that have shown anti-tumor effects on some human cancers (such as breast cancer, lung cancer and gastric adenocarcinoma, etc.) of the polyphenolic treatments with grape seed extracts (Kaur *et al*., 2009; Savin *et. al.,* 2009; Singh *et al.,* 2004; Tyagi *et al*., 2003). However, others researchers have registered an enhancement of the growth and viability of normal human gastric mucosal cells, indicating a lack of toxicity (Ray *et al.*, 2001; Yamakoshi *et al.,* 2002).

Recent studies of Liu *et al*. (2009), Engelbrecht *et al.* (2007), Kaur *et al*. (2006) on cancer cells cultures highlighted the induction of cell apoptosis with proanthocyanidinic extracts from *Vitis vinifera* seeds.

Consequently, it has been evaluated, by flow cytometry, the effect of the seeds extract upon the apoptosis process and viability of HeLa cancerous cells. The sense and scale of the cell apoptosis and viability reactivity can be observed in Fig. 2.

It can be observed, from the Fig. 2, that in the composition of the control HeLa cells cultures a lot of live cells, very few dead cells, extremely rare apoptotic cells and no pre-apoptotic cells are found, thus untreated cultures are characterized by great cell viability. Contrary, the HeLa cells cultures treated for 48 hours with a dose of 30 μg/mL seeds polyphenolic extract have presented a very great number of dead cells, a very low number of live cells, extremely rare pre-apoptotic cells and very few apoptotic cells.

Generally speaking, the highlighting of a negligible number of apoptosis and pre-apoptosis cells in the composition of the treated HeLa cell cultures or their weak representation in the analyzed cells population suggest the lack of a direct interaction between the seeds polyphenolic by-product and the molecular structures implied in triggering of the cell apoptosis process. In other words, its cytostatic effect was not induced by an apoptotic mechanism.

The present study has also evidenced a great number of dead cells in the composition of the treated HeLa cell cultures, their existence pointing out that the bioactive agent induced a major decrease of the cells viability. Thus, its cytotoxic potential is proved.

The bulk ofthe present results has demonstrated the *in vitro* antitumoral property of the seeds polyophenolic by-product-expressed by regression of the HeLa cell cultures-which is conditioned by the complementarity of its cytostatic and cytotoxic actions, they being induced by inhibitory effect upon cell protein synthesis and mitosis, as well as by decrease of cell viability degree.

Conclusions

The obtained results in the context of the complex evaluation of the *in vitro* antitumoral property of the extracts, obtained from seeds, skins, grape pomace and wine lees from the 'Arcaş' grape variety, on HeLa cells cultures, have demonstrated the significant cytostatic and cytotoxic potential of the seeds polyphenolic biopreparate.

Its antitumoral action, expressed by regression of the cell cultures, is due to the inhibitory impact upon the cell protein synthesis and mitosis, as well as to the decrease of the cell viability.

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