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Anthocyanin determination in blueberry extracts from various cultivars and their antiproliferative and apoptotic properties in B16-F10 metastatic murine melanoma cells

Andrea Bunea [a], Dumitriţa Rugină [a,*], Zoriţa Sconţa [a], Raluca M. Pop [a], Adela Pintea [a], Carmen Socaciu [a], Flaviu Tăbăran [a], Charlotte Grootaert [b], Karin Struijs [b], John VanCamp [b]

[a] Department of Chemistry and Biochemistry, University of Agricultural Sciences and Veterinary Medicine, Mănăştur 3-5, 400372 Cluj-Napoca, Romania
[b] Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

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ABSTRACT

Blueberry consumption is associated with health benefits contributing to a reduced risk for cardiovascular disease, diabetes and cancer. The aim of this study was to determine the anthocyanin profile of blueberry extracts and to evaluate their effects on B16-F10 metastatic melanoma murine cells. Seven blueberry cultivars cultivated in Romania were used. The blueberry extracts were purified over an Amberlite XAD-7 resin and a Sephadex LH-20 column, in order to obtain the anthocyanin rich fractions (ARF). The antioxidant activity of the ARF of all cultivars was evaluated by ABTS, CUPRAC and ORAC assays. High performance liquid chromatography followed by electrospray ionization mass spectrometry (HPLC–ESI-MS) was used to identify and quantify individual anthocyanins. The anthocyanin content of tested cultivars ranged from 101.88 to 195.01 mg malvidin-3-glucoside/100 g fresh weight. The anthocyanin rich-fraction obtained from cultivar Torro (ARF-T) was shown to have the highest anthocyanin content and antioxidant activity, and inhibited B16-F10 melanoma murine cells proliferation at concentrations higher than 500 μg/ml. In addition, ARF-T stimulated apoptosis and increased total LDH activity in metastatic B16-F10 melanoma murine cells. These results indicate that the anthocyanins from blueberry cultivar could be used as a chemopreventive or adjuvant treatment for metastasis control.

1. Introduction

1.1. Introduction and structures

Blueberry fruits belong to the genus *Vaccinium*, fam. Ericaceae and are known for their beneficial health effects against chronic diseases such as cancer and diabetes (Grace et al., 2009; Kong et al., 2003; Seeram et al., 2006; Zafra-Stone et al., 2007). A mechanisms involved in the mode of action is based on their antioxidant (Guerra et al., 2005), anti-inflammatory (Triebel et al., 2012) and chemopreventive properties (Bowen-Forbes et al., 2010). The antioxidant activity of blueberry fruits is dependent on their phytochemical content, being mainly represented by anthocyanins, procyanidins, chlorogenic acid and other flavonoid compounds (Moyer et al., 2002). Anthocyanins are secondary metabolites of plants, and are the most important subclass of flavonoids (He and Giusti, 2010). The de-glycosylated or aglycone forms of anthocyanins are known as anthocyanidins. The most common anthocyanidins found in blueberries are cyanidin, delphinidin, petunidin, paeonidin, and malvidin (Fig. 1). The anthocyanidins in blueberries are mostly glycosylated with glucopyranose, galactopyranose or arabinopyranose at position 3 of the C ring (Müller et al., 2012). A high variation exists between different *Vaccinium* species (Prior et al., 1998) and for some cultivars (such as Hannah's Choice) the individual anthocyanin content has not been studied yet.

1.2. Biological relevance

The anthocyanin content, structure and antioxidant activity of different varieties of berries are important in the field of nutritional intake and are of interest for the alimentary, and pharmaceutical industry (Espin et al., 2007). Anthocyanins with different aglycones and sugar moieties have different bioavailability and potential health effects (Wu et al., 2006). It has been shown that blueberries compounds function as strong antioxidants (Schantz et al., 2010), inhibit the growth of tumor cells (Liu et al., 2010; Seeram et al., 2006) and induce apoptosis (Srivastava et al., 2007). Pure anthocyanins such as delphinidin (30–240 μM), as well as paeonidin 3-glucoside (30–100 μM) and cyanidin 3-glucoside (30–100 μM)

* Corresponding author. Tel.: +40 264 596384/126; fax: +40 264 593792.
E-mail addresses: dumitrita.rugina@usamvcluj.ro, oliviapreda@gmail.com (D. Rugină).

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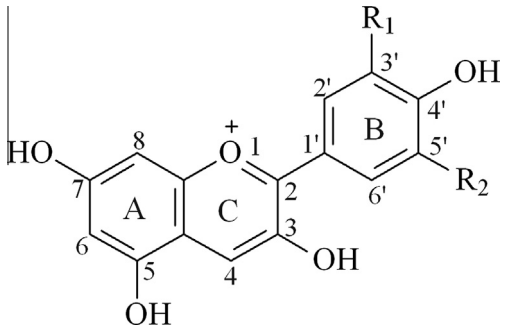

Fig. 1. Anthocyanins commonly present in blueberries, their sugar moieties and structures with A, B, C aromatic rings and R_1 and R_2 substitution sites.

suppressed growth of human tumor cells by causing G2/M cell cycle arrest and apoptosis of HCT116 colon and HS578T breast cell lines (Chen et al., 2005; Yun et al., 2009). Only a limited number of studies are available for the antiproliferative and the proapoptotic effects of blueberry cultivars on tumor cells. A bog bilberry anthocyanin rich extract was found to decrease cell proliferation, to increase the accumulation of sub-G1 cells and lactate dehydrogenase (LDH) activity in malignant cancer cell lines Caco-2 and Hep-G2 (Liu et al., 2010). In another study, black raspberry, strawberry and blueberry extracts, containing significant amounts of anthocyanins, were shown to stimulate apoptosis in a HT-29 colon cancer cell line (Seeram et al., 2006).

1.3. Bioactivity on skin

The mulberry anthocyanin fraction, obtained from lyophilized fruits, had an antiproliferative effect and modulated the metastasis signaling pathways in B16-F1 murine melanoma cells (Huang et al., 2008). Recently it was demonstrated that delphinidin pretreatment protected the non-tumorigenic human immortalized HaCaT keratinocytes and the SKH-1 hairless mice skin from UVB-induced apoptosis, by inhibition UVB-mediated oxidative stress and reduction of DNA damage (Afaq et al., 2007). In another study, a pomegranate extract containing anthocyanins, ellagitannins and hydrolyzable tannins was applied on the skin of CD-1 mice, and resulted in decreased 12-*O*-tetradecanoylphorbol-13-acetate-induced skin tumor incidence (70% reduction) and tumor multiplicity (64% reduction) after 16 and 30 weeks of the bioassay (Afaq et al., 2005). A correlation might be found between anthocyanins and markers for skin cancer development.

1.4. Purpose of the study

Therefore, the objectives of this study are (i) to quantify and identify individual anthocyanins in different Romanian blueberry cultivars, (ii) to evaluate their antioxidant activity, in order to select the richest anthocyanin fraction with the highest antioxidant activity and (iii) to test the selected fraction for its ability to inhibit proliferation and stimulate apoptosis in a B16-F10 metastatic murine melanoma cell line.

2. Material and methods

2.1. Reagents

In Vitro Toxicology Assay Kit (LDH based TOX7), glutamine, penicillin and streptomycin, amphotericin, 2,9-dimethyl-1,10-phenanthroline (Neocuproine), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, Amberlite XAD-7 and Sephadex LH-20 were purchased and malvidin-3-glucoside from Sigma Chemical Co. (St. Louis, MO). Cyanidin 3-glucoside standard was bought from Polyphenols (Sandnes, Norway). Fetal bovine serum (FBS)-Lonza, Dulbecco's Modified Eagle Medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Lonza Group Ltd. (Basel, Switzerland). Methanol (MeOH), trifluoroacetic acid (TFA), ethylacetate (EtOAc), formic acid, acetonitrile, $CuCl_2$, Trolox, dimethylsulfoxide (DMSO), ethidium bromide/acridine orange (EB/AO) and paraformaldehyde were purchased from Merck (Darmstadt, Germany). ApopTag® Red In Situ Apoptosis Detection Kit was from Chemicon (Millipore, Bedford, MA) and Draq5 from Cell Signaling Technology, Inc. (Beverly, MA).

2.2. Preparation of anthocyanin fraction

Seven cultivars of highbush blueberries (Bluegold, Nui, Darrow, Legacy, Nelson, Hannah's Choice and Toro) were purchased in August, 2011 from local farms situated in north-west of Romania. The protocol for obtaining anthocyanin fractions from fresh blueberries was adapted to some recent literature studies (Cuevas-Rodriguez et al., 2010; Grace et al., 2009). Anthocyanin and non-anthocyanin compounds were extracted from fresh blueberries (1 g) in acidified methanol (0.3% HCl (v/v)) using a homogenizer (Miccra D-9 KT Digitronic, Bergheim, Germany). Re-extraction was done until the blueberry residue was colorless, the final extraction was done overnight at 4 °C in the dark. Acidified methanol (0.3% HCl) was used to prevent anthocyanins from degradation (Grace et al., 2009). The colored extract obtained was filtered through multiple layers of cotton, and then concentrated under vacuum at 35 °C to remove methanol. The extract containing anthocyanin and non-anthocyanin compounds was subjected to a liquid–liquid extraction using ethyl acetate, to remove the less polar compounds. The remaining aqueous fraction was then applied to an Amberlite XAD-7 column (1 × 0.5 cm) preconditioned by 6 volumes of 0.3% TFA in H_2O (v/v). After loading of the extract, the Amberlite XAD-7 resin was washed with 4 volumes of H_2O (0.3% TFA (v/v)) in order to remove free sugars, pectins and other impurities. The anthocyanins and procyanidins were then eluted with 4 volumes of MeOH (0.3% TFA (v/v)). This anthocyanins and procyanidins containing fraction was further purified on a Sephadex LH-20 column (2.5 × 0.5 cm), preconditioned and eluted with 10 volumes H_2O:MeOH (0.3% TFA (v/v)) (8:2) obtaining only the anthocyanin rich fraction (ARF). The volume of ARF was made up to 5 ml with distilled water, filtered through 0.45 μm and analyzed by HPLC–DAD–ESI-MS and for antioxidant activity.

2.3. HPLC–DAD analysis of anthocyanins

Analyses were performed on a Shimadzu HPLC system equipped with a binary pump delivery system LC-20 AT (Prominence), a degasser DGU-20 A3 (Prominence), diode-array SPD-M20 A UV–vis detector. A Luna Phenomenex C-18 column (5 μm, 25 cm × 4.6 mm) was used. The mobile phase used was 4.5% formic acid in bidistilled water (solvent A) and acetonitrile (solvent B). The gradient elution system started with 10% B for 9 min. The percent of B increased linearly to 12% at 17 min and continued up to 25% B at 30 min. Between 30 and 50 min the percentage of B was 90%. The flow rate was 0.8 ml/min and the analyses were performed at 35 °C. Data were collected at 520 nm. Anthocyanin quantification was done using malvidin-3-glucoside as standard, in the concentration range 2.5–500 μg/ml, and the calibration curve linearity was excellent ($r^2 > 0.998$). Chromatograms were recorded at 520 nm.

2.4. HPLC–ESI-MS analysis of anthocyanins

Samples were analyzed on an Agilent Technologies 1200 HPLC system (Chelmsford, MA) equipped with G1311A Quaternary Pump, G1322A degasser, G1329A autosampler and G1315D photo-diode array (PDA) detector. Volumes of 10 μl were injected on a Luna Phenomenex C-18 column (5 μm, 25 cm × 4.6 mm). The mobile phase was composed of 1% formic acid in bidistilled water (solvent A) and acetonitrile (solvent B). The flow was maintained at 500 μl/min. The gradient elution system started with 10% B for 9 min. The percent of B increased to 12% at 17 min and continued up to 25% B at 30 min. Between 30 and 50 min the percentage of B was 90%. PDA recorded full spectra. *In-line* MS data were recorded by directing the LC flow to a Quadrupole 6110 mass spectrometer (Agilent Technologies, Chelmsford, MA) equipped with an ESI probe. Most settings were optimized using Flow Injection Analysis (FIA) (Agilent ChemStation) via automatic tuning with cyanidin 3-glucoside in positive ion mode. The spray voltage was set at 3000 V. Nitrogen was used as nebulizer gas and nebulizer pressure was set to 40 psi with a source temperature of 100 °C. Desolvation gas (nitrogen) was heated at 350 °C and delivered at a flow of 8 l/min. Mass spectra were acquired in positive ion and full scan mode in a range of 260–1000 *m*/*z*. Molecular ions and fragment ions were determined by setting the fragmentation voltage at 70 and 130 eV. When the lower voltage was applied (70 eV) the ions passed unchanged through the fragmentation region. When the higher voltage was applied (130 eV), fragmentation was achieved to obtain molecular fragments further used for structural determination. Identification of anthocyanins was carried out based on molecular mass determination, masses and occurrence of fragments, elution order and literature data reported previously (Gavrilova et al., 2011; Latti et al., 2009; Nicoué et al., 2007; Prior et al., 2001).

2.5. Cupric reducing antioxidant capacity (CUPRAC) assay

The cupric ion reducing antioxidant capacity of all ARF samples was determined according to the method of Apak et al. (2007). The absorbance was recorded using the spectrophotometer (JASCO V-630 series, International Co., Ltd., Japan) at 450 nm against the blank reagent. A standard curve was prepared using different Trolox concentrations and the results were expressed as μmol Trolox per gram fresh weight (fr.wt).

2.6. Scavenging effect on ABTS radical

The scavenging ability of all ARF samples against the radical anion $ABTS^{\cdot+}$ was determined in 96-well plates according to the procedure described by Arnao et al. (2001). Absorbance was measured after 6 min of incubation in the dark at room temperature, using the microplate reader at 734 nm (BioTek Instruments, Winooski, VT). Results were expressed as μmol Trolox/g fr.wt.

2.7. Oxygen radical absorbance capacity (ORAC) assay

The antioxidant activity of ARF samples was measured and calculated by the oxygen radical absorbance capacity assay, as described previously (Huang et al., 2002). ORAC values were expressed as μmol Trolox/g fr.wt.

2.8. Cell and cell culture

The B16-F10 metastatic murine melanoma cell line was obtained from American Type Culture Collection (Rockville, MD, USA). B16-F10 cells were grown in DMEM containing 1 g/l glucose, supplemented with 10% FBS, 2 mM glutamine, 1% penicillin and streptomycin, 0.1% amphotericin. Cells were cultured in a humidified, 5% CO_2 atmosphere at 37 °C. For microscopic analysis, cells were grown on coverslides.

2.9. Analysis of cell viability

B16-F10 cells (8×10^3 cells/well) were seeded on a 96-well plate and cultured in DMEM containing 10% FBS for 24 h. The medium was then replaced with complete medium with or without ARF-T (cultivar Toro anthocyanin fraction) at various concentrations (0, 200, 400, 500, 550, 600, 650, 700, 750 μg/ml). A stock solution of ARF-T was prepared with serum-free medium containing 0.3% DMSO. Anthocyanin treatment was applied for 24 h at 37 °C and 5% CO_2. The supernatant was removed and MTT reagent in HBSS buffer (0.5 mg/ml) was added to each well. After 2 h of incubation MTT solution was removed and the formazan crystals were dissolved in DMSO. The solubilized formazan formed in viable cells was measured at 550 nm and 630 nm (for sample and background, respectively) using the microplate reader HT BioTek Synergy (BioTek Instruments, USA). The results were expressed as percent survival relative to an untreated control.

2.10. Detection of LDH activity

Damage of the plasma membrane was evaluated in B16-F10 cells by measuring lactate dehydrogenase (LDH) leakage. B16-F10 cells (8×10^3 cells/well) cultivated on a 96-well plate with or without ARF-T (550, 600, 650 μg/ml) for 24 h, were centrifuged at 250 g for 4 min at room temperature prior to the assay and 50 μl of the supernatants was transferred into new wells. The enzymatic analysis was done according to the manufacturer's instructions Toxicology Assay Kit (TOX7 Sigma, St. Louis, MO). Absorbance values, measured at 490 nm, were translated into LDH leakage percents relative to untreated B16-F10 cells.

2.11. Apoptotic index by 96-well-based EB/AO staining method

In order to assess the apoptotic index and the cell membrane integrity, acridine orange (AO)/ethidium bromide (EB) staining was performed. B16-F10 cells (8×10^3 cells/well) were seeded on a 96-well plate and cultured in DMEM containing 10% FBS for 24 h. The medium was then replaced with complete medium containing ARF-T at a concentration of 600 μg/ml. After incubation for 24 h, B16-F10 cells were stained according to a previously reported method for apoptotic quantification (Ribble et al., 2005). Cells were viewed under the Carl Zeiss Observer A1 microscope, with AxioVision image processing software (Jena, Germany). Early apoptotic cells have a bright green nucleus with condensed or fragmented chromatin. The late apoptotic cells nucleus with condensed and fragmented chromatin appears orange. The cells that have died from necrosis have a red nucleus (Ribble et al., 2005).

2.12. TUNEL assay and analysis

B16-F10 cells (8×10^4 cells/well), cultured on two-well chamber slides, were treated with 600 μg/ml of blueberry anthocyanins for 24 h. Prior to the confocal microscopy TUNEL assay, the adherent cells B16-F10 were fixed with 4% paraformaldehyde for 15 min. The slides were processed for a TUNEL assay using an ApopTag® Red In Situ Apoptosis Detection Kit (Chemicon, Millipore, Billerica, MA) according to the manufacturer's instructions. Nuclei were counterstained with 5 mM Draq5 diluted 1:1000 in distilled water for 5 min at room temperature. Fluorescent images were acquired with a confocal laser scanning microscope (Zeiss LSM 710). The number of TUNEL-positive cells per 1000 cells was counted in

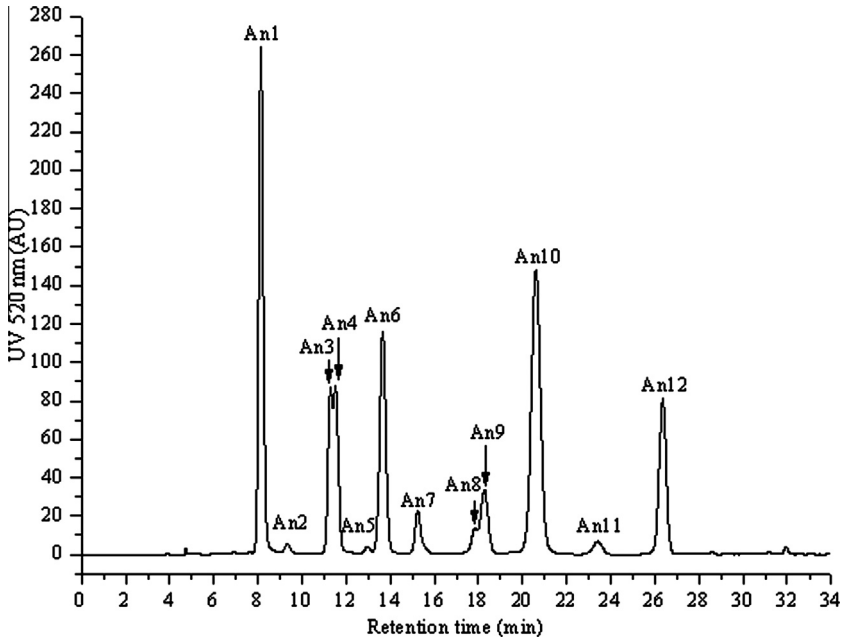

Fig. 2. Chromatogram of *Vaccinium corymbosum* L. cv. Toro anthocyanin-rich fraction (ARF-T) (peak numbers correspond to those from Table 1).

various areas and expressed as an apoptotic index with the percentage apoptotic cells of the total cells counted.

2.13. Statistical analysis

The data were expressed as mean ± standard deviation (SD) from three replicates for each sample. For the cell culture experiments, one replication means the average of five wells containing ARF-T treated or non-treated (control) cells. In order to determine significant differences between values, analysis of variance (ANOVA) and Duncan multiple range tests (MRT) were performed. Significance of difference was defined at the 5% level ($p < 0.05$).

3. Results and discussions

3.1. Identification and quantification of blueberry anthocyanins

Highbush blueberry (*Vaccinium corymbosum* L.) varieties and lowbush blueberries (*Vaccinium angustifolium* Ait.) were extensively studied, and besides the anthocyanins, were found to be a good source of flavanol, procyanidins, vitamin C and chlorogenic acid, (Castrejón et al., 2008; Ehlenfeldt and Prior, 2001; Garzón et al., 2010; Gavrilova et al., 2011; Giovanelli and Buratti, 2009; Rodriguez-Mateos et al., 2012). Prior et al. (1998) reported a lower anthocyanin content in lowbush blueberry relative to highbush blueberry, in contradiction to data from another study (Gao and Mazza, 1994a). Recently, it has been observed that the anthocyanin levels are similar between highbush and lowbush varieties. Lowbush bilberries (*Vaccinium myrtillus* L.) have a great importance in the field as well, containing higher amounts of anthocyanins than the wild blueberry (*V. angustifolium* L.) and the cultivated highbush (*V. corymbosum* L.) (Lätti et al., 2008). The anthocyanin profile of all varieties showed that delphinidin was the dominant anthocyanidin in all varieties, followed by malvidin and petunidin (Rodriguez-Mateos et al., 2012).

For our study seven highbush blueberry cultivars grown in Romania were choosed in order to analyze the anthocyanin content and composition of ARF extracts obtained. To our knowledge, this is the first time that the anthocyanin composition of Nui and Hannah's Choice highbush blueberry extracts are reported. The HPLC profile of the ARF-T sample is presented in Fig. 2. In total 12 peaks were identified as described in Table 1. Peak 1, 2 and 4 were assigned as delphinidin glycosides because for these 3 peaks a fragment ion with an m/z of 303 $[M]^+$ was found. Peaks 3, 5 and 7 showed a fragment ion with an m/z of 287 $[M]^+$ and thus these peaks were assigned as cyanidin glycosides. In a similar way, the peak of petunidin glycosides, paeonidin glycosides and malvidin glycosides were assigned

Table 1
Retention times, UV–vis and mass spectral data of anthocyanins in analyzed ARF samples obtained from blueberry cultivars.

Peak	t_R	Maximum absorptions (nm)	Molecular ion (m/z)	Fragment ions (m/z)	MW	Compound
An 1	8.1	276; 526	465.2	303.4	465	Delphinidin-3-*O*-galactoside
An 2	9.3	276; 524	465.2	303.4	465	Delphinidin-3-*O*-glucoside
An 3	11.2	279; 517	449.2	287.3	449	Cyanidin-3-*O*-galactoside
An 4	11.5	276; 524	435.4	303.4	435	Delphinidin-3-*O*-arabinoside
An 5	12.9	280; 517	449.2	287.3	449	Cyanidin-3-*O*-glucoside
An 6	13.6	276; 526	479.1	317.3	479	Petunidin-3-*O*-galactoside
An 7	15.2	279; 517	419.1	287.3	419	Cyanidin-3-O-arabinoside
An 8	18.0	276; 526	463.2	301.4	463	Paeonidin-3-*O*-galactoside
An 9	18.2	276; 526	449.2	317.3	449	Petunidin-3-*O*-arabinoside
An 10	20.5	276; 527	493.3	331.2	493	Malvidin-3-*O*-galactoside
An 11	23.4	276; 526	493.3	331.2	493	Malvidin-3-*O*-glucoside
An 12	26.3	276; 528	463.2	331.2	463	Malvidin-3-*O*-arabinoside

Table 2
The anthocyanin content in blueberries (*Vaccinium corymbosum* L.) determined by HPLC–DAD and expressed in mg per 100 g fr.wt. Data are expressed as mean ± SD, $n = 3$. Different letters between columns denote statistically difference at $p < 0.05$.

Peak	Compound	Anthocyanin content in *Vaccinium corymbosum* L. berries (mg/100 g fr.wt)						
		Bluegold	Nui	Darrow	Legacy	Nelson	Hannah 's Choice	Toro
	Anthocyanins: Total	101.88 ± 2.36	150.27 ± 2.01	168.50 ± 2.95	189.26 ± 2.70	161.31 ± 4.66	147.12 ± 2.21	195.01 ± 2.65
An 1	Delphinidin-3-*O*-galactoside	9.51 ± 3.29^{e}	12.50 ± 1.06^{e}	29.55 ± 1.10^{c}	50.41 ± 6.27^{a}	36.52 ± 1.48^{b}	21.23 ± 5.67^{d}	35.57 ± 4.36^{b}
An 2	Delphinidin-3-*O*-glucoside	3.64 ± 0.15^{de}	23.76 ± 1.95^{a}	14.05 ± 1.58^{b}	4.37 ± 0.35^{cd}	3.65 ± 1.65^{de}	3.00 ± 0.87^{e}	5.01 ± 0.17^{c}
An 3	Cyanidin-3-*O*-galactoside	6.17 ± 0.01^{c}	7.62 ± 1.77^{c}	nd	19.00 ± 0.84^{a}	11.64 ± 5.12^{b}	7.29 ± 0.77^{c}	13.72 ± 1.45^{b}
An 4	Delphinidin-3-*O*-arabinoside	5.61 ± 1.46^{e}	12.78 ± 1.99^{cd}	28.34 ± 0.79^{a}	19.32 ± 6.27^{b}	17.47 ± 7.27^{bc}	9.33 ± 0.45^{de}	14.79 ± 3.85^{bc}
An 5	Cyanidin-3-*O*-glucoside	3.33 ± 0.18^{b}	13.91 ± 5.68^{a}	4.73 ± 0.64^{b}	3.53 ± 0.39^{b}	3.17 ± 1.34^{b}	2.65 ± 0.32^{b}	3.68 ± 0.50^{b}
An 6	Petunidin-3-*O*-galactoside	10.78 ± 3.84^{d}	7.22 ± 0.67^{d}	20.52 ± 9.84^{bc}	25.28 ± 4.23^{ab}	21.22 ± 7.58^{bc}	19.94 ± 5.43^{c}	27.85 ± 3.43^{a}
An 7	Cyanidin-3-*O*-arabinoside	4.68 ± 0.05^{e}	21.78 ± 1.54^{a}	13.78 ± 0.19^{b}	9.16 ± 1.28^{c}	6.88 ± 3.07^{d}	4.50 ± 0.65^{e}	6.09 ± 0.95^{de}
An 8	Paeonidin-3-*O*-galactoside	4.92 ± 0.28^{bc}	3.08 ± 0.47^{c}	nd	5.14 ± 0.28^{ab}	4.49 ± 2.15^{b}	3.93 ± 0.78^{bc}	6.27 ± 0.30^{a}
An 9	Petunidin-3-*O*-arabinoside	5.97 ± 1.33^{c}	6.02 ± 0.89^{c}	12.02 ± 1.31^{b}	10.31 ± 2.54^{bc}	9.74 ± 3.55^{bc}	48.73 ± 8.99^{a}	9.97 ± 1.73^{bc}
An 10	Malvidin-3-*O*-galactoside	29.33 ± 12.69^{b}	11.95 ± 2.21^{de}	19.18 ± 6.14^{cd}	25.66 ± 5.74^{bc}	28.44 ± 4.91^{b}	4.30 ± 0.88^{e}	48.33 ± 12.77^{a}
An 11	Malvidin-3-*O*-glucoside	4.43 ± 0.56^{d}	24.66 ± 3.63^{a}	13.17 ± 2.76^{c}	4.42 ± 0.17^{d}	4.31 ± 1.80^{d}	19.93 ± 1.23^{b}	5.21 ± 0.93^{d}
An 12	Malvidin-3-*O*-arabinoside	13.53 ± 4.43^{b}	5.01 ± 2.28^{c}	13.15 ± 5.11^{b}	12.67 ± 4.06^{b}	13.78 ± 2.62^{b}	2.29 ± 0.56^{c}	18.53 ± 1.86^{a}

nd, not detected.

(Table 1). According to literature, anthocyanins can be glycosylated with galactose, glucose or arabinose. The elution order of these glycosidic derivatives is galactosides, glucosides and arabinosides (Gavrilova et al., 2011; Müller et al., 2012) and therefore, the first peak of each anthocyanin was assigned as being galactoside, the second as glucoside and the third as arabinoside. Based on literature, the sugars in blueberries are linked to the C3 position (Kader et al., 1996). Among the identified compounds, delphinidin, petunidin and malvidin galactoside represents the anthocyanins identified in all the cultivars. Data on the content of individual anthocyanins in blueberry cultivars is presented in Table 2. The highest anthocyanin content was found in Toro cultivar (195.01 mg/100 g fr.wt), followed by Legacy, while the lowest amount was obtained in Bluegold cultivar (101.88 mg/100 g fr.wt). Delphinidin-3-*O*-galactoside (peak 1) was identified as one of the major compounds in cultivar Toro and one of the minor compounds in Bluegold cultivar, while malvidin-3-*O*-galactoside (peak 10) was identified as one of the major compounds in Toro, Bluegold, Legacy and Nelson cultivars, and a minor compound in Hannah' Choice cultivar. Malvidin-3-*O*-galactoside was the major anthocyanin followed by delphinidin-3-*O*-galactoside and petunidin-3-*O*-galactoside, which represent together more than 56% of the total anthocyanins of Toro cultivar. All anthocyanins identified in our study are in agreement with other papers (Gavrilova et al., 2011; Müller et al., 2012; Prior et al., 2001). Two recent studies in crude extracts obtained from *V. corymbosum* fruits, found lower quantities for anthocyanins such as petunidin-3-glucoside and paeonidin-3-arabinoside (Gavrilova et al., 2011; Müller et al., 2012). The total anthocyanin content reported in a recent study for Toro and Legacy cultivars was 6.35 mg/100 g fr.wt and 68.55 mg/100 g fr.wt (Gavrilova et al., 2011). Other studies indicate that total anthocyanin contents of wild blueberries determined by HPLC analysis varied between 200 and 705 mg/100 g fr.wt while for cultivated blueberries these varied between 36 and 529 mg/100 g fr.wt (Gao and Mazza, 1994b; Kraus et al., 2010; Wu et al., 2006). Total anthocyanin content for 87 highbush blueberries measured by spectrophotometry varied between 0.89 and 3.31 mg cyanidin 3-*O*-glucoside/g fr.wt (Ehlenfeldt and Prior, 2001). The differences between total anthocyanin content could be due to the different extraction solvent used. Also it has to be taken into account that the growing location, season and environmental factors could influence the anthocyanin composition of blueberries (Garzón et al., 2010).

3.2. Determination of antioxidant activity

Since different antioxidant assays give different results, various methods, based on different mechanisms can be used in parallel to evaluate the antioxidant activity of the extracts. Taking into account the assay reaction mechanism (HAT or SET) three antioxidant methods were chosen for this study. SET (Single Electron Transfer) based assays, such as ABTS and CUPRAC, measure the capacity of the antioxidants to reduce an oxidant. ORAC, an HAT (Hydrogen Atom Transfer) based assay measures the capability of antioxidants to quench peroxyl radicals, by hydrogen donation. The antioxidant activity of blueberry anthocyanin-rich extracts was determined using the CUPRAC, ABTS and ORAC assays and expressed as Trolox equivalents (μmol TE/g fr.wt) as shown in Table 3. The ability of blueberry ARF to reduce cupric ion (Cu^{2+}) was measured by the CUPRAC method. The CUPRAC assay was used since it has also been classified as an electron transfer assay (Apak et al., 2007). CUPRAC values for the blueberry cultivars were within the range of 134.76–185.78 μmol TE/g fr.wt and the highest antioxidant activity was obtained for the cultivar Toro. The authors did not find published literature on the antioxidant activity of blueberry extracts using the CUPRAC method. There is one study who reported CUPRAC values for raspberry and blackberry cultivars ranging between 69 and 127 μmol TE/g fr.wt (Sariburun et al., 2010).

The ABTS measures the antioxidants ability to scavenge the radical $ABTS^{+\cdot}$ compared with Trolox, a vitamin E analog. The blueberry radical scavenging activity as shown in Table 3 ranged from 6.05 to 11.96 μmol TE/g fr.wt with a statistically significant ($p < 0.05$) value obtained for the cultivar Toro. The values for scavenging activity toward the $ABTS^{+\cdot}$ radical were about 4 times lower than those reported previously for *Vaccinium meridionale* berries (Garzón et al., 2010) and for *Vaccinium floribundum* berries (Vasco et al., 2009). One explanation for these lower antioxidant values is that in this study only the anthocyanin fraction scavenging activity was evaluated instead of all polyphenols from berries as the literature studies reported before.

Table 3
Antioxidant activity results obtained by complementary assays ABTS, CUPRAC and ORAC on selected cultivar of blueberry. The data expressed as mean ± SD. Different letters in each column denote statistical difference at $p < 0.05$.

Blueberry cultivar	Antioxidant activity (μM TE/g fr.wt)		
	CUPRAC	ABTS	ORAC
Bluegold	134.76 ± 53.03^{b}	6.05 ± 1.34^{c}	21.21 ± 3.26^{d}
Nui	163.04 ± 23.45^{ab}	7.91 ± 3.67^{b}	22.35 ± 6.77^{d}
Darrow	168.65 ± 30.33^{ab}	8.43 ± 2.18^{b}	25.43 ± 2.19^{c}
Legacy	171.15 ± 46.49^{ab}	8.73 ± 2.22^{b}	28.99 ± 5.31^{b}
Nelson	175.25 ± 69.06^{ab}	9.12 ± 1.23^{b}	30.27 ± 4.74^{b}
Hanna's Choice	153.89 ± 57.12^{ab}	7.68 ± 1.09^{bc}	20.36 ± 2.23^{d}
Toro	185.78 ± 92.06^{a}	11.96 ± 3.45^{a}	34.58 ± 3.25^{a}

The ORAC assay measures the scavenging capacity of antioxidants against the peroxyl radical. ORAC values for ARF samples varied from 21.2 to 34.5 μmol TE/g fr.wt among blueberry cultivars. The ARF-T has the highest antioxidant ORAC value. According to the literature data, blueberry ORAC values were in the range of 4.6–76.9 μmol TE/g for different cultivars (Ehlenfeldt and Prior, 2001; Konczak et al., 2010; You et al., 2011). Ehlenfeldt and Prior (2001) compared the antioxidant activity (ORAC) of berry samples from a large collection of cultivars including those tested in this study. The cultivar Toro had the highest ORAC value followed by Nelson, BlueGold, Darrow, Nui and Legacy cultivars (Ehlenfeldt and Prior, 2001). According to a study conducted by Elisia et al. (2007), SPE purification using a Bio-Gel P2 gel filtration column resulted in an increase by a factor 7.3 (from 4885 μmol TE/g to 674.2 μmol TE/g) in antioxidant activity of an anthocyanin-enriched blackberry extract. Another study showed that due to the loss of sugars, organic acids, glutathione and other water soluble compounds, the ORAC values decreased from 28.9 to 23.2 μmol TE/g fr.wt for blueberry extracts that have passed through the C18 Sep-Pak cartridge (Zheng and Wang, 2003). Furthermore, along with differences in anthocyanin content and antioxidant activity among blueberry cultivars, differences at variety levels within other *Vaccinium* sp. were also observed (Lohachoompol et al., 2008; Prior et al., 1998). The results from this study demonstrated that the antioxidant activity was dependent on the anthocyanin content of the extracts of the cultivars tested. ARF-T was the richest anthocyanin fraction with the highest antioxidant activity from all cultivars tested in this study and we chose it to evaluate its effect on B16-F10 metastatic murine melanoma cells.

3.3. Inhibition of tumor cell proliferation

Due to its highest content in anthocyanins, the cultivar Toro extract (ARF-T) was used as treatment for metastatic B16-F10 melanoma murine cells. ARF-T inhibited the proliferation of B16-F10 cells in a dose dependent manner as shown in Fig. 3. ARF-T treatment with 200 and 400 μg/ml for 24 h stimulated the B16-F10 cell proliferation with 20%. A similar effect was observed for HepG2 cell line treated with 200 μg/ml *Vaccinium uliginosum* L. anthocyanins for 72 h (Liu et al., 2010). Treatment with 550, 600 and 650 μg/ml ARF-T for 24 h resulted in decreased B16-F10 cell proliferation with 16.5%, 46.9%, and 59.0% respectively (Fig. 3). Higher concentrations than 650 μg/ml decreased cell proliferation by more than 70%. The calculated IC_{50} value was 615.2 μg/ml and was obtained from the dose–response curve shown in Fig. 3. Therefore, a concentration of 600 μg/ml ARF-T for 24 h was the standard treatment

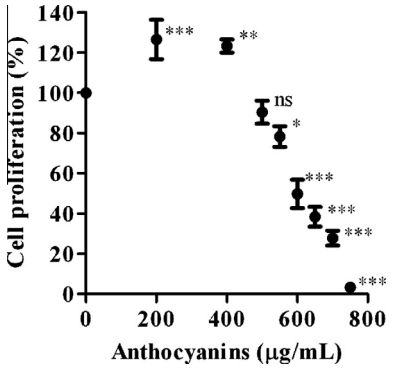

Fig. 3. Blueberry anthocyanins reduces the proliferation of melanoma murine cells B16-F10 treated with 500, 550, 600, 650, 700, 750 μg/ml ARF-T for 24 h. Cell viability was assessed by MTT assay. Data are expressed as mean ± SEM ($n = 5$). Statistically significant differences: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control.

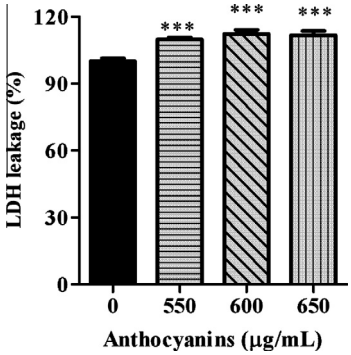

Fig. 4. LDH leakage from B16-F10 melanoma murine cells after 24 h incubation with the 550, 600, 650 μg/ml ARF-T. Results are presented as mean ± SEM ($n = 5$). Statistically significant differences: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control.

chosen for the microscopy experiments. A recent study regarding the effect of a *V. uliginosum* L. anthocyanin extract on cell growth malignant cancer cells reported a IC_{50} calculated for Hep-G2 cell line of 563 μg/ml and for Caco-2 of 390 μg/ml (Liu et al., 2010) which are similar to the value obtained in this experiment. A higher IC_{50} value (3100 μg/ml) was obtained for the low metastatic variant of murine melanoma B16-F1 cell viability after 24 h of mulberry anthocyanins treatment (Huang et al., 2008). The cell growth was inhibited by 15% after 75 μg /ml of monomeric bilberry anthocyanins treatment on human colorectal HT-29 adenocarcinoma cells incubated for 24 h (Zhao et al., 2004).

3.4. Cellular membrane integrity

The leakage lactate dehydrogenase (LDH) was measured to asses cellular membrane integrity after 24 h of incubation of B16-F10 cells with or without ARF-T treatment. ARF-T doses of

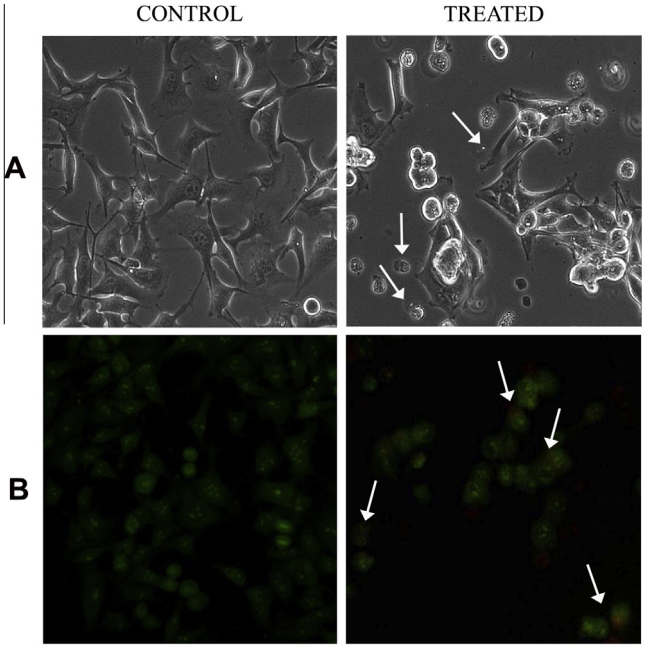

Fig. 5. Effects of blueberry anthocyanins on B16-F10 murine melanoma cells (A). Phase contrast microscopy on B16-F10 cells treated by 600 μg/ml ARF-T for 24 h. (B) 96-well-based EB/AO staining without ARF-T treatment and after treatment of B16-F10 cells for 24 h with 600 μg/ml ARF-T. Cells were observed under inverted fluorescence microscope. The white arrows indicate apoptotic cells (original magnification: ×40).

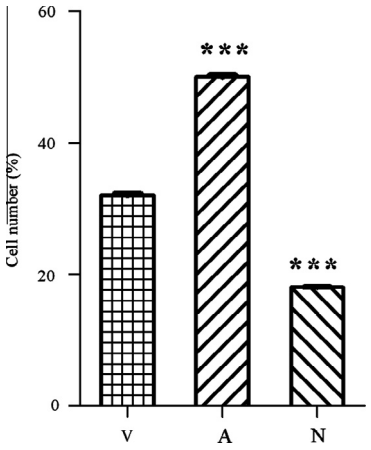

Fig. 6. Quantification of apoptosis induction in B16-F10 cells treated with 600 μg/ml ARF-T for 24 h, by AO/EB staining assay. The results are shown as percentage of total cells counted, calculated based on stained cells in 6 fields, each field with 50 cells; V, viable cells; A, early apoptosis; N, necrosis. Data are expressed as means ± SD. $^{*}p < 0.05$, and $^{***}p < 0.001$ in comparison to untreated cells, respectively to viable ARF-T treated cells.

550, 600, 650 μg/ml increased the leakage lactate dehydrogenase (LDH) from B16-F10 cells by 9%, 10% and 12% compared to control (Fig. 4), indicating that the cellular membranes were strongly affected by anthocyanins. Data reported in a recent study showed that a *V. uliginosum* L. anthocyanin extract treatment increased the LDH release over time in Caco-2 cell line with 21% after 48 h and in a Hep-G2 cell line by 66% after 48 h incubation (Liu et al., 2010).

3.5. Cell morphology changes

Apoptosis was identified as the major mode of anthocyanin-induced cell death (Chen et al., 2005; Seeram et al., 2006; Srivastava et al., 2007). In our study, characteristic morphological changes of apoptosis such as cell detachment, rounding up and shrinkage were observed by contrast-phase microscopy after 24 h of exposure to ARF-T (600 μg/ml) in B16-F10 cells as shown in Fig. 5A. The cell membranes blebbing and the formation of the apoptotic bodies in B16-F10 cells exposed to ARF-T treatment were observed. Acridine orange (AO)/ethidium bromide (EB) staining showed that after ARF-T treatment, about 50% of cells counted were colored orange being in apoptosis and about 18% of cells counted were colored red, which after a time of sustained apoptosis could be in necrosis (Fig. 6). These results suggest that ARF-T (600 μg/ml) can induce apoptosis in B16-F10 melanoma murine cells after 24 h treatment (Figs. 5B, 6). Literature data sustains that blueberry anthocyanins induced apoptosis in HT-29 and Caco-2 cells, resulting in a 2–7 times increase in DNA fragmentation (Yi et al., 2005).

3.6. Apoptotic cell death

Apoptosis leads to nuclear DNA breakdown into multiples of 200–500 base pair oligonucleosomal size fragments. This was determined by the TUNEL assay after anthocyanins addition to the cells. Fig. 7 shows that a high number of ARF-T treated B16-F10 murine melanoma cells were found TUNEL positive compared to untreated cells. Treatment with 600 μg/ml ARF-T for 24 h increased the number of TUNEL positive cells with 14%, whereas only a 2% increase in TUNEL positive cells was observed in untreated samples (Fig. 8). Data obtained in this study demonstrate that ARF-T is able to induce apoptosis in B16-F10 treated melanoma cells. With the same assay, (Wang et al., 2009) showed that black raspberries anthocyanins also induced apoptosis in tumor rat esophagus cells. In addition, the TUNEL assay was also used to confirm the induction of apoptosis by the anthocyanin fraction from potato extract, showing a significant induction of apoptosis in LNCaP and PC-3 cells after treatment with 5 μg chlorogenic acid/ml anthocyanins (Reddivari et al., 2007). The major blueberry anthocyanins responsible for the inhibitory effects on tumor cell growth are those found in large amounts, such as delphinidin and malvidin-glycosides (Katsube et al., 2003).

3.7. Suggested apoptotic mechanism

The results of this study using EB/AO and TUNEL assays for apoptosis demonstrated that ARF-T induced apoptosis in B16-F10. These data are consistent with the already demonstrated

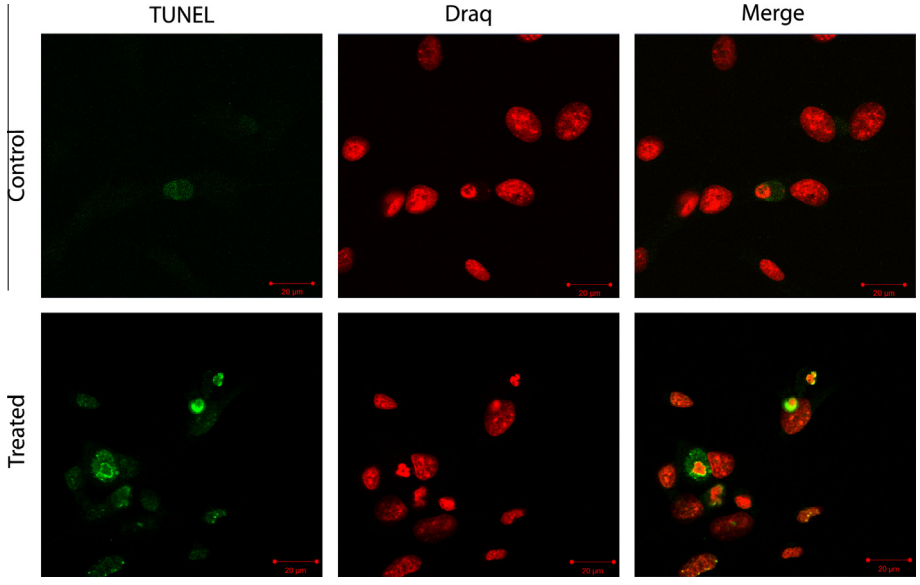

Fig. 7. Confocal microscopy of B16-F10 cells TUNEL staining with or without 600 μg/ml ARF-T treatment for 24 h. TUNEL-positive cells are shown as green fluorescence. Numerous normal nuclei are stained in red with Draq5. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

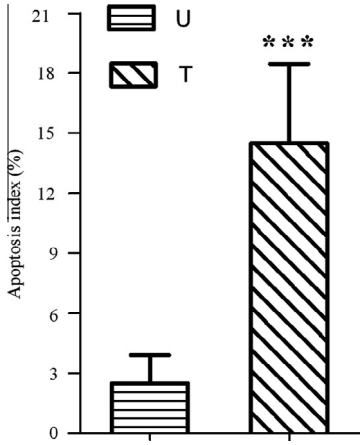

Fig. 8. Quantification of apoptosis induction in B16-F10 cells treated with 600 μg/ml ARF-T for 24 h. Quantification by TUNEL assay. Data are presented as percentage of TUNEL-positive cells per 1000 cells; U, untreated cells; T, ARF-T treated cells.

proapoptotic effect of anthocyanins in other cancer cell lines (Chen et al., 2005; Katsube et al., 2003; Olsson et al., 2004; Seeram et al., 2006). Data regarding the effects of blueberry anthocyanins on cell proliferation murine melanoma cells have not been reported previously and the mode of action of anthocyanins remains unknown. Recently, it was suggested that mulberry anthocyanins could mediate B16-F1 cell metastasis by reduction of MMP-2 and MMP-9 activities, which are involved in the suppression of the Ras/PI3K signaling pathway (Huang et al., 2008). In both PI3K/Akt and RAF/MAPK cellular signaling cascades the reactive oxygen species (ROS) can participate as second messengers. Paradoxically to their antioxidant activity in vitro, treatment of cancer cells, but not normal cells, with anthocyanins leads to an accumulation of ROS and subsequent apoptosis. This suggests that the ROS-mediated mitochondrial caspase-independent pathway is important for anthocyanin-induced apoptosis (Feng et al., 2007; Ruginǎ et al., 2011).

4. Conclusion

In this study 12 individual anthocyanins were identified and quantified. Malvidin-3-*O*-galactoside, petunidin-3-*O*-galactoside and delphinidin-3-*O*-galactoside were the major anthocyanins in all cultivars studied. The highest antioxidant scores were found for cultivar Toro, consistent with the highest anthocyanins concentration. Anthocyanins purified from *V. corymbosum* cv. Toro could inhibit melanoma tumor cell proliferation and induce apoptosis. According to Cuevas-Rodriguez et al. (2010), the extraction method is suitable to have a strongly enriched anthocyanin fraction, but for our tested ARF we cannot exclude the side effects induced by small amounts of impurities like chlorogenic acid, some procyanidins or flavonoid compounds. The amounts of anthocyanins needed in our in vitro experiments are exceeding than the amounts observed in human plasma in vivo after 2 h post-consumption of 1.3 g anthocyanins in the serum was found 591.7 nmol/L anthocyanin metabolites (Kay et al., 2004). Pharmacokinetic data proved that the anthocyanins absorption into human blood is minimal (He et al., 2006; Prior and Wu, 2006). As such, further studies are required to clarify the mechanisms of action and the bioavailability of anthocyanins in melanoma cells. In addition, topical application of anthocyanins in tissues, such as the skin, should be studied further, since they might be more efficient in the treatment of melanoma cancer. However further studies are required in order to clarify this statement, the mechanisms of action and the bioavailability of anthocyanins in melanoma cells.

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