Antiproliferative Activity of Anthocyanins Pure Extracts from Mulberries and Raspberries on HeLa and A2780 Human Cancer Cell Lines

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Abstract

Red berries are important sources of bioactive compounds and they are known to provide unique health benefits. Lately, it has been proved that anthocyanins have health benefits against degenerative diseases such as cardiovascular disease, cancer or diabetes. Accordingly, the aim of this study was to characterize the anthocyanin content of anthocyanins pure extracts (APEs) obtained from raspberries (Rubus sp.) and mulberries (Morus sp.) and to evaluate their antiproliferative effect in vitro. Upon chromatographic analysis, three anthocyanins were identified in purified extracts of mulberries (M-APEs), with cyanidin-3-O-glucoside being more abundant. On the other hand, purified extracts of raspberries (R-APEs) contained 2 anthocyanins, both identified as cyanidin-derivatives. The in vitro test demonstrated that APEs decreased the proliferation on both HeLa and A2780 human cancer cell lines in a dose dependent manner, demonstrating that these two different berries are both rich sources of anthocyanins and are able to exert antiproliferative proprieties toward cervical and ovarian cancer.

Keywords: anthocyanins, berries, cancer, HPLC

Introduction

The most conspicuous member of flavonoids is anthocyanins, which are the water-soluble pigments found in many plants, including fruits, vegetables, grains and flowers. Anthocyanin rich foods have gained a lot of attention in the past years due to their positive effects on the human and animal health. Several in vivo studies have proved their antioxidant potential and anti-inflammatory properties against different degenerative illnesses such as diabetes, cardiovascular diseases or cancer (He and Giusti, 2010). Epidemiological investigations showed that moderate consumption of red wine, berry extracts or other anthocyanin containing products have the potential to decrease the risk of developing cardiovascular or visual impairments (Hou, 2003). The anticarcinogenic effect of berries has also been under investigation since berries are rich sources of anthocyanins, making this group of fruits increasingly hot...
topic in the field. In a recent study, the phenolic constituents from six berries were obtained (among which raspberries and mulberries were included) and tested on different tumor cell lines in order to evaluate the growth inhibitory ability, concluding that cell proliferation was inhibited in human oral, breast, colon and prostate cancer cell lines (Seeram et al., 2006).

The raspberry extract was also previously tested on human cervical cancer cell line (HeLa). The anthocyanin rich extract reduced the tumor cells viability and proliferation in a dose-dependent manner (Ross et al., 2007). Similar results have been observed by McDougall (2008), attributed to ellagitannins, phenolic constituents of the fruits (McDougall et al., 2008). Anthocyanins are also antiangiogenic, because of their cytotoxic effect towards Helicobacter pylori, a pathogen that can cause gastric cancer (Zafra-Stone et al., 2007). Besides the anti-proliferative, pro-apoptotic and inhibitory effects of anthocyanins from berries on in vitro tumor cells, it has been proven that mulberry extract hinders the migration of human lung carcinoma cells by inhibiting the activities of oncogenic transcription factors (MMP-2 and u-PA) (Chen et al., 2006). Anthocyanins have antioxidant effect as demonstrated in various in vitro tests previously conducted on tumoral cell lines (colon, liver, breast, endothelial, leukemic and skin cells). The beneficial activity of the anthocyanins lays in their chemical structure, specifically the phenolic ring that allows them to scavenge the reactive oxygen species (ROS) (Xia et al., 2010).

Besides the antioxidant effect, they can also inhibit the proliferation of tumoral cells by blocking cellular pathways that are important in the development of regulatory proteins of the cell cycle process, like p53, p21, p27, cyclidin A and D1, etc., revealed by an in vitro study done on different cancer cell lines like stomach, colon, breast, lung and SNC cells (Zhang et al., 2005). Anthocyanins have many putative effects as well, due to their antioxidant and anti-inflammatory properties. They are absorbed from stomach as well as in the small intestine. However, there are some limitations regarding the transportation into the blood circulation, which makes anthocyanins to have low bioavailability (He and Giusti, 2010).

Several anthocyanin extracts tests have been conducted on cancer and normal cell lines with highly positive results. The normal cells showed a normal growth and a proliferation rate unaffected by the anthocyanins, whereas the tumoral cells suffered morphological changes, turning into spherical shape, and the proliferation rate was a lot decreased (Hakimuddin et al., 2004). This could be linked to the anthocyanins selective cytotoxic effect in vitro.

In this context, the aim of this work was to obtain data about chemical composition of two common berries consumed and also to evaluate their antiproliferative potential.

**Material and Methods**

**Chemsicals.** HPLC grade water, LC-MS grade methanol (99.9%), formic acid (88%), certified ACS grade ethyl acetate, certified ACS grade hydrochloric acid (12N) were purchased from Sigma-Aldrich (Darmstadt, Germany). Standards of cyanidin (95%), cyanidin-3-O-arabinoside (97%), cyanidin-3-O-glucoside (95%), cyanidin-3-O-galactoside, petunidin-3-O-glucoside (90%) were acquired from Polyphenols Laboratories AS (Sandnes, Norway). Dulbecco’s Modified Eagle’s Medium (DMEM) was acquired from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS), streptomycin, penicillin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sodium pyruvate were purchased from Gibco, Romania.

**Berries samples.** Raspberries (Rubus idaeus) and mulberries (Morus nigra) were harvested in 2016 from an area located in Cluj-Napoca, Romania (Coordinate: 46°57′23″N 23°46′50″E). Samples were then washed 3 times with water and kept at - 20°C until further analyses. Plant material was identified by professional botanists of the Department of Botany, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania using available scientific literature comparison with properly identified herbarium specimens.

**Extraction and purification of anthocyanins pure extracts (APes).** Berries samples were homogenized using an Ultraturax homogenizer (Ultra-Turrax, Model Miccra D-9 KT, Digitronic GmbH, Bergheim, Germany) and 5 g of it was used for the further analyses. Acidified methanol (0.3 % HCl (v/v)) was added and the mixture was shaken for 20 min on a magnetic stirrer in dark. The supernatant was collected and the extraction procedure was repeated until the samples were
colorless. The collected extracts were evaporated at 35°C under reduced pressure (Rotavapor R-124, Buchi, Switzerland) and redissolved in acidified water and filtered through 0.45 μm millipore filter prior to HPLC and antioxidant analyses. To avoid any anthocyanin pigment loss during the experiment, all the analytical methods steps were carried out in subdued light and under controlled conditions.

**Preparation of APEs.** The semipurification of the obtained extracts was done by solid-phase extraction (SPE) using C₁₈ Sep-Pak cartridges (Waters Corp., Milford, MA, USA) based on a recent protocol (Diaconeasa et al., 2015, Giusti et al., 1999). Briefly, anthocyanins and other polyphenolics were adsorbed onto the Sep-Pak column, while sugars, acids, and other water-soluble compounds were removed by washing with aqueous HCl. The flavonoids fraction was eluted with ethyl acetate (EtOAc). Anthocyanins were eluted and collected in acidified methanol.

**HPLC-PDA-ESI/MS analysis of APEs.** HPLC analyses were performed on a Shimadzu HPLC-PDA system equipped with a binary pump delivery system LC-20 AT (Prominence), a degasser DGU-20 A3 (Prominence), a diode-array SPD-M20 and a UV-VIS detector. The separation was achieved on a Luna Phenomenex C₁₈ column (5µm, 25 cm x 4.6 mm) and column temperature was maintained at 25°C. The mobile phases were 4.5% formic acid in bidistilled water (solvent A) and acetonitrile (100%) (solvent B) with a flow rate set at 0.5 mL/min. The gradient elution system started with 10% B for 9 minutes. The percent of B increased to 12% at 17 min and continued up to 25% B at 20 min. Between 20 and 55 min, the percentage of B was 90%. The chromatograms were monitored at 520 nm. The compounds identification and peak assignments were done based on their retention times, UV-VIS spectra as well as comparisons to standards and published data. As a confirmation, the samples were analyzed by HPLC-ESI-MS according to our protocol published recently (Bunea et al., 2013).

**Cell proliferation.** The cells were grown in standard conditions: 37°C, 95% humidity, 5% CO₂. Cells were cultivated with high-glucose DMEM supplemented with 10% fetal calf serum, penicillin, streptomycin and amphotericin. MTT test was applied in order to measure the proliferation rate of the cells. The assay is based on the reduction of MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to formazan, reaction that only occurs in the mitochondria of the living cells. Cells were plated (10,000 cells per well) in 96-well plates and treated with APEs (0-400µg/mL). The cells were washed using PBS and MTT solution was added in each well. After 2 h of incubation, the MTT reagent was removed and the formazan particles were solubilized with DMSO. The absorbance was read at 550 nm and at 630 nm (for background) with a microplate plate reader HT BioTek Synergy (BioTek Instruments, VT). Cell viability was expressed as a percentage of control (cells incubated in normal medium only).

**Statistical Analysis.** All data were expressed as mean ± 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 Discussions**

**HPLC-PDA-ESI/MS analysis of anthocyanins pure extracts (APEs)**

Anthocyanins identification and peak assignments were done based on their retention times, UV-VIS spectra, MS data comparing with standards and literature. Three anthocyanins were detected in mulberries while raspberries contain only 2 types of anthocyanin, both cyanidin derivatives (Table 1).

**Raspberries.** In R-APEs, two anthocyanins were detected, both being cyanidin derivative. Peak 1 at the Retention time 10.31 min (Rt ) had a molecular ion at m/z 611 and a fragment ion at m/z 287, which corresponds to cyanidin. Based on this and literature review, we concluded that peak 1 is cyanidin-3-O-sophoroside. This peak was found to be the major one quantified. A previous study published by Seeram et al., 2006, (Seeram, Adams, Zhang, Lee, Sand, Scheuller and Heber 2006) reported similar results, but have also identified the presence of pelargonidin-3-O-glucoside. The second peak (Rt = 13.16 min) identified in raspberries was cyanidin-3-O-sophoroside-5-rhamnoside, having a mass spectrum with molecular ion at m/z 625 and a fragment ion at m/z 317. Another paper (Borges et al., 2007), reported that raspberry juice contained nine anthocyanins, two ellagittannins and ellagic acid. The main raspberry anthocyanins
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identified by them were di- and triglycosides with the disaccharide cyanidin-3-sophoroside being the major anthocyanin in the juice followed by the trisaccharide cyanidin-3-O-(2G-O-glucosylrutinoside) and the monosaccharide cyanidin-3-O-glucoside. Regarding quantitative analysis, the obtained values are shown in Table 1. The results obtained for raspberries were comparable with those obtained previously (De Ancos et al., 1999). They evaluated the anthocyanins from 4 Spanish raspberry cultivars and the reported values ranging from 31.13 to 122.88 mg /100 g of fresh berries.

**Mulberries.** As we mention before, mulberry extract was found to contain 3 anthocyanins mainly cyanidin derivatives (Figure 1; Table 1), all of them were identified by comparing their MS data and retention times with published data. The major peak (Rt = 9.8 min) showed a molecular ion at m/z 449, which fragmented to give ions at m/z 287 corresponding to a cyanidin aglycone. The fragment loss [M - 162] + indicated a hexose moiety. Thus, the major peak was tentatively identified as cyanidin-3-O-glucoside. Peak 2 (Rt = 11.6 min) was cyanidin-3-rutinoside with molecular ion at m/z 595. The fragment ion was at m/z 287 for

**Table 1.** Retention times, UV-Vis, tentative identification and contents of anthocyanins in purified extracts

<table>
<thead>
<tr>
<th>Peak no</th>
<th>Rt (min)</th>
<th>Molecular ion [M-H]^+ (m/z)</th>
<th>Fragment ion [M-H]^+ (m/z)</th>
<th>Tentative identification</th>
<th>Quantity (mg cy-3-O-gal/100 g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Raspberries</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11.9</td>
<td>611</td>
<td>287</td>
<td>Cyanidin-3-O-sophoroside</td>
<td>40.32±0.22</td>
</tr>
<tr>
<td>2</td>
<td>15.2</td>
<td>757</td>
<td>611/430/286</td>
<td>Cyanidin-3-O-sophoroside-5-rhamnoside</td>
<td>22.05±0.56</td>
</tr>
<tr>
<td><strong>TOTAL:</strong></td>
<td><strong>62.37</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mulberries</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13.5</td>
<td>449</td>
<td>287</td>
<td>Cyanidin-3-O-glucoside</td>
<td>70.44±0.26</td>
</tr>
<tr>
<td>2</td>
<td>15.6</td>
<td>595</td>
<td>287</td>
<td>Cyanidin-3-O-rutinoside</td>
<td>12.55±0.45</td>
</tr>
<tr>
<td>3</td>
<td>-17.9</td>
<td>625</td>
<td>317</td>
<td>Petudin-3-(6-O-p-coumaryl-glucoside)</td>
<td>2.55±0.12</td>
</tr>
<tr>
<td><strong>TOTAL:</strong></td>
<td><strong>85.54</strong></td>
<td></td>
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</tbody>
</table>

*Figure 1.** HPLC chromatograms of analysed samples recorded at 520 nm
cyanidin, and -308 for loss of rutinoside. There was one more anthocyanin present in our samples (petunidin-3-(6-O-p-coumaryl-glucoside). However, the amount of this anthocyanin was very low compared to other two anthocyanins. Our results were in accordance with the study by Dugo and others (2001) regarding the anthocyanin composition of mulberry extract. However, contrary to our results, a recent study identified 4 different anthocyanins in mulberry water extracts: cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, pelargonidin-3-O-glucoside and pelargonidine-3-O-rutinoside (Liu, et al. 2008), cyanidins being the major ones quantified. Moreover, Zou at el. 2011 identified 8 anthocyanins in mulberry: cyanidin-3-(2G-glucosylrutinoside), delphinidin-3-O-rutinoside-5-glucoside, cyanidin-3,5-diglucoside, cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, pelargonidin-3-O-glucoside, pelargonidine-3-O-rutinoside, and delphinidin-3-O-rutinoside (Zou, et al. 2011). Their results showed that cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside were the major anthocyanins in mulberry. The difference reported till now in mulberry anthocyanin is probably linked to different mulberry varieties, cultivation conditions or harvesting time.

Cell proliferation

The in vitro test demonstrated that APEs decreased the proliferation on both cell lines (HeLa and A2780) in a dose dependent manner. Many studies conducted studies on these cell lines HeLa (Diaconeasa, Leopold, Rugina, Ayvaz and Socaciu 2015, Ross, McDougall and Stewart 2007), A2780 (Lee, et al. 2016) or on other tumoral cell lines reported similar results (Seeram, Adams, Zhang, Lee, Sand, Scheuller and Heber 2006, Zhang, Vareed and Nair 2005).

In our experiment, A2780 cell line was found to be more sensitive to the treatment. At concentrations higher than 250 µg/mL, cytotoxic effects were observed. Comparing the purified extracts, raspberries extract was more effective on both cell lines. The obtained results are comparable.
with those published previously (Bagchi et al., 2004, Stoner et al., 2010).

A recent in vitro study evaluated the antiproliferative and apoptotic effect of mulberries extract on human prostate cancer cells (Turan, et al. 2017). The authors suggested that mulberries extract treatment arrested the cell cycle of PC-3 cells at the G1 phase, induced apoptosis via increased caspase activity and reduced mitochondrial membrane potential. Moreover, mulberries extracts were used as a supplement to synergize with the effects of paclitaxel in the treatment of the TSGH 8301 human bladder cancer cell line (Chen, et al. 2016). The treatment with paclitaxel combined with mulberries showed an enhancement of paclitaxel cytotoxicity by inducing severe G2/M arrest, mitotic catastrophe and subsequent apoptosis. These findings clearly demonstrated the synergistic effect on the anticancer efficacy of paclitaxel/mulberries, providing a novel and effective therapeutic option for bladder cancer treatment management. Several studies have focused on the anthocyanins as bioactive components responsible for raspberries-mediated antiproliferative potential. Zhang et al., reported that a black raspberry extract inhibits proliferation and regulates apoptosis in cervical cancer cells (Zhang, et al. 2011). Recently, another study conducted by Camille S. Bowen-Forbes et al. (Bowen-Forbes, et al. 2010) demonstrated that raspberry extracts moderate COX inhibitory activity (27.5–33.1%) at 100 μg/mL, and exhibited potential to inhibit cancer cell growth, inhibiting colon, breast, lung, and gastric human tumor cells by 50, 24, 54 and 37%, respectively.

Conclusions
Out of three anthocyanins determined in M-APEs, the major compounds detected were cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside. On the other hand, only two anthocyanins were identified and quantified in R-AREs. The purified anthocyanin extracts were tested on two different cell lines (HeLa, human cervical cancer cells and A2780, human ovarian cancer cells) and both extracts exhibited antiproliferative effects, due to the well-known antioxidant and anti-inflammatory property of the anthocyanins. Based on our findings, these fruits demonstrated to contain high concentrations of bioactive compound such as anthocyanins with antiproliferative potential in vitro.

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