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EFFECTS OF POLYPHENOLS ON CELL VIABILITY OF SELECTED VARIETIES OF GRAPES BERRIES AND POMACE

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ABSTRACT

The effect on cell viability and content of PhC of three grapes varieties – Moravian Muscat, Blue Burgundy and Lemberger is presented. The effect of polyphenols from wine and grapes was studied for many times, but the effect of pomace, the by-product of wine production, was neglected. Thus study is devoted to compare the effect of berries and pomace on cell viability in context of their utilization as source of bioactive compounds. Effect on viability of human keratinocytes (HaCaT) was investigated *in vitro* using following concentrations of PhC in cultivation medium: 25, 50, 75 and 100 $\mu g \cdot ml^{-1}$. The results show that the content of PhC in berries and pomace was similar and the cell viability decreased with increasing concentrations of PhC, in most cases. The impact on cell viability also depends on individual variety of grapes.

Key words: proliferation, grapes, pomace, phenolic compound, cancer

INTRODUCTION

Phenolic compounds (PhC) have a variety of chemical and biochemical properties as antioxidants [Skrovankova et al. 2016], anticarcinogenic, anti-inflammatory activity [Yang et al. 2001, Jurikova et al. 2012] or cytoprotective effects which is caused by either ameliorating the toxic effects of chemotherapeutic agents or by influencing gene expression [Bonfili et al. 2008]. Positive effect of PhC can be used in treatment and prevention of cancer [Zinov'eva et al. 2011] in combination with standard procedures as surgery [Gatek et al. 2013], neurodegenerative disorders [Mandel et al. 2004], coronary heart diseases [Sharif et al. 2010] and strokes [Neto 2007]. Mentioned effects are connected to the attribute of PhC which affect signal transduction pathways, causing inhibition of cell growth, enhanced apoptosis [Lambert et al. 2005], arresting cell cycle progression or triggering cell death [Sharif et al. 2010].

Present study is focused on the detection of content of PhC in grapes berries and pomace and their effect on cell viability. The second, related aim, is to detect the possible use of pomace in food industry.

MATERIALS AND METHODS

Extraction conditions. PhC were extracted from three grape varieties from Czech Republic – Moravian Muscat, Lemberger and Blue Burgundy, specifically pomace of Moravian Muscat (MM_P), berries of Moravian Muscat (MM_B), pomace of Lemberger (L_P), berries of Lemberger late harvest (L_B_LH), pomace of Blue Burgundy (BB_P), berries of Blue Burgundy (BB_B) and berries of Blue Burgundy late harvest

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(BB_B_LH). Grapes berries and pomace were mixed and fermented with yeast Oenoferm (Erbslöh, Geisenheim). Than they (10 g) were homogenized in methanol (100 ml) and subsequently extracted on vortex at –4°C for 3 hours. After extraction the centrifugation at 3,000 × g was employed for 10 minutes to separate the supernatant. Sediment was subjected to new extraction. This process was repeated three times. The supernatants containing PhC were dried using Laborota4011 digital (Heidolph, Germany). Finally, the extracts were concentrated to the final concentration of 1000 mg·ml^{-1} [Hakimuddin et al. 2008].

Cell cultivation. The human immortalized non-tumorigenic keratinocyte cell line (HaCaT) [Boukamp et al. 1988] supplied by Cell Lines Service (Catalog No. 300493, Eppelheim, Germany) was used. Dulbecco's Modified Eagle Medium (DMEM) – high glucose, with added fetal bovine serum (10%) and penicillin/streptomycin (100 U·ml^{-1}) (100 µg·ml^{-1}) (PAA Laboratories GmbH, Pasching, Austria) was used as the culture medium.

Antiproliferation tests. The methanolic extracts (containing PhC) were diluted in culture medium (DMEM) to obtain dilutions with concentrations of 25, 50, 75 and 100 µg of PhC per ml of cultivation medium. All dilutions were used immediately. Cells were pre-cultivated for 24 hrs and the culture medium was subsequently replaced by dilutions. As a control experiment, pure medium without PhC was used. To assess antiproliferative activity on HaCaT cells, the MTT assay (Invitrogen Corporation, Carlsbad, California, USA) [Mosmann 1973] was performed after seven-day cultivation in dilutions. The absorbance was measured at 540 nm using a Sunrise microplate absorbance reader (Tecan, Männedorf, Switzerland). The cell proliferation expressed as MTT absorbance measured in respective dilutions relative to control is presented. All the tests were performed in quadruplicate. The photomicrographs were taken using an inverted Olympus CKX41 phase contrast microscope (Olympus, Hamburg, Germany). The differences between observed absorbance were detected by Student's t-test using Statistica for Windows [Kucekova et al. 2011].

Determination of total PhC. A standard solution of tannin (Sigma Aldrich) was prepared from tannin (50 mg) dissolved in water (100 ml). The standard solution of tannin was added using a pipette to six 50 mL flasks in volumes of 0.2, 0.3, 0.4, 0.5 ml. Extract (1 ml) was added to the seven flasks and dissolved as needed. Distilled water (20 ml) and the Folin-Ciocalteu reagent (1 mL) was added to every flask. After three minutes 20% solution Na_2CO_3 (5 ml) was added. The solutions were mixed and the distilled water was added to a volume of 50 ml. After 30 minutes the color intensity compared to control (no tannin) was measured at 700 nm.

HPLC analysis of individual PhC. For analysis of individual polyphenols was used method by de Quiros et al. [2010] with some modifications: Polyphenols were analysed by HPLC Dionex 3000, USA with UV-VIS detection and an Chromeleon 7 (systems software). Chromatographic separation was carried out on a Column: Phenomenex Kinetex C18 150 × 4.6 mm. The flow rate was 1 ml·min^{-1}. The injection volume was 10 µl. The separation was performed at room temperature (30°C). Detector was set at 275 nm. The data obtained were analyzed statistically by the analysis of variance (ANOVA) and Tukey's multiple range test for comparison of means.

Detection of apoptosis by flow cytometry. Cells were pre-cultivated for 24 hrs and the culture medium was subsequently replaced by dilutions of PhC in conncentration 100 µg·ml^{-1}. As a control experiment, pure medium without PhC was used. After 24 hrs of incubation with PhC, trypsinized cells were stained by adding propidium iodide solution (50 µg·ml^{-1} in PBS). Stained cells were analyzed using FACSCanto II flow cytometer.

The data presented are the average values calculated from three measurements.

RESULTS AND DISCUSSION

The content of PhC in grapes berries or pomace is influenced by many factors, for example by ground, species, harvest time, temperature or climatic conditions of the region [Soleas at al. 1997]. The content of total PhC in extracts were in berries of Moravian Muscat (MM_B) 730.0 µg·ml^{-1}; pomace of Moravian Muscat (MM_P) 807.5 µg·ml^{-1}; pomace of Blue Burgundy (BB_P) 1402.5 µg·ml^{-1}; berries of Blue Burgundy

(BB_B) 1440.0 $\mu g \cdot ml^{-1}$; berries of Blue Burgundy late harvest (BB_B_LH) 1302.5 $\mu g \cdot ml^{-1}$, pomace of Lemberger (L_P) 747.5 $\mu g \cdot ml^{-1}$ and berries of Lemberger late harvest (L_B_LH) 905.0 $\mu g \cdot ml^{-1}$. The content of PhC was similar in both parts of grapes (berries and pomace), which indicate that pomace could be suitable source of PhC like berries.

Table 1 shows the phenolic compounds identified and quantified in the berries and pomace extracts. These compounds included gallic acid, catechin, vanillic acid and caffeic acid were the most abundant compounds in the extracts. Generally we can say that the pomace contains more individual polyphenols than grape berries. This is caused mainly higher content of phenolic compounds found in skin and seed than in the pulp [Di Lecce et al. 2014]. De la Cerda-Carrasco et al. [2014] states, that pomaces from the white grape varieties showed significantly higher contents of polyphenols than pomaces from the red grape varieties. This wasn't confirmed in our work. But the average results are consistent with this study. In particular, BB_B_LH showed the highest levels and BB_B the lowest levels of most of those polyphenols. Catechin was the most abundant compound identified in the grape pomace for all varieties. Similar statement presents number of authors [Iacopini et al. 2008, Rockenbach et al. 2011].

In this study the human immortalized non-tumorogenic keratynocite cell line was used to determine the effect on cell viability of PhC from grapes berries and pomace at concentrations that range from 25 $\mu g \cdot ml^{-1}$, 50 $\mu g \cdot ml^{-1}$, 75 $\mu g \cdot ml^{-1}$ and 100 $\mu g \cdot ml^{-1}$. The results are shown in table 1. It is evident that different varieties of grapes as well as concentrations of PhC have different effects on the viabilities of the cells. Moreover, it was determined if the PhC affect the cell viability by cytotoxic or anti-proliferative effect by staining with propidium iodide (PI) (tab. 2). Concentration 25 $\mu g \cdot ml^{-1}$ seems to be a critical concentration because only PhC extracted from BB_P influenced the cell viability significantly in this concentration. In three cases the cell viability even slightly increased compare to control. It is in case of L_P, L_B_LH and BB_B (tab. 2).

As mentioned, remarkable differences in cell viability between different concentrations were observed. Different cell viability between the concentrations of PhC 50 and 75 $\mu g \cdot ml^{-1}$ are about 20 to 40%. These differences were not detected only in extracts BB_B and BB_P. In case of extracts of BB_P, concentrations higher than 50 $\mu g \cdot ml^{-1}$ have similar impact on proliferation of cells. In others cases, the proliferation decreases with increasing concentrations of PhC. The lowest effect on cell viability showed extract from BB_B in all concentrations, it only slightly influence the proliferation on HaCaT cells. On the other hand, BB_B reaches 13% of apoptic cells after one-day treatment, so this effect could be considered as slightly cytotoxic. Similar proportion of apoptic cells (16%) reached also cells after treatment with MM_P.

Table 1. Phenolic compounds ($mg \cdot kg^{-1}$) of grape berries and pomace

Polyphenol	MM_P	MM_B	L_P	L_B_LH	BB_P	BB_B	BB_B_LH
Gallic acid	12.18 ±2.4^{c}	8.16 ±1.03^{b}	19.23 ±1.21^{d}	8.62 ±1.24^{b}	8.26 ±1.16^{b}	3.13 ±0.80^{a}	13.78 ±2.05^{c}
Catechin	77.57 ±5.91^{a}	80.47 ±6.59^{a}	81.49 ±6.31^{a}	80.33 ±4.81^{a}	167.24 ±5.27^{b}	77.58 ±1.18^{a}	170.96 ±4.75^{b}
Vanillic acid	3.64 ±0.66^{a}	3.76 ±0.55^{a}	3.61 ±0.55^{a}	5.94 ±0.09^{b}	4.01 ±0.16^{ab}	3.74 ±0.06^{a}	7.22 ±0.25^{c}
Caffeic acid	1.10 ±0.04^{b}	0.18 ±0.02^{a}	11.46 ±2.01^{c}	11.06 ±1.92^{c}	11.26 ±2.59^{c}	1.28 ±0.06^{b}	1.79 ±0.09^{b}
Coumaric acid	0.14 ±0.01^{a}	0.10 ±0.02^{a}	0.57 ±0.09^{bc}	0.14 ±0.02^{a}	0.67 ±0.03^{c}	0.17 ±0.04^{a}	0.48 ±0.05^{b}
Ferrulic acid	0.04 ±0.01^{a}	0.09 ±0.04^{a}	1.44 ±0.06^{e}	1.01 ±0.06^{d}	0.25 ±0.01^{b}	0.44 ±0.03^{bc}	0.54 ±0.02^{c}
Rutin	0.17 ±0.03^{b}	0.12 ±0.06^{a}	0.12 ±0.01^{a}	0.31 ±0.02^{bc}	0.38 ±0.05^{c}	0.10 ±0.01^{a}	1.03 ±0.04^{d}
Resveratrol	0.28 ±0.08^{c}	0.42 ±0.15^{e}	0.36 ±0.01^{d}	0.13 ±0.05^{a}	0.12 ±0.01^{a}	0.28 ±0.05^{c}	0.21 ±0.08^{b}
Cinnamic acid	nd	nd	nd	nd	nd	nd	0.01 ±0.00^{a}
Quercetin	nd	nd	1.48 ±0.15^{d}	1.24 ±0.01^{c}	0.52 ±0.06^{b}	0.33 ±0.07^{a}	0.31 ±0.05^{a}

nd – not detected. Different superscripts in each row indicate the significant differences in the mean at $P < 0.05$

Table 2. Effect on cell viability of grapes' PhC (average absorbance ± SD)

Sample μg·ml^{-1}	Absorbance	%	Sample μg·ml^{-1}	Absorbance	%
MM_P 100	0.2181 ±0.0148**	33	L_B_LH 25	0.6656 ± 0.1045	101
MM_P 75	0.1956 ±0.0323**	30	BB_P 100	0.2281 ± 0.0091**	34
MM_P 50	0.4029 ±0.0475**	61	BB_P 75	0.2571 ± 0.0203**	39
MM_P 25	0.6240 ±0.0929	94	BB_P 50	0.2257 ± 0.0204**	34
MM_B 100	0.3037 ±0.0345**	46	BB_P 25	0.4269 ±0.0280**	65
MM_B 75	0.3860 ±0.0382**	58	BB_B 100	0.5839 ±0.0481**	88
MM_B 50	0.5317 ±0.1008**	80	BB_B 75	0.6082 ±0.0949	92
MM_B 25	0.6538 ±0.0438	99	BB_B 50	0.6832 ±0.1068	103
L_P 100	0.2553 ±0.0082**	39	BB_B 25	0.8790 ±0.1550**	133
L_P 75	0.2598 ±0.0680**	39	BB_B_LH 100	0.2350 ±0.0208**	36
L_P 50	0.5478 ±0.1204	83	BB_B_LH 75	0.2796 ±0.0133**	42
L_P 25	0.8287 ±0.0742**	125	BB_B_LH 50	0.4386 ±0.0540**	66
L_B_LH 100	0.2637 ±0.0426**	40	BB_B_LH 25	0.5970 ±0.0640	90
L_B_LH 75	0.3981 ±0.0454**	60	Control	0.6612 ±0.0774*	100
L_B_LH 50	0.6176 ±0.0727	93			

Values with different superscripts show significance level within column: $P < 0.01$ (*,**)

Table 3. Flow cytometry results

Sample	All events (%)	PI- (events/%)	PI+ (events/%)
MM_P	7137**	5988/99	1149/16
MM_B	14920**	14744/84	176/1
L_P	6837**	6562/96	275/4
L_B_LH	6865**	6484/94	381/6
BB_P	8761**	6485/74	2276/26
BB_B	7996**	6956/87	1040/13
BB_B_LH	6692**	6082/91	610/9
Control	25769*	25340/98	429/2

Values with different superscripts show significance level within column: $P < 0.01$ (*,**). PI- propidium negative cells, PI+ propidium positive cells

The flow cytometric method proved that there are statistically significant differences between treated cells and control in cell viability, what confirm also the MTT results. In addition, PI staining demonstrated the character of different levels in cell viability. From the table 3 is obvious that lower values than 10% of apoptic cells reached followed samples: MM_B, L_P, L_B_LH and BB_B_LH. These results confirmed that these samples do not have cytotoxic effect in used concentrations and they suppress the proliferation of cells.

The highest ratio of apoptic cells (26%) was observed in cells treated with BB_P, sample that achieved the highest impact on cell viability after seven-day treatment. Hence, this effect could be specified as cytotoxic.

The differences in cell viability between flow cytometry and MTT assay is caused by different exposure time by PhC. MTT assay shows the influence of cell viability and flow cytometry the induction of apoptosis, which occurs after 24hrs.

If consider all extracts, the lowest amount of apoptic cells was observed after treatment with MM_B, this sample reached 46% of cell viability by MTT assay. After treatment with samples L_P and L_B_LH were obtained moderate amount of apoptic cells as well. These samples increased cell viability to 39% and 40% by MTT assay, respectively.

Described results can be associated with suppressing the inflammatory processes that lead to transformation, hyperproliferation, and initiation of carcinogenesis. One of many possible mechanisms as can PhC influence the proliferation is their effect on the phosphoinositide 3-kinase (PI3K)/Akt, glycogen synthase kinase-3 (GSK-3) pathway. The PI3K/Akt pathway, one of the apoptotic pathways, is an important pathway involved in cell survival. PI3K is a lipid kinase and it plays role in cell cycle progression, differentiation, survival, invasion and metastasis [Roy et al. 2008]. Also, PI3K/Akt signalling pathway occurs in human cancer [Poolman et al. 2005]. It has been reported that PhC affects PI3K/Akt signalling pathway. According Roy et al. [2008], resveratrol, which is contained in grapes, can block signal transduction pathway through the PI3K/Akt. PI3K/Akt pathway also inhibits quercetin [Kang et al. 2011]. Effect of gallic acid, epigallocatechingallate and epicatechin on PI3K/Akt pathway was described by Mustafi et al. [2010] or Lu et al. [2010]. These PhC affected upstream signalling through PI3K/Akt pathway. GSK-3 is a serine/threonine protein kinase that mediates oxidative-stress-mediated apoptosis through caspase-3 by releasing cytochrome c from mitochondria [Lu et al. 2010]. GSK-3 regulates the function of structural and signalling proteins, such as activator protein-1. Activation of GSK-3, activates the mitochondrial death pathway [Ali et al. 2001]. Treatment with PhC inhibit GSK-3 phosphorylation, the effect of PhC on GSK-3 was studied by Han et al. [2007] or Patel et al. [2008].

Our results suggest that the antiproliferative effect depends on each particular variety of grape and different composition of PhC, but is independent on the part of grape. Pomace, which are by-product, reach similar content of PhC and antiproliferation effect as berries, this make pomace useful for prevention and treatment of tumour diseases. Presence of other biological active compounds in extracts can also influence the proliferation effect, but these compounds have not been determined in this study.

CONCLUSION

Grapes contain a variety of phytochemicals, including phenolic compounds (PhC), which have among other, anticancer activities. The results shows that PhC extracted from pomace and berries have promising antiproliferative effect on HaCaT cells, because phenolic compounds contained in grapes significantly decrease proliferation of used cell line. It also demonstrates that this effect varies with used variety of grapes and that pomace have similar effect as berries, so they can be used in practical applications. These results suggest that grapes extracts could be useful for treatment and prevention of tumour diseases.

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