

# Melanoma Inhibition by Anthocyanins Is Associated with the Reduction of Oxidative Stress Biomarkers and Changes in Mitochondrial Membrane Potential

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**Abstract** Anthocyanins are water soluble pigments which have been proved to exhibit health benefits. Several studies have investigated their effects on several types of cancer, but little attention has been given to melanoma. The phytochemical content of nine different berry samples was assessed by liquid chromatography followed by electrospray ionization mass spectrometry (LC-ESI<sup>+</sup>-MS). Twenty-six anthocyanins were identified, after a previous C<sub>18</sub> Sep-pak clean-up procedure. Chokeberry and red grape anthocyanins rich extracts (C-ARE and RG-ARE) were selected to be tested on normal and melanoma cell lines, due to their different chemical pattern. C-ARE composition consists of cyanidin aglycone glycosylated with different sugars; while RG-ARE contains glycosylated derivatives of five different aglycones. Both C-ARE and RG-ARE anthocyanins reduced proliferation, increased oxidative stress biomarkers and diminished mitochondrial membrane potential in melanoma cells, having no negative influence on normal cells. A synergistic response may be attributed to the

five different aglycones present in RG-ARE, which proved to exert greater effects on melanoma cells than the mixture of cyanidin derivatives with different sugars (C-ARE). In conclusion, C-ARE and RG-ARE anthocyanins may inhibit melanoma cell proliferation and increase the level of oxidative stress, with opposite effect on normal cells. Therefore, anthocyanins might be recommended as active ingredients for cosmetic and nutraceutical industry.

**Keywords** Anthocyanins · Proliferation · MDA · LDH · Mitotracker · Melanoma

## Introduction

Anthocyanins are natural plant pigments, water-soluble, intensely colored in blue, purple or red, and found in fruit, vegetables or cereals [1]. So far more than 635 anthocyanins have been identified, featuring six common aglycones and various types of glycosylation and acylation. The aglycones are the free forms of anthocyanins (de-glycosylated or de-acyl-glycosylated), called anthocyanidins (cyanidin, delphinin, pelargonidin, malvidin, petunidin, and peonidin). Different sugar components can be attached to an aglycone nucleus such as glucose, galactose, rhamnose, xylose or arabinose, which are usually conjugated to the anthocyanidin skeleton via the C3 hydroxyl group in ring C. In addition, they may be acylated with different organic acids [2]. Anthocyanins and anthocyanidins are known to exhibit various biological effects, such as antioxidant activity [3–5], antidiabetic [6, 7] or anticarcinogenic potential [8, 9].

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Melanoma is a skin cancer that originates in melanocytes, with exposure to ultraviolet light as an environmental risk factor. The potential of anthocyanin to protect the skin from the adverse biological effects of solar radiation has already been proved. A topical administration of cy-3-*O*-gluc (500  $\mu$ M) on mouse skin exposed to ultraviolet light reduced oxidative damage and inflammation [10]. Afaq et al. reported that delphinidin is able to inhibit apoptosis and markers of DNA damage if it is applied topically (1 mg/0.1 mL DMSO/mouse) to SKH-1 hairless mouse skin exposed to UVB radiation [11]. Anthocyanins from blueberries, blackcurrants, strawberries and mulberries conferred remarkable reduction of melanoma cell proliferation, in parallel with a decrease of other markers involved [8, 12–15].

Here we present data about the structure and content of anthocyanins from nine different berry sources (blackberries, blackcurrants, blackthorns, blueberries, chokeberries, cranberries, mulberries, red grapes and dwarf elderberries). From these, chokeberries (C-ARE) and red grapes (RG-ARE) enriched anthocyanin fractions were selected for assessment of their effect on normal and melanoma cells due to the differences in the anthocyanin composition. C-ARE contains cyanidin derivatives with various sugars, while RG-ARE contains glucosylated anthocyanins of different aglycones.

## Materials and Methods

A detailed description of chemicals and used sample can be found as supplementary material.

**Anthocyanin Rich Extract (ARE) Preparation** In order to extract polyphenols from selected berries, we performed the extraction according to the protocol published previously [8]. The semipurification of the obtained extracts was done by solid-phase extraction (SPE), using C<sub>18</sub> Sep-Pak cartridges (Waters Corp., Milford, MA, USA), based on a recent protocol [16].

**HPLC-PDA-ESI/MS Analysis of Anthocyanins** HPLC analysis was performed on a Shimadzu HPLC-PDA system following a previously published protocol [8]. A more detailed description of the HPLC-PDA-ESI/MS method can be found as supplementary material.

**Cell Culture** A375 human melanoma, B16-F10 murine melanoma and Hs27 human fibroblast cells purchased from American Type Culture Collection (ATCC) were maintained in DMEM media, supplemented with FBS (10%), 1 mM glutamine, 1% antibiotics, in standard conditions.

**Cell Proliferation Assay** Cell proliferation was measured by MTT assay. The results were expressed as percent survival

relative to the untreated control. Each treatment was repeated three times and each repetition had five experimental wells for each concentration. A more detailed description of the HPLC-PDA-ESI/MS method can be found as supplementary material.

**Measurement of LDH Release Level** LDH release assay was performed according to kit instructions (Pierce LDH Cytotoxicity Assay Kit, Rockford, IL 61,105 USA) and the results were expressed as percentage variation of LDH release normalized to control. A more detailed description of the used method can be found as supplementary material.

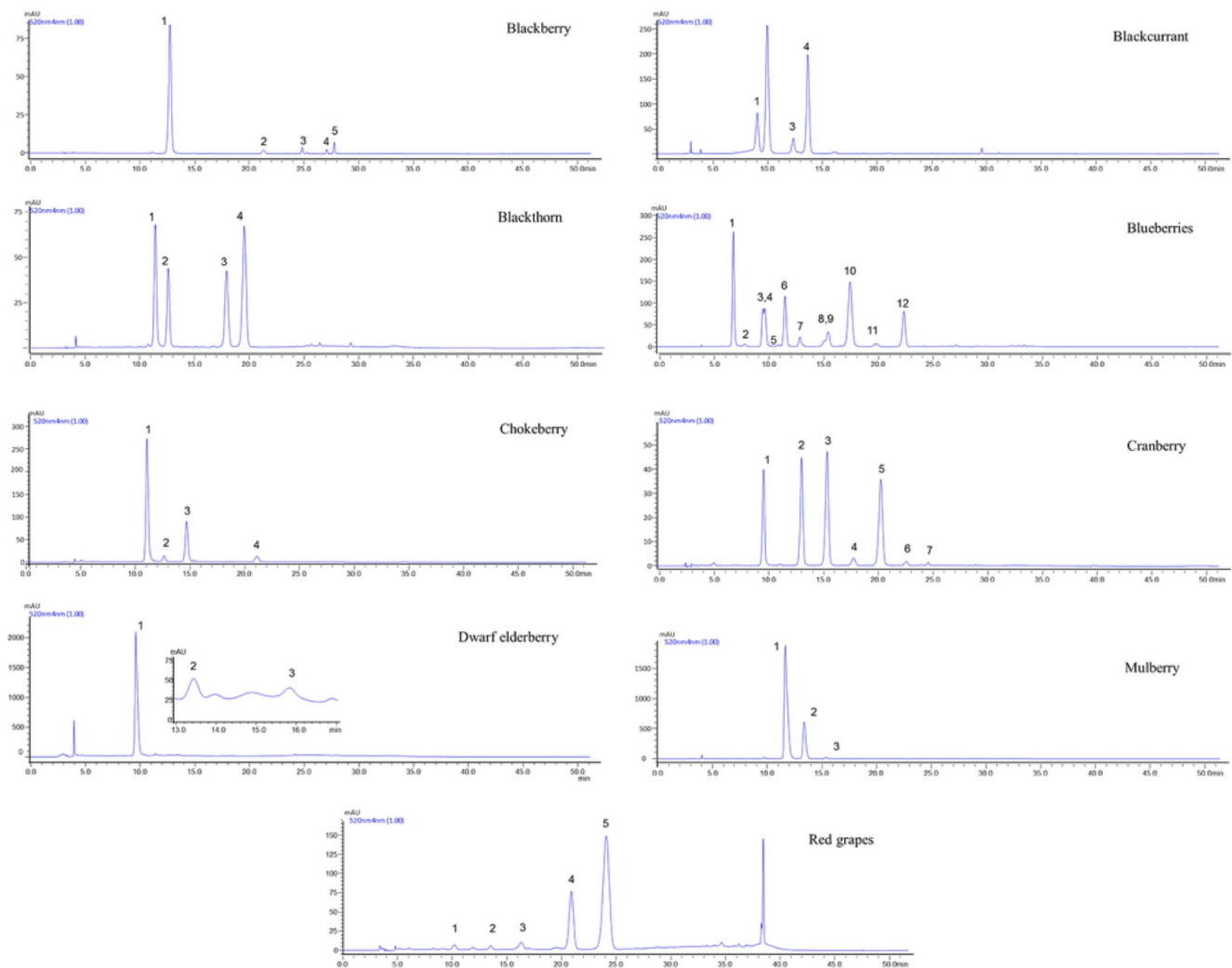
**Measurement of Mitochondrial Membrane Potential** To assess the mitochondrial activity in live cells by MitoTracker (MitoTracker™ Red CMXRos) was used. The images were acquired on a Zeiss LSM 710 confocal laser scanning unit (Oberkochen, Germany) equipped with argon and HeNe laser mounted on an Axio Observer Z1 Inverted Microscope. Fluorescence was quantified by using ImageJ Analysis 1.46r software according to a method published by McCloy et al. [17]. A more detailed description of the used method can be found as supplementary material.

**Assessment of Malondialdehyde Level** In order to evaluate the levels of lipid peroxidation as malondialdehyde (MDA) was quantified by HPLC using a previously published protocol [18]. Data were expressed as  $\mu$ M MDA and were normalized to the protein concentration of cell lysates. A more detailed description of the used method can be found as supplementary material.

**Statistical Analysis** Data were expressed as mean  $\pm$  standard error of mean (SEM) for each sample, analyzed three times. Analysis of variance (ANOVA) and Dunnett's multiple comparisons test were used to determine significant differences between values ( $p < 0.05$ ).

## Results and Discussion

**Anthocyanin Content of Berries** Anthocyanin identification and peak assignments were done based on their retention times, comparison of UV-VIS spectra with those of standard compounds and with literature data (Table 1, [supplementary data](#)). A total of 26 different individual anthocyanins have been identified and quantified from all berries: derivatives of cyanidin (8), malvidin (5), delphinidin (4), petunidin (4), peonidin (3) and pelargonidin (2). Five anthocyanins were detected in blackberries (Fig. 1). The major compound peak 1 ([M]<sup>+</sup>, m/z 449; fragment ion, m/z 287) was assigned as Cy-3-*O*-glu. Wu et al. (2005) reported the presence of nine anthocyanins in blackberries: Cy-3-*O*-glu; Cy-3-*O*-ara; Cy-3-*O*-rut;



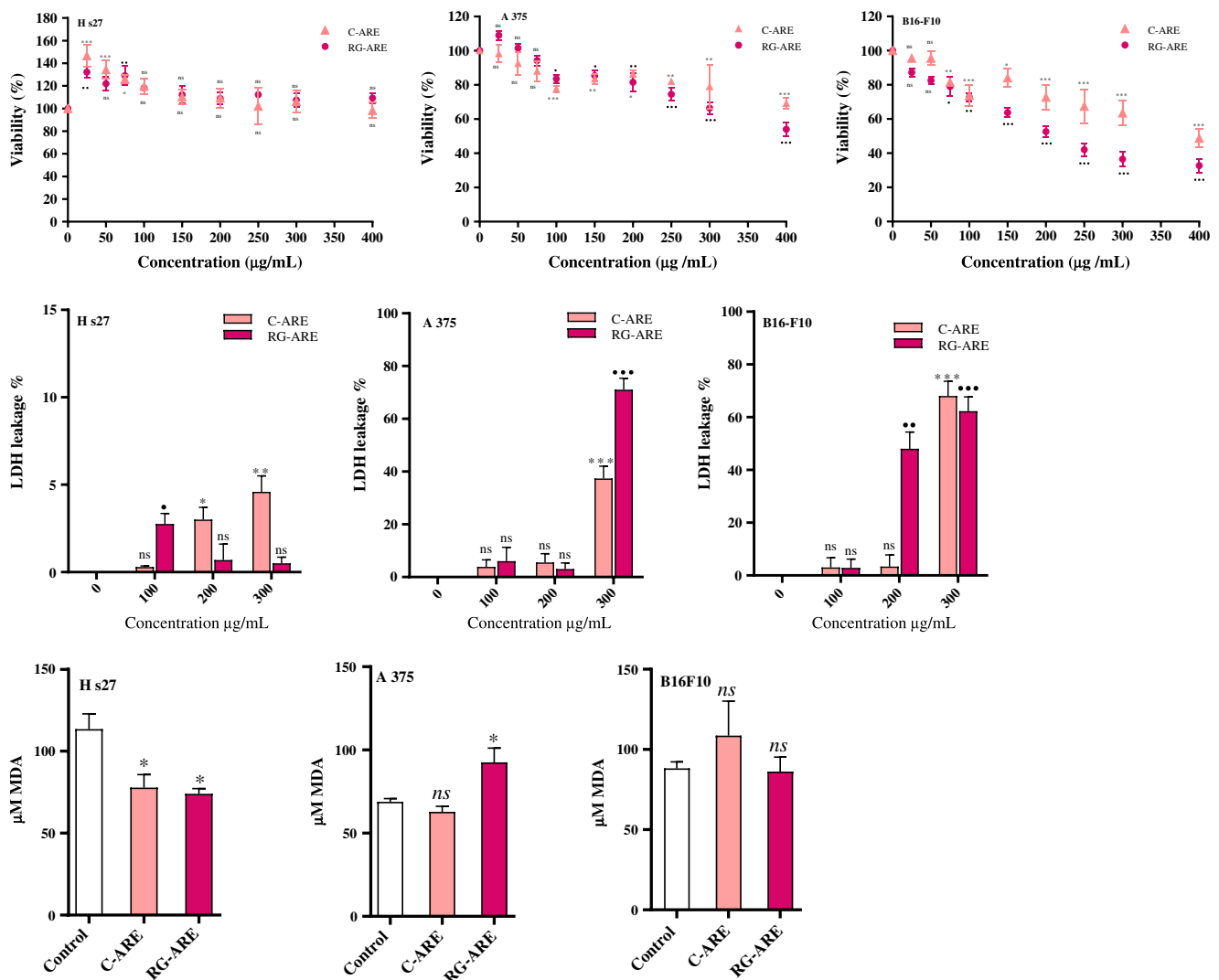
**Fig. 1** Separation of individual anthocyanins from nine different berries. HPLC chromatograms recorded at 520 nm (Peak assignment is shown in Table 1)

Pel-3-*O*-glu; Cy-3-*O*-xyl; Cy-3-(6''-malonoyl)-glu and Cy-3-*O*-dioxaloylglucoside [19]. According to obtained MS data, blackthorns contain four anthocyanins: Cy-3-*O*-glu ([M]<sup>+</sup>, *m/z* 449; fragment ion, *m/z* 287), Cy-3-*O*-rut ([M]<sup>+</sup>, *m/z* 595; fragment ion, *m/z* 287), Peo-3-*O*-glu ([M]<sup>+</sup>, *m/z* 463; fragment ion, *m/z* 301), Peo-3-*O*-rut ([M]<sup>+</sup>, *m/z* 609; fragment ion, *m/z* 301). Our results regarding the blackthorn extracts are in agreement with the literature data reporting the presence of the same anthocyanins with similarly distributed concentration levels [20]. Blueberries represent a complex matrix having the highest number of anthocyanins (12). The profile and content of anthocyanins in blueberries is similar to that reported in our previous study [8]. Five anthocyanins were identified in red grapes (RG-ARE), the major compounds being Pet-3-*O*-glu (peak 4 [M]<sup>+</sup>, *m/z* 479; fragment ion, *m/z* 317) and respectively Pel-3-*O*-glu (peak 5 [M]<sup>+</sup>, *m/z* 433; fragment ion, *m/z* 271). Kallithraka et al.) analyzed 46 red grape samples from 6 common varieties from Greece, and reported that Mal-3-*O*-glu

was the most prevalent anthocyanin [21]. In blackcurrant berries, cyanidin and delphinidin having rutin or glucose as a sugar moiety were identified; data which are in agreement with the literature [22]. Based on molecular weight cations (M<sup>+</sup>) and fragmentation pattern, monoglycosylated cyanidins with hexose (glucose and galactose) or pentose (arabinose, xylose) at the 3-position were identified in chokeberries (C-ARE). A similar profile was previously reported [7]. Seven anthocyanins were identified in cranberries by comparing their MS data with those of anthocyanins discussed above. The major anthocyanins are represented by: Cy-3-*O*-gal, Cy-3-*O*-ara, Pet-3-*O*-ara, Peo-3-*O*-gal and the total anthocyanins content was close to that of blackberries. Prior et al. reported that cranberries contain Cy-3-*O*-ara, Cy-3-*O*-gal, Peo-3-*O*-ara Peo-3-*O*-gal as major compounds and Cy-3-*O*-glu and Pet-3-*O*-gal as minor constituents [23]. Additionally, in the present study, we have identified three malvidin derivatives. Cy-3-*O*-gal, Cy-3-*O*-samb and Pel-3-*O*-sam were the three anthocyanins identified

in dwarf elderberries. Cy-3-*O*-gal (Peak 1 [M]<sup>+</sup>, m/z 449; fragment ion, m/z 287) is the major compound with 309 mg/100 g FW from total anthocyanin of 320.2 Mikulic-Petkovsek et al. identified a total of 16 anthocyanins in four different elderberry species and eight interspecific hybrids [24]. The major compounds in mulberries are Cy-3-*O*-glu ([M]<sup>+</sup>, m/z 449; fragment ion, m/z 287) and Cy-3-*O*-rut ([M]<sup>+</sup>, m/z 595; fragment ion, m/z 287). Other studies reported the presence of cyanidin and pelargonidin derivatives (Cy-3-*O*-glu, Cy-3-rut and Pel-3-glu) as major anthocyanins in mulberries [20, 25]. The chemical composition of two samples, C-ARE and RG-ARE, were considered to have considerable relevance, and were selected to be further evaluated *in vitro* for their potential on normal and melanoma cells. As seen in Table 1, C-ARE composition consists of cyanidin derivatives with different sugars, while RG-ARE contains glucosylated derivatives of five different aglycones.

*Cell Proliferation* was assessed by measuring the mitochondrial succinate dehydrogenase activity in normal (Hs27) and melanoma (A375, B16-F10) cells treated with AREs. Both C-ARE and RG-ARE stimulated proliferation of Hs27 cell line for the entire concentration range. In the case of the most resistant melanoma cell line A375, AREs reduced cell proliferation with 25% for C-AREs, respectively with 50% for RG-ARE for the highest concentration tested (400 µg/mL) (Fig. 2a). Both AREs decreased the proliferation of melanoma murine cell line B16-F10 in a dose dependent manner (Fig. 2a). C-ARE, containing only cyanidin derivatives, showed a lower growth inhibition than that of RG-ARE, which contains 3-*O*-glucosides with different aglycones. The IC<sub>50</sub> of C-ARE was 352 µg/mL, while the IC<sub>50</sub> of RG-ARE was 183 µg/mL. These data suggest that the antiproliferative effects of RG-ARE are twice that of C-ARE, when administrated to A375 or B16-F10 melanoma cells.



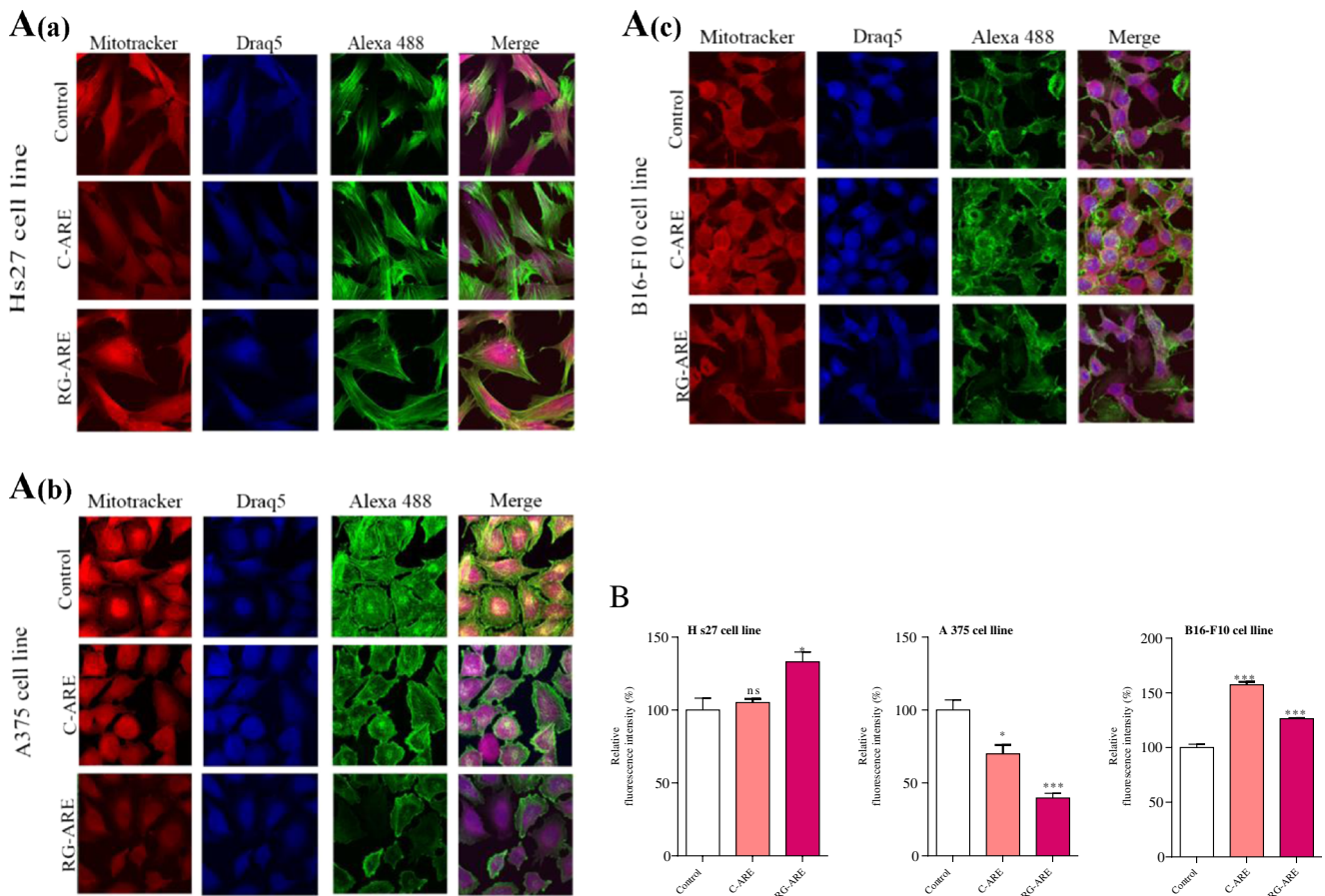
**Fig. 2** Effects of C-ARE and RG-ARE on Hs27, A375 and B16-F10 cell viability (a), LDH release (b) and MDA level (c) after 24 h of exposure. Data represents the means  $\pm$  SEM of at least three independent experiments (significant differences, \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001)

**Detection of LDH Activity** Lactate release into the cell culture medium was determined as an indicator of a damaged cell membrane. In normal Hs27 cells, the treatment with AREs induced an insignificant release of LDH, suggesting no damage of the cell membrane. Conversely, in melanoma cells treated with AREs, substantial LDH release was recorded. For example, the LDH release from A375 cells treated with the highest concentration of the extract (300  $\mu\text{g}/\text{mL}$ ) was approximately 71% for RG-ARE and 37% for C-ARE (Fig. 2b). In the same way, the LDH release increased dose-dependently after RG-ARE treatment of B16-F10 cells, exceeding the level of LDH released after C-ARE treatment (Fig. 2b). Therefore 3-*O*-glucoside anthocyanins (containing different aglycones) found in RG-ARE is more efficient in both melanoma cell lines.

**Determination of Mitochondrial Membrane Potential** In order to assess the function of mitochondria after AREs treatment, MitoTracker CMXRos, a cell-permeant dye that passively diffuses across the plasma membrane and accumulates in active mitochondria was used to monitor the changes of the mitochondrial membrane potential ( $\Delta\Psi\text{m}$ ). A decrease of

$\Delta\Psi\text{m}$  indicates an inhibition of the mitochondrial respiration which occurs as a consequence of cellular stress and can be used as a marker for the antiproliferative efficiency of anthocyanin treatment on melanoma cells. AREs did not reduce  $\Delta\Psi\text{m}$  in normal Hs27 cells but decreased it significantly in A375 melanoma cell, especially in the case of RG-ARE (Fig. 3). This collapse of membrane potential could be associated with a similar decrease in cell respiration, ultimately followed by cytochrome c release, inducing a swelling and rupture of the mitochondrial outer membrane and initiating apoptosis [26]. Although melanoma B16-F10 cells were expected to have a decrease of  $\Delta\Psi\text{m}$ , no depolarization after AREs treatment occurred. We find that there are in literature other studies which reported an increase in  $\Delta\Psi\text{m}$  after a death stimulus, but a decrease of  $\Delta\Psi\text{m}$  occurred later in the death process [27, 28]. A human amelanotic melanoma cell line A375, carrying the BRAF mutation seems to be more resistant than B16F10 murine melanoma cell line.

**Assessment of Malondialdehyde Level** It is already known that reactive oxygen species (ROS) are generated primarily in



**Fig. 3** Images of (A) Hs27 (a), A375 (b) and B16-F10 (c) cells exposed to C-ARE and RG-ARE treatments for 24 h were observed using a Zeiss LSM 710 confocal laser scanning microscope fitted with a 63 $\times$ /1.40NA oil immersion objective. Nuclei were stained with Draq 5 (blue channel),

the cytoskeleton with Phalloidin Alexa488 (green) and mitochondria with MitoTracker Red (red). (B) Fluorescence was quantified by using ImageJ Analysis 1.46r software

the mitochondria of the cell exposed to a stress stimulus. ROS generated in A375 and B16-F10 melanoma cells after AREs treatment (300  $\mu\text{g/mL}$ ), produced toxic free radicals resulting in lipid peroxidation in the form of malondialdehyde (MDA). Therefore, MDA can be used as an indicator of lipid peroxidation and of cellular oxidative stress. RG-ARE was the only extract to cause an increase of MDA level in the A375 cell line, from 68.9  $\mu\text{M}$  in the control to 92.57  $\mu\text{M}$  in treated cells. In the case of the B16-F10 cell line, the RG-ARE treatment had no significant effect but the treatment with C-ARE increased MDA level from 88.23  $\mu\text{M}$  in control to 108.5  $\mu\text{M}$  in treated cells. However in normal cells (Hs27), AREs treatment reduced lipid peroxidation acting as antioxidants (Fig. 2c).

## Conclusions

In summary, all the berries tested in this study are rich sources of anthocyanins and their effects on melanoma cells are dependent on their structure and quantity. Therefore, it is obvious that on normal Hs27 cells anthocyanins from C-ARE and RG-ARE do not have any negative effect. Conversely, when delivered to melanoma cells, anthocyanins are able to reduce cell proliferation, diminished mitochondrial membrane potential and to induce oxidative damage. RG-ARE proved to be more effective at inducing these effects than C-ARE, and this may be attributable to either to the presence of different aglycones (delphinidin, cyanidin, malvidin, petunidin, pelargonidin) acting in a synergistic way or to the differences in the structure of sugar moieties. It is known that cancer cells require high levels of sugars, which are utilised for either fast energy production through the glycolytic pathway, or via the pentose 5-phosphate pathway [29]. Melanoma cells most likely obtain energy only from glucose, as it is an easier and faster way than using the aforementioned four carbohydrates as sources.

Our findings that AREs are effective on melanoma cells at concentrations similar to those achieved by food intake ( $\mu\text{M}$  concentrations) but with no toxicity on normal skin cells are very encouraging and these compounds may be utilized as anti-cancer therapies in the future. A very recent study that used a multistable-isotope labeled Cy-3-*O*-gluc demonstrated a significantly higher relative bioavailability of anthocyanin (about 12%) than previously reported [30]. Anthocyanins absorption could be more efficient locally, for example at gastrointestinal or skin level. Literature data contain few studies about the efficacy of some anthocyanins applied topically [11, 31]. As far as we know there is no other study about the antiproliferative effect of anthocyanins from chokeberries and red grapes on melanoma cells. Our data provide not only a new perspective in the development of novel strategies for melanoma treatment, but also wishes to encourage the

population to consume anthocyanin rich food as a part of an appropriate diet for cancer prevention.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Abbreviations** AREs, Anthocyanin rich extract; LC–ESI-MS, Liquid chromatography followed by electrospray ionization mass spectrometry; DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal Bovine Serum; MTT; 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; RG-ARE, red grape anthocyanin rich extract; C-ARE, chokeberry anthocyanin rich extract; Cy-3-*O*-glu, Cyanidin-3-*O*-glucoside; Cy-3-*O*-ara, Cyanidin-3-*O*-arabinoside; Cy-3-*O*-mal-glu, Cyanidin-3-*O*-malonylglucoside; Cy-3-*O*-dio-glu, Cyanidin-3-*O*-dioxalylglucoside; Cy-3-*O*-rut, Cyanidin-3-*O*-rutinoside; Peo-3-*O*-glu, Peonidin-3-*O*-glucoside; Peo-3-*O*-rut, Peonidin-3-*O*-rutinoside; Delp-3-*O*-gal, Delphinidin-3-*O*-galactoside; Delp-3-*O*-glu, Delphinidin-3-*O*-glucoside; Cy-3-*O*-gal, Cyanidin-3-*O*-galactoside; Delp-3-*O*-ara, Delphinidin-3-*O*-arabinoside; Pet-3-*O*-gal, Petunidin-3-*O*-galactoside; Peo-3-*O*-gal, Peonidin-3-*O*-galactoside; Pet-3-*O*-ara, Petunidin-3-*O*-arabinoside; Mal-3-*O*-gala, Malvidin-3-*O*-galactoside; Mal-3-*O*-glu, Malvidin-3-*O*-glucoside; Mal-3-*O*-ara, Malvidin-3-*O*-arabinoside; Pet-3-*O*-glu, Petunidin-3-*O*-glucoside; Pel-3-*O*-glu, Pelargonidin-3-*O*-glucoside; Delp-3-*O*-rut, Delphinidin-3-*O*-rutinoside; Cy-3-*O*-xyl, Cyanidin-3-*O*-xyloside; Mal-3-*O*-gluc 4 vinylcatechol, Malvidin-3-*O*-glucoside-4-vinylcatechol; Mal-6-acetyl-3-gal, Malvidin-6-acetyl-3-galactoside; Cy-3-*O*-sam, Cyanidin-3-*O*-sambubioside; Pel-3-*O*-sam, Pelargonidin-3-*O*-sambubioside; Pet-3-(6-*O*-p-coumarylglu), Petunidin-3-(6-*O*-p-coumarylglucoside); LDH, Lactate dehydrogenase; MDA, Malondialdehyde

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