MECHANISM OF ACTION OF SOME NEW CYTOSTATIC/CYTOTOXIC POLYPHENOLIC EXTRACTS FROM *VITIS VINIFERA* SEEDS

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Abstract: *Vitis vinifera* seeds are very rich in bioactive compounds, especially polyphenols, representing a generous and available source for obtainment of new active biopreparations with agricultural, biomedical and ecological valorification. In this paper are presented the results focused on identification of the possible action mechanisms of some total polyphenolic bioproducts, extracted from grape seeds (P4, P5 in doses of 200 and 300 µg/mL) and proven already in our previous tests as cytostatic/cytotoxic agents. For this purpose were analyzed, on tumoral HeLa and normal Vero cells, the cell apoptosis process (Annexin V-PI assay) as well as the levels of ROS (DCFH-DA assay). It was registered, after the polyphenolic treatment period (48 hours), a stimulation of the apoptosis in HeLa cell cultures, the maximum effect being in the case of the bioproduct coded P4, at a dose of 300 µg/mL. In Vero cell cultures, the intensity of apoptosis was similar to the control group. No variations in ROS levels, as compared with control group, were registered in both cell cultures. Therefore, the cytotoxic effect of the studied polyphenolic extracts seems to be induced by activation of the cell apoptosis process and not by oxidative mechanism.

INTRODUCTION

In the last decades, the cancerous disease has become one of the most frequent cause of the human mortality, its presence being very difficult and insecure to diagnosis in his early stages. Despite the fact that the understanding of the mechanisms underlying the initiation, progression and spreading of the cancer in the organisms has registered important progressions, its treatment with nowadays methods is still ineffective in many cases. So, the actual chemotherapeutic research is oriented to the identification of new sources for antineoplastic compounds, with a more pronounced selectivity for cancerous cells, paying a special attention to the natural resources.

Wastes from wine industry are representing a large source of possible new bioactive products, useful in the organic agriculture, in animal farming or even in human therapy. Development of a highthroughput technology able to release, identify and concentrate the biological active compounds from wine waste could respond, among other things, at two important problems: identification and validation of new antineoplastic compounds and waste depletion.

Currently, there are several developments regarding winery waste valorification that should be taken into account to select the best available technique for the winery waste recovery and recycling by different methods as presented in table 1 (Oliveira & Duarte, 2016)

Table 1. Treatment of different solid waste from wine industry and their possible use (after Oliveira & Duarte, 2016)

	Treatment	Use
grape marc	fractionation of grape seed	polyphenol production
	hydrolysis and fermentation	lactic acid production
	hydrolysis and fermentation	biosurfactants and bioemulsifiers production
	destillation	ethanol and tartaric acid production
	extraction	tannins, polyphenols and oil production
	fermentation of grape seed	laccase production
	composting	plant substrate
lees	solubilization and precipitation	tartaric acid production
	composting	plant substrate

stalks	composting	plant substrate
	lyophilisation and extraction	polyphenol production
sludge	co-composting	plant substrate
	anaerobic digestion	biogas production

By applying different treatment methods, the seed waste from the wine industry has recently become a natural resource useful in obtaining of some bioproducts with practical capitalization as fertilizer, antimicrobial, antifungal, cytostatic, healing, imunomodulating, antioxidant agents, etc. Beside the benefits of obtaining new bioactive compounds, the environmental cleaning impact of the wastes is also an important objective acquired.

The polyphenolic substances are secondary metabolites abundant in different vegetables, fruits, cereals, including and dimers, oligomers and polymers of catechins (monomers of flavan-3-ols, the most chemically complex subclass of flavonoids) (Fantini et al., 2015).

The broad pharmacological spectrum and medicinal properties of the polyphenols from grape seed include benefits against the cardiovascular dysfunctions, acute and chronic stress, gastrointestinal distress, neurological disorders, pancreatitis, various stages of neoplastic processes (carcinogenesis). A significant cytotoxicity towards human breast, lung and gastric adenocarcinoma cells in parallel with the improvement of the growth and viability of normal cells was noticed (Bagchi, Bagchi & Stohs, 2002; Bagchi, Swaroop, Preuss, & Bagchi, 2014). Also, these chemical compounds are agents useful in the detoxification of carcinogenic metabolites, in the scavenging of free radicals, released as a result of oxidative stress. The antioxidant ability is significantly better than of vitamins C, E and beta-carotene oxidative stress scavangers.

Therefore, Vitis vinifera grapes represent a generous and available source for obtaining new actively biopreparations with agricultural, biomedical and ecological capitalization. In this paper are presented our results focused on the identification of the possible action mechanisms of some new total polyphenolic bioproducts, extracted from grape seeds (P4, P5 in doses of 200 and 300 µg/mL) and proven already in our previous tests ascytotoxic agents, like other polyphenolic preparations obtained in the past (Savin et al, 2009; Nechita et al., 2011).

Thus, the *in vitro* experimental approach was oriented to the cell apoptosis identification and oxidative stress levels assesement in normal and neoplastic cells treated with polyphenolic extracts, obtained from grape waste, in order to identify and understand their mechanism of action.

MATERIALS AND METHODS

The polyphenolic biopreparations were obtained from Vitis vinifera seeds - from grape marc after the oil removal by cold pressing - by several extraction methods with: supercritical fluids (liquid CO_2 and 98% ethanol, P1); water, at 15 bar (P2) or 3 bar (P3); **78 % ethanol** at 15 bar (P 4) and 3 bar (P5) pressures.

Neoplastic HeLa and normal Vero cells were seeded in DMEM medium supplemented with fetal bovine serum (FBS) in 24 well plates at a density of 50.000 cells / well. After 24 hours from cell cultures initiation, the growth medium was replaced with fresh complete medium containing P4 and P5 compounds, in doses of 200 and 300 µg/mL. After 48 hours from adding the compounds, the levels of reactive oxygen species (ROS, by DCFH-DA assay) as well as of apoptosis processes (by Annexin V-FITC assay) were investigated.

DCFH-DA assay. The cells (both from control and treated groups) were harvested by trypsinization, washed twice with PBS and finally resuspended in cold PBS. The cells were stained with 5 µL of 5 mM DCFH-DA solution and incubated 30 minutes at 37ºC. Before flow cytometer analysis, to the cell suspension was added 0.5 µL of propidium iodide solution (1 mg/mL). The experimental model consisted in the discrimination between alive and dead cells and the determination of ROS levels only in the alive cells.

Apoptosis assay. After treatment, the cultures were trypsinized, the cells being washed with cold PBS, resuspended in binding buffer and successively marked with Annexiv V-FITC and propidium iodide.

The registration of ROS and apoptosis levels was performed with a Beckman Coulter Cell Lab QuantaSC flow cytometer, equipped with a 488 nm laser and with specific excitation and collection filters suitable for the selected fluorochromes.

The collected data were exported as LMD files and analyzed with Flowing Software (developed by Perttu Terho, Turku University, Finland).

All of the experiments were carried out with at least three independent repetitions and all data were expressed as the mean value and standard error of mean (SEM). The statistical analysis was performed using Student's "t" test and the differences were expressed as significant at the level of $p < 0.05$ [Cann, 2002].

RESULTS AND DISCUSSIONS

The dynamic levels of the reactive oxygen species, produced by the metabolic reactions, are permanently maintained in equilibrium by different mechanisms, playing important roles in the cellular metabolism and cell signaling. The effect of different reactive species is dependent by their specific concentrations. Thus, it can be either positive, modulating different signaling networks, or negative, influencing DNA integrity or lipid peroxidation.

Table 2. Percentage distribution of ROS negative and positive cells after incubation with different doses of the P4 and P5 products.

	Vero cells				**HeLa cells**			
	ROS -		ROS +		ROS -		ROS +	
Group	X±SEM	p<	X±SEM	p<	X±SEM	p<	X±SEM	p<
Control	99.92±0.05	-	0.08±0.05	-	99.95±0.02	-	0.05±0.02	-
H_2O_2 5 mM	83.50±1.14	0.001	16.50±1.1	0.001	82.42±1.50	0.001	17.58±1.50	0.001
P4 200 µg/mL	99.94±0.02	NS	0.06±0.02	NS	99.96±0.01	NS	0.04±0.01	NS
P4 300 µg/mL	99.96±0.02	NS	0.04±0.02	NS	100.00±0.00	<0.05	0.00±0.00	<0.05
P5 200 µg/mL	99.20±0.33	0.05	0.80±0.33	NS	99.98±0.01	NS	0.02±0.01	NS
P5 300 µg/mL	99.25±0.24	0.05	0.75±0.24	<0.05	100.00±0.00	NS	0.00±0.00	NS

Prolonged period of incubation (48 hours) of normal and cancerous cells with P4 and P5 didn't significantly influenced the intracellular levels of ROS, those being similar to the control group, as it can be noticed from above table (table 2). In the positive control, in which cells were treated with a solution of 5 mM H_2O_2 within 20 minutes before readings, the production of ROS was intensified with about 17% in both cell lines.

Apoptosis is a dynamic process, essential for the normal development of the multi- and pluricellular organisms, being strictly controlled by different cellular mechanisms, maintaining the normal cellular homeostasis (Elmore, 2007) (Häcker, 2000) (Danial & Korsmeyer, 2004) (Moquin & Chan, 2010).

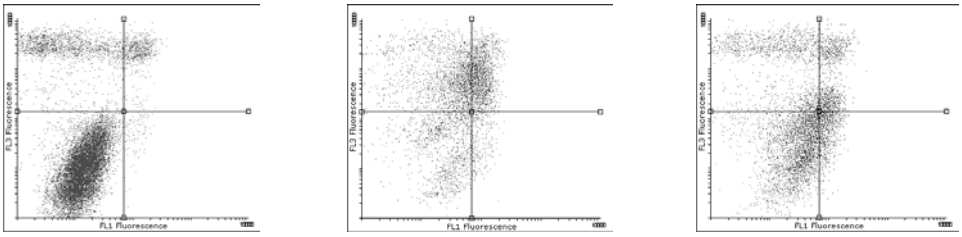
Investigation of apoptosis by annexin V / propidium iodide resides in the strong affinity of annexin V for phosphatidylserine residues (normally hidden within the plasma membrane) on the surface of the cell. During apoptosis, phosphatidylserine is translocated from the cytoplasmic face of the plasma membrane to the cell surface. Propidium iodide is used to discriminate between dead and alive cells, allowing also separation between preapoptotic and apoptotic cells in combination with Annexin V.

The apoptotis intensity in tumoral HeLa and normal Vero cells was assayed after 48 hours of incubation with different doses of the P4 and P5 polyphenolic extracts, the experimental results being included in the figures 1 and 2.

In HeLa cells, the frequency of preapoptotic and apoptotic cells was significantly increased, above the specific frequency of the control group. Due to increase of the apoptosis intensity, the frequency of the dead cells has also augmented in the HeLa cell cultures.

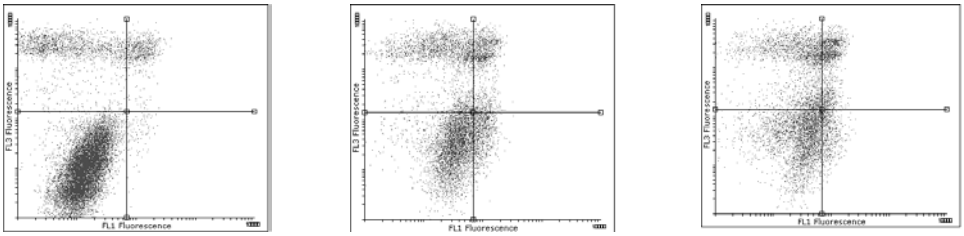
In the case of the P4 biopreparation, the increase of dose determined the amplification of the preapoptotic and cytotoxic effects, as shown in the figure 1**A**. The P5 compound didn't achieved a higher preapoptotic or cytotoxic effect as related to the increase of the dose, the values between

both doses being similar (figure 1**B**).



Control group | P4 200 µg/mL | P4 300 µg/mL

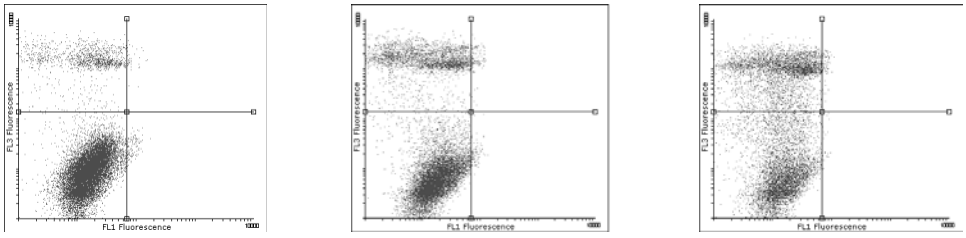
(A) Q1: 91.2% Q2: 5.2% Q3: 3.1% Q4: 0.4% | Q1: 44.6% Q2: 25.8% Q3: 16.5% Q4: 13.0% | Q1: 26.4% Q2: 41.4% Q3: 28.2% Q4: 4.0%



Control group | P5 200 µg/mL | P5 300 µg/mL

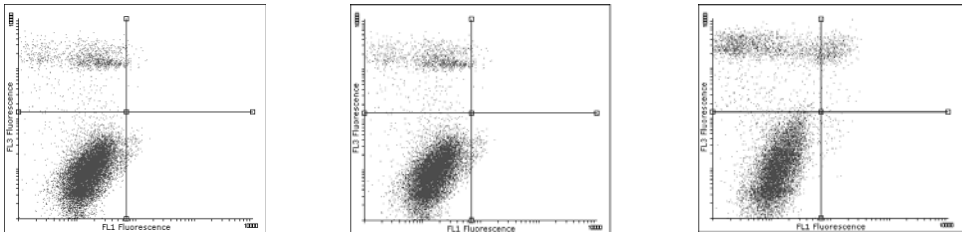
(B) Q1: 91.2% Q2: 5.2% Q3: 3.1% Q4: 0.4% | Q1: 51.7% Q2: 25.1% Q3: 12.7% Q4: 10.5% | Q1: 51.5% Q2: 31.4% Q3: 9.1% Q4: 8.0%

Figure 1. Cytograms of bivariate analysis (Ann V-Fitc vs PI) for cell apoptosis and viability identification in HeLa cultures treated with different doses of P4 **(A)** and P5 **(B)** polyphenolic extracts. Q1 (left-down quarter) denotes viable cells , Q2 (left-up quarter) – dead cells , Q3 (right-up quarter) – apoptotic cells and Q4 (right-down quarter) – preapoptotic cells.



Control group | P4 200 µg/mL | P4 300 µg/mL

(A) Q1: 94.8% Q2: 5.0% Q3: 0.03% Q4: 0.16% | Q1: 76.1% Q2: 23.6% Q3: 0.19% Q4: 0.06% | Q1: 41.9% Q2: 58.0% Q3: 0.01% Q4: 0.00%



Control group | P5 200 µg/mL | P5 300 µg/mL

(B)

Q1: 94.8% Q2: 5.0%
Q3: 0.03% Q4: 0.16%

Q1: 67.2% Q2:32.5%
Q3: 0.21% Q4: 0.01%

Q1: 64.2% Q2: 35.5%
Q3: 0.23% Q4: 0.01%

Figure 2. Cytograms of bivariate analysis (Ann V-FITC vs PI) for cell apoptosis and viability identification in Vero cultures treated with different doses of P4 **(A)** and P5 **(B)** compounds. Q1 (left-down quarter) denotes viable cells, Q2 (left-up quarter) – dead cells, Q3 (right-up quarter) – apoptotic cells and Q4 (right-down quarter) – preapoptotic cells.

It can be seen, from the above figure (figure 2, A and B), that in the case of normal Vero cells both bioproducts have actioned as cytotoxic agents, determining a significant decrease of the cell viability, illustrated by the increase of the dead cell number and the decrease of the alive cells number, the P4 extract inducing a great cytotoxic impact on normal cells, as compared to P5. The apoptosis process was very little amplified in this type of normal cells as compared with the control cultures.

The way of cell malignant transformation from normal to cancerous cell implies a cell to go through complete cycle of progressive changes at cellular, genetic, and epigenetic level that ultimately reprogram a cell to undergo uncontrolled cell division. The complex process of carcinogenesis will assure a number of characteristics (sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis) essential for acquirement of the malignant phenotype (Hanahan & Weinberg, 2011). Due to gained functions, the cancerous cells are able to survive even in the worst conditions or in the case of treatment assault.

Achievement of a targeted therapy, in the conditions of that each individual cancer cell carries no recognizable molecules or structures that make them consistently distinguishable from normal cells (Sonnenschein & Soto, 2011), is a desirable aim.

Addressing to improvement of the antitumoral chemotherapy selectivity, the polyphenols are very promising regarding to prevention of the DNA damages (by their high antioxidant potential) and to impairment of the steady state of the malignant transformed cells.

Vitis vinifera seeds are very rich in bioactive compounds, especially polyphenols, they representing a generous and available source for obtaining new actively biopreparations with agricultural, biomedical and ecological capitalization. In our paper, have been included the results focused on the reactivity of the cell ROS status and apoptosis to the action of some new total polyphenolic bioproducts, extracted from grape seeds.

Analysis of the normal and cancerous cells treated with these compounds doesn't revealed any modifications in the reactive oxygen species levels as compared with the control group. Also, between the cell types weren't registered differences.

The study of cellular apoptosis phenomenon in the same cell cultures has shown that neoplastic cells were more sensitive to the tested compounds as compared with the normal cells. The apoptosis was highly expressed in the case of P4 compound in a dose relationship manner, with the maximum impact at the dose of 300 µg/mL.

Small variations in the oxidative status of the treated cells, as compared with the control group, and apoptosis triggering by polyphenolic compounds proves that cytotoxic effect of the tested compounds is especially due to the proapoptotic effect.

Our investigations, presented in this paper, have revealed that the impact of the polyphenolic extracts , isolated from Vitis vinifera seeds, on the normal and cancerous cells is selective, the cytotoxic effect being higher in neoplastic cells than in normal ones.

CONCLUSIONS

Apoptosis triggering is the main effect of the polyphenolic extracts and was highly expressed in the neoplastic cells treated with P4 biopreparation.

Tested biopreparations were cytotoxically selective with a high degree on HeLa cancerous cells.

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