ZORIȚA MARIA SCONȚA

EXTRACTION, PURIFICATION, CHARACTERIZATION AND IN VITRO TESTING OF ANTHOCYANIN RICH FRACTIONS OBTAINED FROM ARONIA MELANOCRAPA AND VACCINIUM SP.

PhD THESIS ABSTRACT

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CONTENT

INTRODUCTION: AIMS AND OBJECTIVES ........................................................................... V
EXPERIMENTAL RESULTS ............................................................................................... VII
CHAPTER I ............................................................................................................................. IX
CHEMICAL CHARACTERIZATION OF DIFFERENT VARIETIES OF ARONIA
MELANOCARPA SP. AND VACCINUM MYRTILUS SP. AND THEIR
ANTIOXIDANT ACTIVITY ................................................................................................. IX
MATERIALS AND METHODS ............................................................................................ IX
    Extraction .................................................................................................................... IX
    High-performance liquid chromatography (HPLC) ................................................... IX
    Total polyphenolic content ..................................................................................... IX
    Total flavonoid content ......................................................................................... IX
    Quantification of total anthocyanins ...................................................................... IX
    Scavenging effect on ABTS radicals ....................................................................... X
    Ferric reducing antioxidant power (FRAP) Assay ............................................... X
    Cupric reducing antioxidant potential assay CUPRAC ...................................... X
    Oxygen radical absorbance capacity assay (ORAC) ........................................ X
    Scavenging effect on hydrogen peroxide (HPS) ................................................ X
RESULTS AND DISCUSSIONS ......................................................................................... X
    Quantitative analysis of anthocyanins by HPLC ................................................ X
    Total polyphenolic content .................................................................................. XV
    Total flavonoid content ....................................................................................... XV
    Total anthocyanin content .................................................................................. XV
    Total antioxidant activity ................................................................................... XVII
    Trolox equivalent antioxidant capacity (ABTS) assay .................................. XVII
    Ferric reducing antioxidant potential (FRAP) assay ................................ XVII
    Cupric reducing antioxidant potential (CUPRAC) assay ................................ XVII
    Oxygen radical absorbance capacity (ORAC) assay .................................... XVII
    Hydrogen peroxide scavenging activity (HPS) ................................................ XVII
    Conclusions ........................................................................................................... XIX
CHAPTER II ...................................................................................................................... XXI
PURITY EVALUATION OF ANTHOCYANIN RICH-FRACTIONS OBTAINED
FROM CHOKEBERRIES AND BLUEBERRIES USING DIFFERENT SOLID PHASE
EXTRACTION METHODS ............................................................................................... XXI
MATERIALS AND METHODS .......................................................................................... XXI
    Preparation of anthocyanin rich-fraction .......................................................... XXI
    C18 Sep Pack SPE ................................................................................................ XXI
    Oasis- MCX SPE .................................................................................................... XXI
    Amberlite XAD-7 and Sephadex LH-20 SPE ....................................................... XXI
    Purity evaluation .................................................................................................. XXI
HPLC-PDA and MS analysis ................................................................. XXI
RESULTS AND DISCUSSIONS ............................................................ XXII
Purity evaluation by HPLC-PDA .......................................................... XXII
Purity evaluation by molar absorptivity ............................................... XXIV
SPE methods comparison ................................................................ XXIV
Purity determination methods comparison .......................................... XXIV
Confirmation of purity with HPLC-MS analysis .................................. XXV
CONCLUSIONS .............................................................................. XXVII
CHAPTER III. .................................................................................. XXVIII
CHEMOPROTECTIVE EFFECTS OF AN ANTHOCYANIN-RICH FRACTION
FROM ARONIA PRUNIFOLIA ON B16-F10 MELANOMA MURINE AND HELA
TUMOR CERVICAL CELL LINES ......................................................... XXVIII
MATERIALS AND METHODS ............................................................... XXVIII
   Extraction preparation of anthocyanin fraction ................................... XXVIII
   Cell culture ................................................................................... XXVIII
   Cellular proliferation ..................................................................... XXVIII
   Intracellular reactive species assay ............................................... XXVIII
RESULTS AND DISCUSSION ............................................................... XXVIII
   Inhibition of B16F10 and HeLa tumor cells survival ......................... XXIX
   Intracellular ROS ......................................................................... XXX
CONCLUSIONS .............................................................................. XXXI
CHAPTER IV. .................................................................................. XXXIII
ANTHOCYANINS DETERMINATION IN VARIOUS CULTIVARS OF HIGHBUSH
BLUEBERRIES AND THEIR ANTIPROLIFERATION AND APOPTOSIS
PROPERTIES IN B16-F10 METASTATIC MURINE MELANOMA CELLS .... XXXIII
Materials and methods ................................................................. XXXIII
   Preparation of anthocyanin fraction .............................................. XXXIII
   HPLC – DAD analysis of anthocyanins .......................................... XXXIII
   HPLC-ESI-MS analysis of anthocyanins ...................................... XXXIII
   Cupric reducing antioxidant capacity (CUPRAC) assay .................. XXXIV
   Scavenging effect on ABTS radical .............................................. XXXIV
   Oxygen radical absorbance capacity (ORAC) assay ..................... XXXIV
   Cell and cell culture .................................................................... XXXIV
   Analysis of cell proliferation ......................................................... XXXIV
   Detection of LDH activity ............................................................. XXXIV
   96-well-based EB/AO staining ...................................................... XXXIV
   TUNEL assay and analysis ............................................................ XXXV
Results and discussions ................................................................. XXXV
   Identification and quantification of blueberry anthocyanins ............ XXXV
   Determination of antioxidant activity .......................................... XL
   Inhibition of tumor cell proliferation ............................................. XL
INTRODUCTION: AIMS AND OBJECTIVES

Anthocyanins are the exceptional natural pigments, not only due to their wonderful range of red to blue colours of plants, flowers or fruits, but to their strong antioxidant potential in vitro and in vivo, with specific health benefits. The richest sources of anthocyanins are the small berries like chokeberries, blueberries, elderberries, cranberries and many others fruits and vegetables.

As abundant polyphenol type of antioxidants in our diets, anthocyanins have received an increased interest from consumers and food manufacturers (Scalbert and Williamson, 2000). There are many studies which had suggested a potential health benefit of anthocyanins (Jing and Giusti, 2011). Also they can be used in technological field, as natural colorants.

Despite the great potential of anthocyanins use in food, pharmaceutical and cosmetic industries, this was limited by their relative instability and low extraction percentages. Currently, most investigations on anthocyanins are focused on solving these problems, as well as their purification, identification and antioxidant effect in cell culture (Castaneda-Ovando et al., 2009).

The aim of this PhD thesis was:

Our aim was to investigate the metabolic profile of four Romanian chokeberry varieties and nine Romanian blueberry varieties regarding anthocyanins, total phenolic and flavonoid content by High Performance Liquid Chromatography (HPLC) and spectrophotometric assays. The antioxidant capacity of analyzed samples was evaluated through five, complementary assays (ABTS, HPS, ORAC, FRAP, CUPRAC). HPS assay being a new method to evaluate antioxidant activity of foods.

The second major proposal of this work was to obtain a rich fraction of anthocyanins and to evaluate its antioxidant and chemopreventive potential against tumor cells. HeLa (human ovarian cancer) and B16F10 (melanoma murine) cells line where chosen in order to demonstrate the proapoptotic effect of anthocyanins.

Based on recent data literature which reveals the anti-tumor proprieties of anthocyanins such as inhibition of cell growth, cell cycle progression, induction of apoptosis, inhibition of angiogenesis, tumor invasion and metastasis we developed an experiment to evaluate their antiapoptotic and control the cancer cells proliferation.

The main objectives are:
1. The evaluation of the metabolic profile of four Romanian chokeberry varieties three varieties of cultivated highbush blueberries and two types of wild blueberries by extraction, spectral and chromatographic characterization and identification of individual anthocyanins
2. The evaluation of the comparative antioxidant activity (by complementary assays ABTS, HPS, ORAC, FRAP, CUPRAC) of chokeberry and blueberry extracts and quantification of their total polyphenol content.
3. Purification of an anthocyanin-rich fractions from blueberry and chokeberry extracts and purity evaluation by HPLC and molar absorptivity methods
4. The evaluation of the chemopreventive potential and the prooxidative effect of the anthocyanin-rich fraction on HeLa tumor cervical and B16-F10 melanoma murine cell lines, using MTT proliferation assay and Intracellular reactive species assay
5. Investigation of blueberry anthocyanins potential in melanoma cancer cells, by MTT proliferation assay, LDH activity, 96-well-based EB/AO staining and TUNEL assay

**Thesis structure.** The thesis is structured into two main parts, first one contains a literature survey and second one is focused on the original contributions (including results and discussions, as well presentations of experimental measurements).

The first part includes two chapters:

**Chapter 1** summarizes the chemical characterization of anthocyanins regarding their stability, biosynthesis, antioxidant activity and analytical methods used for purification.

**Chapter 2** describes the pathology of cancer disease and anthocyanins implications in tumoral process. Briefly, recent research studies are summarized on antocyanins as inducers of apoptosis and inhibitors of tumor invasion on different types of cancer.

The second part includes four chapters (4-6):

**Chapter 3** reveals a comparative study on antioxidant activity and their anthocyanin content from the all studied blueberry varieties

**Chapter 4** contains an experiment realized in order to evaluate and compare different anthocyanins solid phase extraction methods

**In chapter 5** an experiment regarding evaluation of the chemopreventive effects of chokeberry anthocyanin-rich fraction on cancer cell lines (B16-F10 melanoma murine and HeLa tumor cervical cell lines) is shown.

**In chapter 6** a study on anthocyanins identification and their antioxidant activity from blueberry cultivars is presented. The antitumoral and proapoptotic effects of anthocyanin-rich fraction on B16-F10 melanoma murine cells were also evaluated.

A part of the experimental results are due to the collaborative agreement between our University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania, Department of Chemistry & Biochemistry with the Ohio State University, Columbus, OH, Department of Food Chemistry.

The PhD program was funded by the European Social Fund Doctoral Program POSDRU/88/1.5/S/49598, National Project PN-II-RU-TE-109-2010 and European Social Fund Postdoctoral Program POSDRU/89/1.5/S/60746.
EXPERIMENTAL RESULTS

Anthocyanins belong to the class of plant secondary metabolites named flavonoids and they are responsible for red, orange or blue colors in many vegetables and fruits (Giusti and Wrolstad, 2003) (Fig. 2). These pigments are found to be more complex in flowers and ornamental plants than anthocyanins found in fruits and vegetables, blueberries and grapes make exception because they contain a variety of anthocyanins (Cara et. al., 2008).

They are stable and soluble in aqueous media, properties which makes them interesting for the use as natural water-soluble colorants (Ahmadiani, 2012, Castanelo Osvaldo et al., 2009; Socaciu ,2007; Pazmino-Duran et al., 2001).

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Name} & \text{Substitution} & \text{Visible color} & \text{Visible max. (nm) in MeOH-HCl} \\
\hline
\text{Cyaniding (Cy)} & \text{OH} & \text{magenta} & 535 \\
\text{Peonidin (Pn)} & \text{OCH}_3 & \text{red} & 532 \\
\text{Pelargonidine (Pg)} & \text{H} & \text{purple} & 520 \\
\text{Malvidin (Mv)} & \text{OCH}_3 & \text{OCH}_3 & 542 \\
\text{Delphinidin (Dp)} & \text{OH} & \text{OH} & 546 \\
\text{Petunidin (Pt)} & \text{OCH}_3 & \text{OH} & 543 \\
\hline
\end{array}
\]

Fig. 1. Structure of the most commonly anthocyanidins (adapted from Harborne , 1998)

Fig. 2. Structura chimică a celor mai importanţi antociani anthocyanidins (adaptată după Harborne , 1998)

Anthocyanins stability can be affected also by several factors such as pH, temperature, light, chemical structure, co-pigmentation, acylation, oxygen, solvents,
enzymes, flavonoids, proteins and metallic ions. Chemical structure of anthocyanins plays an important role in their stability.

Being found in many edible berries anthocyanins offer some hope in the struggle to prevent and treat cancer. Studies performed on cell cultures, animal cells or on human intervention cells have shown that berry fruits have great potential to reduce the risk of many cancer types. This is because they contain phytochemicals and anthocyanins in their composition. (Kähkönen et al., 2001)
CHAPTER I.
CHEMICAL CHARACTERIZATION OF DIFFERENT VARIETIES OF ARONIA MELANOCARPA SP. AND VACCINUM MYRTILUS SP. AND THEIR ANTIOXIDANT ACTIVITY

The identification and quantification of anthocyanin founded in chokeberry and blueberry where evaluated through HPLC analysis. Various antioxidant activity methods have been used evaluate and compare the antioxidant activity of analyzed extracts: ABTS, FRAP, CUPRAC, ORAC and H$_2$O$_2$.

MATERIALS AND METHODS

Extraction. Berries analyzed (5 g) were homogenized in methanol containing HCl (0.3%) using an ultraturax (Micra D-9 KT Digitronic, Germany). Extracts were concentrated at 35°C under reduced pressure (Rotavapor R-124, Buchi, Switzerland) and filtered through 0.45 μm Millipore filter for HPLC and antioxidant assays.

Quantitative HPLC analysis of anthocyanins

High-performance liquid chromatography (HPLC) is now one of the most used technique for chromatographic separation in analytical chemistry. HPLC analyses were performed on a Shimadzu, equipped with a diode-array detector (DAD) using a Luna Phenomenex C-18 column (5µm, 25 cm x 4.6 mm) The mobile phase consisted in: solvent A - formic acid (4.5%) in bidistilled water and solvent B - acetonitrile. The gradient elution system was: 10% B, 0-9 min; 12% B, 9-17 min; 25% B 17-30 min; 90% B, 30-50 min; 10% B, 50-55 min.

Total polyphenolic content. Folin's method measures -OH groups in a sample based on the fact that light absorption increases as OH groups in a sample increase. Phenolic compounds react with Folin-Ciocâlteu’s reagent only under basic conditions (adjusted by a sodium carbonate solution to pH =10). Absorption at 765 nm was measured. Total phenol contents were expressed in gallic acid equivalents.

Total flavonoid content. The total flavonoid content of chokeberry and blueberry extracts was determined by a colorimetric method as described previously in other studies (Kim et al., 2003; Zhishen et al., 1999).

Quantification of total anthocyanins. Chokeberry and blueberry total anthocyanins were determined by the differential pH method based on the property of anthocyanin pigments to change the color with pH. Total monomeric anthocyanins (mg cyanidin 3-galactoside equivalent/ 100 g FW) were calculated as follows:

\[
\text{Anthocyanin content (mg/L)} = \frac{(A \times \text{MW} \times \text{DF} \times 1000)}{\varepsilon \times L},
\]

where \( A = (A_{510\,\text{nm}} \, \text{pH} \, 1.0 \, -A_{700\,\text{nm}} \, \text{pH} \, 1.0) - (A_{510\,\text{nm}} \, \text{pH} \, 4.5 \, - A_{700\,\text{nm}} \, \text{pH} \, 4.5), \quad \text{MW}= \text{cyanidin 3-galactoside molecular weight (484.84); DF = dilution factor; } \varepsilon =
cyanidin 3-galactoside molar absorbptivity in methanol/ HCl (34300 M⁻¹cm⁻¹); L = cell pathlength (1 cm) (Giusti and Wrolstad, 2001).

**Scavenging effect on ABTS radicals.** The ABTS assay (2, 2-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid) diammonium salt) is also called TEAC (Trolox®-equivalent antioxidant capacity) and is based on the capacity of a sample to scavenge the ABTS radical cation (ABTS. +) compared to a standard antioxidant (Trolox).

**Ferric reducing antioxidant power (FRAP) Assay.** Antioxidants are evaluated as reducers of Fe³⁺ to Fe²⁺, which is chelated by TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) to form Fe²⁺-TPTZ complex, with a maximum absorbance at 593 nm (Benzie and Strain, 1999).

**Cupric reducing antioxidant potential assay CUPRAC** was determined according to a method reported before (Apak et al., 2004).

**Oxygen radical absorbance capacity assay (ORAC)** measure the peroxyl radical scavenging activity using as standard 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) (Huang et al., 2005).

**Scavenging effect on hydrogen peroxide (HPS)** this assay was carried out following the procedure of Ruch et al., 1989.

**RESULTS AND DISCUSSIONS**

**Quantitative analysis of anthocyanins by HPLC.** Anthocyanins identification and peak assignment are based on their retention times, UV-VIS spectra comparing with standards (cyanidin-3 galactoside, cyanidin-3 glucoside, delphinidin-3 rhamnoside, pelargonidin-3 glucoside, delphinidin, paeonidin-3-glucoside, malvidin-3-glucoside, cyanidin, malvidin) and the literature data. The total anthocyanin content in chokeberries varied from 290.69 mg/ 100 g FW in AmA to 1319.18 mg/ 100 g FW in AmN (Table 1). The HPLC chromatograms of the chokeberry extracts are presented in Fig. 2.

![HPLC chromatogram](image)

### Table 1

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>Chokeberry samples</th>
<th>Ap</th>
<th>AmA</th>
<th>AmN</th>
<th>AmV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/100g %</td>
<td>mg/100g %</td>
<td>mg/100g %</td>
<td>mg/100g %</td>
<td>mg/100g %</td>
</tr>
<tr>
<td>Cy-3-O-galactoside</td>
<td>576.7 ± 18 a</td>
<td>67.1</td>
<td>200.9 ± 13 a</td>
<td>69.1</td>
<td>906.9 ± 20 a</td>
</tr>
<tr>
<td>Cy-3-O-glucoside</td>
<td>23.1 ± 4 d</td>
<td>2.6</td>
<td>6.8 ± 2 c</td>
<td>2.3</td>
<td>14.9 ± 3 d</td>
</tr>
<tr>
<td>Cy-3-O-arabinoside</td>
<td>209.5 ± 12 b</td>
<td>24.3</td>
<td>68.1 ± 7 b</td>
<td>23.4</td>
<td>352.4 ± 15 b</td>
</tr>
<tr>
<td>Cy-3-O-xylloside</td>
<td>49.9 ± 9 c</td>
<td>5.8</td>
<td>14.7 ± 3 c</td>
<td>5.0</td>
<td>44.8 ± 8 c</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>859.41</strong></td>
<td>100</td>
<td><strong>290.69</strong></td>
<td>100</td>
<td><strong>1319.18</strong></td>
</tr>
</tbody>
</table>

Notes: means with different letters in the same column are statistically significant at P≤ 0.05
Based on their retention time, UV-VIS spectra compared with standards and literature data, 13 anthocyanins were identified in bluebery extracts.(Fig. 3). In Vaccinium myrtillus extracts, petunidin-3-glucoside and delphinidin-3-glucoside have the highest contribution to the anthocyanin content. In Vaccinium corymbosum, in Elliot and Duke variety the major anthocyanin content was peonidin-3-galactoside but in BlueCrop this anthocyanin was absent. (Table 2)

Fig. 2. HPLC chromatograms of chokeberry Aronia melanocarpa var. Nero (A), Aronia melanocarpa var. Viking (B), Aronia prunifolia (C) Aronia melanocarpa var. Aron (D). Peak identification 1-cyanidin 3-O-galactoside, 2- cyanidin 3-O-glucoside, 3-cyanidin 3-O-arabinoside, 4- cyanidin 3-O-xyloside.

Fig. 2. Cromatogramele HPLC ale extractelor de Aronia melanocarpa var. Nero (A), Aronia melanocarpa var. Viking (B), Aronia prunifolia (C) Aronia melanocarpa var. Aron (D). Peak identification 1-cyanidin 3-O-galactoside, 2- cyanidin 3-O-glucoside, 3-cyanidin 3-O-arabinoside, 4- cyanidin 3-O-xyloside.
Aron (D). Identificarea peak-urilor 1-cianidin 3-O-galactozid, 2- cianidin 3-O-glucozid, 3- cianidin 3-O-arabinozid, 4- cianidin 3-O-xilozid
Anthocyanin concentrations in blueberry extracts (mg/100 g FW), calculated as cy-3-galactoside equivalent

<table>
<thead>
<tr>
<th>Elution order</th>
<th>Compounds</th>
<th>BlueCrop</th>
<th>Wild 1</th>
<th>Wild 2</th>
<th>Elliot</th>
<th>Duke</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Delphinidin-3-galactoside</td>
<td>53.29</td>
<td>113.67</td>
<td>73.43</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>2.</td>
<td>Delphinidin-3-glucoside</td>
<td>24.53</td>
<td>119.86</td>
<td>87.14</td>
<td>53.62</td>
<td>23.69</td>
</tr>
<tr>
<td>3.</td>
<td>Cyanidin-3-galactoside</td>
<td>9.96</td>
<td>91.85</td>
<td>37.95</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>4.</td>
<td>Delphinidin-3-arabinoside</td>
<td>31.78</td>
<td>66.64</td>
<td>76.35</td>
<td>41.07</td>
<td>13.64</td>
</tr>
<tr>
<td>5.</td>
<td>Cyanidin-3-glucoside</td>
<td>2.08</td>
<td>96.48</td>
<td>43.34</td>
<td>3.09</td>
<td>0.11</td>
</tr>
<tr>
<td>6.</td>
<td>Petunidin-3-galactoside</td>
<td>28.54</td>
<td>38.36</td>
<td>17.53</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>7.</td>
<td>Petunidin-3-glucoside</td>
<td>25.14</td>
<td>146.27</td>
<td>85.19</td>
<td>21.35</td>
<td>8.18</td>
</tr>
<tr>
<td>8.</td>
<td>Paeonidin-3-galactoside</td>
<td>0.00</td>
<td>8.71</td>
<td>0.00</td>
<td>125.79</td>
<td>37.11</td>
</tr>
<tr>
<td>9.</td>
<td>Petunidin-3-arabinoside</td>
<td>12.70</td>
<td>12.80</td>
<td>9.66</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>10.</td>
<td>Paeonidin-3-glucoside</td>
<td>54.37</td>
<td>108.81</td>
<td>28.91</td>
<td></td>
<td>12.14</td>
</tr>
<tr>
<td>11.</td>
<td>Malvidin-3-galactoside</td>
<td>37.97</td>
<td>119.53</td>
<td>39.00</td>
<td>67.45</td>
<td>27.55</td>
</tr>
<tr>
<td>12.</td>
<td>Malvidin-3-glucoside</td>
<td>34.75</td>
<td>17.51</td>
<td>6.04</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>13.</td>
<td>Malvidin-3-arabinoside</td>
<td>0.00</td>
<td>tr</td>
<td>tr</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>315.11</strong></td>
<td><strong>940.50</strong></td>
<td><strong>504.55</strong></td>
<td><strong>312.38</strong></td>
<td><strong>122.43</strong></td>
</tr>
</tbody>
</table>
Fig. 3. HPLC chromatogram of blueberry extracts: (A) Bluecrop, (B) Duke, (C) Elliot, (D) Wild 1, (E) Wild 2. Peak identification: 1-Delphinidin-3-O-galactoside, 2-Delphinidin-3-O-glucoside, 3-Cyanidin-3-O-galactoside, 4-Delphinidin-3-O-arabinoside, 5- Cyanidin-3-O-glucoside, 6-Petunidin-3-O-galactoside, 7-Petunidin-3-glucoside, 8- Cyanidin-3-O-arabinoside, 9- Paeonidin-3-O-galactoside, 10-Petunidin-3-O-arabinoside,
PhD Thesis Abstract

11-Malvidin-3-O-galactoside, 12- Malvidin-3-O-glucoside, 13- Malvidin-3-O-arabinoside

Fig. 3. Cromatogramele HPLC ale extractelor de afine: (A) Bluecrop, (B) Duke, (C) Elliot, (D) Wild 1, (E) Wild 2. Identificarea peak-urilor: 1- Delphinidin-3-O-galactoside, 2- Delphinidin-3-O-galactoside, 3- Cyanidin-3-O-galactoside, 4- Delphinidin-3-O-arabinoside, 5- Cyanidin-3-O-glucoside, 6- Petunidin-3-O-galactoside, 7- Petunidin-3-glucoside, 8- Cyanidin-3-O-arabinoside, 9- Paeonidin-3-O-galactoside, 10- Petunidin-3-O-arabinoside, 11- Malvidin-3-O-galactoside, 12- Malvidin-3-O-glucoside, 13- Malvidin-3-O-arabinoside

**Total polyphenolic content** The total phenolic (TPC) values for chokeberry cultivars ranged from 1586.5 to 2059.5 mg GAE/100 g FW which are in agreement with values reported previously in literature. AmN had the highest total phenolic content of all investigated chokeberry samples, followed by cultivar. The total phenolic value of AmN 1756.4 mg GAE/100 g FW was two fold higher than 690.2 mg GAE/100 g FW reported previously for the same cultivar (Benvenuti et al., 2004). For blueberry analyzed extracts the total phenolic (TPC) values were in the range of 424.84 - 819.12 mg GAE/100 g FW. Among all the varieties analyzed, the Wild 1 revealed the highest TPC at 819.12 gallic acid equivalents/100 g FW followed by Wild 2 blueberries (672.59 mg GAE/100g). Between the cultivated blueberries, Bluecrop has the highest TPC at 652.27 mg GAE/100 g while the lowest value was found for Duke variety (424.84 mg GAE/100g). (Table 3)

**Total flavonoid content**

The total flavonoid content obtained among chokeberry cultivars ranged from 47.67 mg/100 g FW (Ap) to 75.53 mg/100 g FW (AmN) and the results are shown in Table 3. The total flavonoid content in blueberry ranged from 84.33 mg QE/100 g for Duke variety to 112.5 mg QE/100 g for Wild 2 blueberries. For the others blueberries varieties the TFC were as follows: Wild 1 (110.36 mg QE/100 g), Bluecrop (103.18 mg QE/100 g), Elliot (92.82 mg QE/100 g) and Duke (84.33 mg QE/100 g).

**Total anthocyanin content** Using a rapid and easy screening method for total monomeric anthocyanin quantification, we estimated for the first time the chokeberry anthocyanin content of AmA, AmN and AmV cultivars, ranging from 176 to 437 mg/100g FW (Table 3).

The highest anthocyanin content was found in wild blueberries Wild 1 (300.02 mg/100g), followed by wild blueberries Wild 2 (252.23 mg/100g), Elliot (163.4 mg/100g), Bluecrop (160.76 mg/100g) and the lowest TAC was found in Duke variety (69.58 mg/100g). There were statistically significant differences (p<0.05) in anthocyanin content between Wild 1, Wild 2 and Duke variety.
Total polyphenol, total flavonoid and total anthocyanin content of chokeberry, wild and cultivated blueberries

Polifenolii totali, flavonoidele totale și conținutul total de antociani din aronia, afine cultivate și cele din flora spontană

<table>
<thead>
<tr>
<th>Chokeyberry fruits</th>
<th>Blueberries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aprunifolia</td>
</tr>
<tr>
<td>Total polyphenol GAE mg/100g</td>
<td>2059.5 ± 145.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total flavonoid mg QE/100g</td>
<td>47.67 ± 3.7&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total anthocyanin C3GE mg /100g</td>
<td>366.16 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notes: means with different letters in the same column are statistically significant at P≤ 0.05

a) Expressed in gallic acid equivalence (GAE), (mg GAE/100g FW)
b) Expressed in quercetin equivalence, (mg/100g FW)
c) Expressed in cyanidin-3-galactoside, (mg/100 g FW)
Total antioxidant activity

Trolox equivalent antioxidant capacity (ABTS) assay

The chokeberry radical scavenging activity ranged from 95 to 180 μmol Trolox/g FW, with a statistically significant elevated value for AmN extract. Blueberries Wild 1 extract showed the highest antioxidant activity ABTS assay (56.65 μmol TE/g). The lowest level in both assays was obtained for Duke variety.

Ferric reducing antioxidant potential (FRAP) assay

The effective reducing power of chokeberries and blueberry is summarized in Table 4. AmN extract demonstrated the highest ferric reducing antioxidant potential (341.9±14.2 μmol Fe²⁺/g FW), followed by Ap (300.2±10.6 μmol Fe²⁺/g FW), comparing to other two cultivars AmA and AmV. Towards blueberries, Wild 1 extract showed the highest antioxidant activity based on FRAP assay (73.71 μM Fe2+/g). The lowest level in both assays was obtained for Duke variety. (Table 5)

Cupric reducing antioxidant potential (CUPRAC) assay

Ap and AmN cultivars had the highest reducing potential, more than 200 μmol TE/g FW, values statistically different compared to those obtained for AmA and AmV cultivars, 158.8, respectively 177.8 μmol TE/g FW. The CUPRAC values for the blueberry varieties were within 20.37-44.58 μmol TE/g. The ability of analyzed extracts to reduce cupric ion (Cu²⁺) is shown in Table 4 and 5.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC values for chokeberry extracts ranged from 35.3 to 42.3 μmol Trolox/ g FW and for blueberry extracts the values obtained were not significantly different among samples, ranging from 34.85 to 38.49 μmol TE/g FW.

Hydrogen peroxide scavenging activity (HPS)

AmV, AmN, AmA and Ap exhibited 61.9, 83.6, 53.3 and 65.0 μmol/g FW H₂O₂ scavenging activity (Table 4). AmN cultivar was an effective scavenger toward H₂O₂ and AmV cultivar had the lowest inhibition of H₂O₂. The values obtained for blueberry were between 25.30-44.37 μmol TE/g.
Antioxidant capacity of selected chokeberry cultivars, measured by different complementary assays. Details in *Materials and methods*.

**Table 4**

Activitatea antioxidanță a extractelor din fructe de aronia, prin patru metode complementare

<table>
<thead>
<tr>
<th>Chokeberry cultivar</th>
<th>ABTS^a</th>
<th>FRAP^b</th>
<th>CUPRAC^c</th>
<th>ORAC^a</th>
<th>HPS^a</th>
<th>WA^d</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ap</em></td>
<td>167.6 ± 6.2^a</td>
<td>300.2 ± 10.6^b</td>
<td>232.3 ± 14.2^a</td>
<td>42.3 ± 2.0^a</td>
<td>65.0 ± 2.6^b</td>
<td>1.10</td>
<td>2</td>
</tr>
<tr>
<td><em>AmA</em></td>
<td>95.9 ± 7.3^b</td>
<td>185 ± 8.6^c</td>
<td>177.8 ± 9.6^c</td>
<td>35.3 ± 1.7^c</td>
<td>53.3 ± 1.5^c</td>
<td>0.79</td>
<td>4</td>
</tr>
<tr>
<td><em>AmN</em></td>
<td>180.5 ± 8.1^a</td>
<td>341.9 ± 14.2^a</td>
<td>203.8 ± 8.7^b</td>
<td>37.1 ± 1.3^b</td>
<td>83.6 ± 3.2^a</td>
<td>1.15</td>
<td>1</td>
</tr>
<tr>
<td><em>AmV</em></td>
<td>171.7 ± 6.8^a</td>
<td>206.2 ± 9.4^c</td>
<td>158.8 ± 8.3^d</td>
<td>41.7 ±1.2^a</td>
<td>61.9 ± 1.9^b</td>
<td>0.94</td>
<td>3</td>
</tr>
<tr>
<td>Average capacity</td>
<td>153.9</td>
<td>258.4</td>
<td>193.2</td>
<td>39.2</td>
<td>66.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes: means with different letters in the same column are statistically significant at P≤ 0.05

^a Expressed in TE (μmol /g FW)

^b Expressed in Fe^{2+}, (μmol Fe^{2+}/g FW)

^c Expressed in Cu^{2+}, (μmol Cu^{2+}/g)

^cWA-Weighted average calculation see Results and Discussions section
Table 5

Antioxidant activity for blueberry fruits, using four different complementary assays (FRAP, ABTS, ORAC)

Activitatea antioxidantă a extractelor din fructe de afine, prin patru metode complementare (FRAP, ABTS, ORAC)

<table>
<thead>
<tr>
<th></th>
<th>FRAP</th>
<th>ABTS</th>
<th>ORAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM Fe²⁺/g</td>
<td>TEμmol /g</td>
<td>TE μmol/g</td>
</tr>
<tr>
<td><strong>Vaccinium corymbosum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elliot</td>
<td>50.74 ± 1.9c</td>
<td>36.46 ± 4.26c</td>
<td>38.05±1.56a</td>
</tr>
<tr>
<td>Bluecrop</td>
<td>60.39 ± 1.6b</td>
<td>37.96 ± 2.98bc</td>
<td>37.04 ± 1.7a</td>
</tr>
<tr>
<td>Duke</td>
<td>33.03 ± 2.54d</td>
<td>24.33 ± 3.76d</td>
<td>34.85 ± 1.3b</td>
</tr>
<tr>
<td><strong>Vaccinium myrtillus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild 1</td>
<td>73.71 ± 3.2a</td>
<td>56.65 ± 3.79a</td>
<td>38.49± 1.01a</td>
</tr>
<tr>
<td>Wild 2</td>
<td>64.87 ± 2.9b</td>
<td>43.08 ± 2.3b</td>
<td>37.78± 0.89a</td>
</tr>
</tbody>
</table>

CONCLUSIONS

A comparative evaluation of the metabolic profile considering the phytochemical content of different cultivars of *Aronia melanocarpa* and *Vaccinium sp* berries was performed. Five blueberry and four chokeberry varieties purchased from local Romanian farmers were analyzed by reverse phase HPLC for identification and the quantification of anthocyanins.

The results obtained can be summarized as follows:

1. The HPLC analysis showed that *Aronia melanocarpa* ‘Nero’ cultivar extract was the richest one in phenolics, flavonoids and anthocyanins. Cyanidin glycosides were the major anthocyanin derivatives identified in chokeberry, especially the 3-O-galactoside and 3-O-arabinoside.

2. For blueberry extracts the highest values regarding total phenolics, flavonoids and anthocyanins were obtained for Wild 1 sample. The most representative anthocyanidins in *Vaccinium myrtillus* were delphinidin and petunidin while in *Vaccinium corymbosum*, delphinidin and malvidin.

3. The total phenolics concentration found in chokeberry extracts varied from 1586.5 to 2059.5 mg/g fresh weight (expressed in equivalents of gallic acid). For blueberry the lowest phenolic content was found in Duke variety, while highest phenolic content in Wild 1.

4. The antioxidant capacity of analyzed extracts (as determined by five, complementary assays (ABTS, HPS, ORAC, FRAP, CUPRAC) was positively
correlated with the anthocyanin content. The highest Pearson’s coefficient for blueberry extracts analysed was obtained when it has been compared ABTS and CUPRAC antioxidant methods (0.96) while for the chokeberry samples analyzed the Pearson’s correlation coefficient (r) showed a high correlation between antioxidant activity determined by HPS assay versus total anthocyanin (0.97). The highest antioxidant capacity was obtained for *Aronia melanocarpa* ‘Nero’ cultivar and also high level of antioxidant activity obtained for Wild 1 blueberries, by all the methods.

As a final conclusion, our study demonstrates that the fruits of chokeberry and blueberry (cultivated and wild) commonly consumed in Romania are a good source of anthocyanins. The wild blueberries contain a higher amount of anthocyanins than cultivated ones.  

Aslo this is the first study regarding chemical composition and chemoprotective effects of anthocyanin-rich fractions obtained from *Aronia melanocarpa* cultivated in Romania.
CHAPTER II.

PURITY EVALUATION OF ANTHOCYANIN RICH-FRACTIONS OBTAINED FROM CHOKEBERRIES AND BLUEBERRIES USING DIFFERENT SOLID PHASE EXTRACTION METHODS

The aim of this study was to evaluate and to compare the purity of an anthocyanin rich – fraction (ARF) which was obtained from chokeberry and blueberry crude extracts, by SPE techniques, such as C18 Sep Pack, Oasis- MCX, and Amberlite XAD-7 & Sephadex –LH 20. Subsequent high performance liquid chromatography (HPLC) (% AUC), molar absorbtivity (MA) and electrospray ionization mass spectrometry (LC-ESI-MS) were used to determine the purity of ARF.

MATERIALS AND METHOD
Anthocyanin extraction was done according to the method reported by Abdel-Aal, 2003 with some minor modifications.

PREPARATION OF ANTHOCYANIN RICH-FRACTION
The most convenient method is solid-phase extraction (SPE) because is the simplest, most effective and do not imply any sophisticate equipment low cost (Xiaoke and Zhimin, 2011) 

C18 Sep Pack SPE. C18 sorbent considered being most used, simple and effective for anthocyanin purification. The purification mechanism includes a non-polar stationary phase and a polar mobile phase (Rodriguez-Saona and Wrolstad, 2001). In order to obtain anthocyanin rich fraction the procedure describe by Giusti el al. 1999 was applied.

Oasis- MCX SPE. This is actually a mixed mode between cation exchange and reversed-phase interactions and was developed in 2011 by He and Giusti in order to apply it for the isolation of anthocyanins from edible sources.

Amberlite XAD-7 and Sephadex LH-20 SPE. In this case the anthocyanin purification was done trough 2 different cartridges. First purification was done on Amberlite XAD 7, and the obtained purified was loaded in to a Sephadex LH 20 cartridge in order to separate anthocyanins by proanthocyanidins.

PURITY EVALUATION
HPLC-PDA and MS analysis of analysed samples were performed using using Shimadzu HPLC-Photodiode array (PDA) system equipped with a SPD-M20A PDA detector and also Shimadzu LCMS-2010 EV liquid chromatograph (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with a SPD-M20A PDA detector and a single quadrupole electron spray ionization (ESI) MS detector. A Waters Corp.
Symmetry® C18 column (3.5µm, 4.6×150mm; Milford, MA) was used for anthocyanins separation. The mobile phase was solvent A: 4.5% formic acid in LC/MS grade water, mobile phase B: LC/MS grade acetonitrile.

**Molar absorptivity.** In order to confirm the anthocyanins purity calculated using HPLC-PDA data we also evaluate the purity based on molar absorptivity. Anthocyanins purity was calculated according Lambert-Beer’s law using cyanidin 3 glucoside extinction factor. Dilutions were prepared in triplicate.

\[
\text{(Experimental conc./actual conc.)*100}
\]

**RESULTS AND DISCUSSIONS**

**Purity evaluation by HPLC-PDA**

Four sorbents commonly were used for anthocyanin purification. The highest purity calculated by % AUC for chokeberry was found to be obtained using Oasis MCX (97.7%±2.0) followed by C18 cartridges (93.3% ±2.8), while the lowest value was obtained for Amberlite XAD-7& Sephadex LH-20 (82.6%±4.3).

For blueberry anthocyanin rich fraction analyzed, the calculated purity using those three sorbents were in the range of 99.9% ± 1.9 -75.90% ±4.8. Figure 44 and 45 provides a visual representation of the calculation of purity by % AUC for anthocyanin rich fraction obtained from chokeberry and blueberry.

![Fig. 4.](image)

Fig. 4. Purity evaluation by HPLC-PDA of chokeberry (A) and blueberry (B) ARFs using three different SPE methods: C18, Oasis MCX, Amberlite XAD-7& Sephadex LH-20

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*Fig. 4. Evaluarea purității prin metoda HPLC-PDA a antocianilor obținuți din aronia (A) și afine (B) utilizând trei metode SPE: C18, Oasis MCX, Amberlite XAD-7& Sephadex LH20*
Fig. 5. HPLC-PDA max-plot of purified chokeberry (A) and (B) extracts using three different SPE methods: C18, Oasis MCX, Amberlite XAD-7& Sephadex LH-20

Fig 44. Cromatogramele HPLC-PDA max-plot ale extractelor obținute din Aronia (A) și afine (B), utilizând trei metode SPE: C18, Oasis MCX, Amberlite XAD-7& Sephadex LH-20
Purity evaluation by molar absorptivity

Molar absorptivity is a spectrophotometric method used for the purity calculation by Lambert-Beer’s law.

The highest purity achieved using molar absorptivity analysis for chokeberry ARF was through MCX cartridges for about 79.18 %, comparing to Amberlite XAD-7 & Sephadex LH-20 (63.39 %) and C-18 Sep Pack sorbents (72.46%). The calculated purity values by molar absorptivity for blueberry extracts was found to be higher using Oasis MCX (78.2%) than C18 cartridges and Amberlite XAD-7 & Sephadex LH-20 (68.3%, 59.8%) (Fig. 6).

The purity data values calculated with this method was lower than values reported previously, calculated by HPLC.

Fig. 6. Purity evaluation of chokeberry (A) and blueberry (B) ARFs by molar absorptivity, using three different SPE methods: C18, Oasis MCX, Amberlite XAD-7 & Sephadex LH-20

SPE methods comparison

High-purity (>97%) was achieved for all purified blueberry and chokeberry anthocyanin mixtures trough Oasis MCX SPE cartridge. The comparison of the efficiency of the separation procedures was based on sorbent capacity to remove unwanted compounds.

Purity determination methods comparison

The values for purities evaluation by HPLC-PDA and molar absorptivity are significant different. The purities values calculated by HPLC –PDA (% AUC) as we mention before were higher. The calculation of this can be affected by sensitivity of the
detector. In order to evaluate and for a better understanding of difference between those two methods used for purity calculation further analysis are required.

**Confirmation of purity with HPLC-MS analysis**

To this method we cannot quantified the purity based on intensity of the signal, but we can clearly observed the profile or the shapes of the analyzed compounds comparing to peaks impurities. Figure 6 shows the total ion concentration recorded by a MS detector for chokeberry and blueberry extracts purified.
Fig. 7. Impurities recorded in chokeberry (A) and blueberry (B) ARF by the appearance of noise peaks in the MS chromatogram using three different SPE methods: C18, Oasis MCX, Amberlite & Sephadex.

Fig. 7. Impuritățile înregistrate și evidențiate prin zgomotul peak-urilor în cromatograma MS pentru fracțiile bogate în antociani obținute din aronia (A) și afine (B) utilizând trei metode SPE: C18, Oasis MCX, Amberlite XAD-7 & Sephadex LH-20.
CONCLUSIONS

There were applied three different SPE methods (C18, Oasis-MCX and Amberlite XAD 7&Sephadex LH 120 sorbents) to purify the anthocyanin extract from chokeberry and blueberry.

The Oasis MCX was found to have the higher efficacy than the others SPE methods used for obtaining anthocyanin rich fractions. This fact is related to the adsorption and desorption of anthocyanins on the MCX sorbent which is determined by the pH dependent electric charge. The positively charged anthocyanin molecules interact with strong MCX cation-exchange sorbent, while most other compounds without positive charges could be easily removed.

For evaluation of ARFs purity by HPLC and molar absorptivity assay, the values obtained were different depending on the method used. The values obtained by HPLC for chokeberry extracts were range between 97.7%±2.0 (Oasis MCX) and 82.6%±4.3 (Amberlite XAD-7& Sephadex LH-20) while for blueberry extracts the obtained values were the range of 99.9% ± 1.9 -75.90% ±4.8 (Oasis MCX, Amberlite XAD-7& Sephadex LH-20 respectily). However the purity calculated by molar absorptivity were found to be lower. The highest purity achieved using molar absorptivity analysis for chokeberry ARFs was through MCX cartidges for about 79.18 %, comparing to Amberlite XAD-7 & Sephadex LH-20 (63.39 %). For blueberry extracts the results were similar, the highest purity being obtained for Oasis MCX sorbent. Accurate balances clean and dry containers, properly functioning desiccators, large enough sample, sample evaporated to complete dryness, contamination by compounds that do not absorb in the visible range are only few fact which can affect all those calculation.
CHAPTER III.
CHEMOPROTECTIVE EFFECTS OF AN ANTHOCYANIN-RICH FRACTION FROM ARONIA PRUNIFOLIA ON B16-F10 MELANOMA MURINE AND HEla TUMOR CERVICAL CELL LINES

This study evaluates the cytotoxic action and the prooxidative effect of the anthocyanin fraction on HeLa, respectively B16-F10 cell lines.

MATERIALS AND METHODS

Extraction preparation of anthocyanin-rich fraction. The anthocyanins extract was obtained by homogenization of chokeberries (5 g) in methanol containing HCl (0.3%) using an ultraturax (Micra D-9 KT Digitronic, Germany). Extracts obtained were concentrated at 35°C under reduced pressure (Rotavapor R-124, Buchi, Switzerland) in order to remove methanol. The remaining aqueous extract was loaded into Sep-pak C18 (5ml, 1g sorbent) Waters Corp. Milford. MA) SPE cartridge. Purification conditions were done according to procedure described by Giusti et al., 1999.

HPLC – PDA analysis. HPLC analyses were performed on a Shimadzu, equipped with a diode-array detector (DAD) using a Luna Phenomenex C18 column (5µm, 25 cm x 4.6 mm). The mobile phase consisted in: solvent A - formic acid (4.5%) in bidistilled water and solvent B - acetonitrile. The gradient elution system was: 10% B, 0-9 min; 12% B, 9-17 min; 25% B 17-30 min; 90% B, 30-50 min; 10% B, 50-55 min.

Cell culture. Human tumor cervical HeLa cell line was maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing 1g/L glucose supplemented with 10% fetal bovine serum, 1 mM glutamine, 1% gentamicin, 1% non-essential aminoacids at 37°C, 5% CO₂, and 95% relative humidity. For B16F10 murine melanoma cells the grow media was consistent in Dulbecco’s Modified Eagle Medium (DMEM) containing 1g/L, fetal bovine serum to a final concentration of 10%, 1 mM glutamine, 1% penicillin, streptomycin at 37°C, 5% CO₂, and 95% relative humidity. Cells were plated on 96-well microplates and allowed to attach for 24h, as follow 5 x 10³ cells/ well for HeLa cell line and 8 x 10³ cells/ well for B16F10 cell line. The culture medium was then replaced with complete medium containing different concentrations from 0 to 250 μg/ml anthocyanins for HeLa and 0 to 600 μg/ mL for B16F10 cell lines.

Cellular proliferation. After anthocyanin rich fraction extract treatment was evaluated using MTT assay.

Intracellular reactive species assay. The determination of intracellular ROS uses the fluorescent probe 2’’, 7’’- dichlorodihydrofluorescein diacetate (DCF-DA) which is cell membrane permeable and it is hydrolyzed by cellular esterases to DCF (2’’, 7’’ dichlorofluorescein).

RESULTS AND DISCUSSION

HPLC – DAD analysis. After each purification the obtained anthocyanin rich-fraction was dissolved in a know amount of sterile water, filtered through 0.45µm and analyzed by
HPLC-DAD. The stock solution obtained was diluted with grow media in range 250-800 μg/mL. The chromatogram of obtained extract is showed in Fig. 7.

**Fig. 8.** HPLC chromatogram of purified chokebery extract  
Fig. 8. Cromatograma HPLC a extractului purificat din aronia

**Inhibition of B16F10 and HeLa tumor cells survival**

Cell viability is expressed as mean percentage of control (100%). The C-ARF treatment with anthocyanin-rich fraction inhibited the proliferation of HeLa cells by 40% at a concentration, expresses as cyanidin 3-O-galactoside equivalents, of 200 μg/mL after 48 h (Fig. 9A), respective of B16F10 cells by 51%, at the dose of 400 μg/mL (Fig. 9B). Also the morphology of the cells is affected and can be observed in figure 10.

**Fig. 9.** Results of MTT proliferation assay (proliferation %) of human cervical cancer HeLa (A) and of B16-F10 (B) metastatic murine melanoma cells -treated cells by C-ARF
Fig. 9. Rezultatele testului MTT de proliferare (proliferare%) pentru linia umană de cancer cervical HeLa (A) și pentru linia B16-F10 (B) melanom metastatic după tratamentul cu C-ARF

Fig. 10. Comparative morphology of B16F10 cells non-treated and treated with anthocyanin-rich fraction

Fig. 10. Comparation entre morfologia celulelor B16-F10 tratate și netratate cu extract bogat în antociani

**Intracellular ROS**

To determine the ROS generation in C-ARF HeLa and B16F10 treated cells was used 2′, 7′-dichlorodihydrofluorescein, a dye specific for detection of ROS. A significant suppression of intracellular ROS generation was detected for B16F190-treated cells with the dose of 200, 300, 400 μg/mL anthocyanins (Fig. 11A).

Significant increases in dose and time response effects of intracellular ROS in C-ARF HeLa-treated cells were observed at 30 min (Fig. 11B). An initial reduction of ROS for doses of 25 μg/mL and 50 μg/mL C-ARF after 4 h was noted. For the C-ARF 100, 150, and 200 μg/mL concentrations tested, ROS levels were increased above the control value after 30 min, but the accumulated level of ROS was gradually reduced with time. It is possible that the increased oxidative stress, being toxic, was responsible for the cell
death.

Fig. 11. Intracellular reactive oxygen species level in human cervical cancer HeLa (A) and B16-F10 (B) metastatic murine melanoma cells treated with chokeberry anthocyanins, as determined by the fluorescence test with 2′,7′-dichlorofluorescein (DCF). The cells treated with chokeberry extract showed increased fluorescence compared with untreated cells. Data are mean±SD values (n=3), **P<0.01 (very significant) from corresponding control, ***P<0.001 (extremely significant) from corresponding control.

CONCLUSIONS

In vitro, cell culture studies on human cervical cancer HeLa and B16-F10 metastatic murine melanoma cells showed that the cyanidin glycosides purified from Aronia prunifolia fruits inhibited gradually tumor cell proliferation after 24h, respective 48 h of treatment, proving to be cytotoxic for HeLa and B16-F10 cells, and the intracellular ROS generation was increased in a dose dependent manner.

➢ The tumor cells used in this experiment change their morphology according to the concentration of C-ARF and treatment period.
➢ ARF inhibited growth of the of human cervical cancer HeLa (A) with an IC50 value of about 171 µg/ml whilst for B16-F10 (B) metastatic murine melanoma cells treated cells by C-ARF the IC 50 was 313 µg/ml
➢ The intracellular reactive oxygen species level as determined by the fluorescence test with 2′,7′-dichlorofluorescein (DCF) shows that the cells treated with chokeberry extract had increased fluorescence compared with untreated cells, indicating their proapoptotic effect by ROS accumulation in both tumor cells.

XXXI
The effects were significantly higher when 150 or 300 ug/ml anthocyanin fraction was applied on HeLa and B16-F10 cells, respectively.

The anthocyanins in chokeberries could have a health benefit, acting as antitumor agents, so these fruits can be recommended for daily consumption.
CHAPTER IV.

ANTHOCYANINS DETERMINATION IN VARIOUS CULTIVARS OF Highbush blueberries AND THEIR ANTIproliferation AND APOPTOSIS PROPERTIES IN B16-F10 METASTATIC MURINE MELANOMA CELLS

The main aim of this study was to investigate the potential of blueberry anthocyanins for their protective effect against melanoma cancer cells. Therefore, the objectives of this study were to quantify and identify individual anthocyanins in different Romanian blueberry varieties and to evaluate their antioxidant potential, in order to select the richest anthocyanin fraction with the highest antioxidant activity and to test it for its ability to inhibit the proliferation and to stimulate apoptosis in B16-F10 metastatic murine melanoma cell line.

MATERIALS AND METHODS

Preparation of anthocyanin fraction.
Seven cultivars of highbush blueberries Bluegold, Nui, Darrow, Legacy, Nelson, Hannah’s Choice and Toro were used in order to obtain anthocyanin extract. Blueberries (1 g) were homogenized with acidified methanol (0.3 % HCl (v/v)) using the ultraturax (Miccra D-9 KT Digitronic, Germany), then stored at 4 ºC in dark for 24 h. The extract obtained was filtered through multiple layers of cotton, then concentrated by rotary evaporation at 35°C to remove methanol. The extract containing anthocyanin and non-anthocyanin compounds was subject to partition procedure using ethyl acetate, for the less polar compounds removal. The remaining aqueous fraction was then applied to an Amberlite XAD-7 column (1 x 0.5 cm) chromatography and the obtained purified was also further purified on a Sephadex LH-20 column (2.5 x 0.5 cm).

HPLC-DAD analysis of anthocyanins analyses were performed on a Shimadzu, equipped with a diode-array detector (DAD) using a Luna Phenomenex C-18 column (5µm, 25 cm x 4.6 mm) The mobile phase consisted in: solvent A - formic acid (4.5%) in bidistilled water and solvent B - acetonitrile. The gradient elution system was: 10% B, 0-9 min; 12% B, 9-17 min; 25% B 17-30 min; 90% B, 30-50 min; 10% B, 50-55 min.

HPLC-ESI-MS analysis of anthocyanins. Samples were analyzed on an Agilent Technologies 1200 HPLC system (Chelmsford, MA, USA) equipped with G1311A Quaternary Pump, G1322A degasser, G1329A autosampler, G1315D photo-diode array (PDA) detector and Quadrupole 6110 mass spectrometer (Agilent Technologies, Chelmsford, MA, USA) equipped with an ESI probe. The mobile phase was composed of solvent A – (1 % formic acid in bidistilled water) and solvent B – (acetonitrile). The percent of B increased to 12 % at 17 min and continued up to 25 % B at min 30. Between 30 and 50 % B the percentage of B was 90 %.
Cupric reducing antioxidant capacity (CUPRAC) assay
The cupric ion reducing antioxidant capacity of blueberries 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).

Scavenging effect on ABTS radical
The scavenging ability of blueberry extracts against radical anion ABTS\(^{+}\) was determined in 96-well plates according to procedure described by Arnao et al. (2001). Results were expressed as μmol Trolox/ g FW.

Oxygen radical absorbance capacity (ORAC) assay
The antioxidant capacity was measured and calculated by the oxygen radical absorbance capacity assay, as described previously (Huang et al., 2005). ORAC values were expressed as μmol Trolox /g fruit fr. wt.

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 1g/ 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

Analysis of cell proliferation
Cell proliferation assay was done using MTT reagent. The results were expressed as percent survival relative to an untreated control.

Detection of LDH activity
Damage of the plasma membrane was evaluated in B16-F10 cells by measuring LDH leakage. Absorbance values measured at 490 nm, using the BioTek Synergy HT microplate reader (BioTek Instruments Inc., USA) were translated into LDH leakage percents relative to untreated B16-F10 cells.

96-well-based EB/AO staining
In order to assess the apoptotic index and the cell membrane integrity, the acridine orange (AO)/ ethidium bromide (EB) staining was performed. Early apoptotic cells have bright green nucleus with condensed or fragmented chromatin and 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).
TUNEL assay and analysis

Prior to 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, USA) 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).

RESULTS AND DISCUSSIONS

Identification and quantification of blueberry anthocyanins

To our knowledge this is the first time that the anthocyanin composition of Nui and Hannah’ Choice blueberries are reported. The HPLC chromatograma of analysed sapmles are presened in Fig. 12. In total 12 peaks were identified as described in Table 7. Based on literature data the elution order reported of anthocyanin derivatives was: galactosides, glucosides and arabinosides (Gavrilo et al., 2011). The fragmentation of delphinidin derivatives in MS2 yielded the formation of delphinidin aglycone (m/z = 303 [M+H]⁺), after the characteristic release an 162 indicating the galactoside or glucoside in case of compounds 1 and 2, respectively and the release of 132 indicating the arabinoside unit in case of compound 4. Using a similar procedure, the identification of all other compounds as presented in Table 7.
Fig. 12. HPLC chromatogram of blueberry extracts: Bluegold (A), Nui (B), Darrow (C), Nelson (D), Hanna’s Choice (E), Toro (F), Legacy (G).

Peak identification: 1-Delphinidin-3-O-galactoside, 2-Delphinidin-3-O-glucoside, 3-Cyanidin-3-O-galactoside, 4-Delphinidin-3-O-arabinoside, 5- Cyanidin-3-O-glucoside, 6-Petunidin-3-O-galactoside, 7-Cyanidin-3-O-arabinoside, 8- Paeonidin-3-O-galactoside,
9-Petunidin-3-O-arabinoside, 10-Malvidin-3-O-galactoside, 11- Malvidin-3-O-glucoside, 12-Malvidin-3-O-arabinoside

Fig. 12. Cromatogrammele HPLC ale extractelor de afine Bluegold (A), Nui (B), Darrow (C), Nelson (D), Hanna`s Choice (E), Toro (F), Legacy (G). Identificarea peakurilor: 1-Delfinidin-3-O-galactozid, 2-Delfinidin-3-O-glucozid, 3-Cianidin-3-O-galactozid, 4-Delfinidin-3-O-arabinozid, 5- Cianidin-3-O-glucozid, 6-Petunidin-3-O-galactozid, 7-Cianidin-3-O-arabinozid, 8- Paeonidin-3-O-galactozid, 9-Petunidin-3-O-arabinozid, 10-Malvidin-3-O-galactozid, 11- Malvidin-3-O-glucozid, 12- Malvidin-3-O-arabinozid

Data on the content of individual anthocyanins content in blueberry cultivars are presented in Table 8. The highest anthocyanin content was found in Toro cultivar (195.01 mg/ 100g fr. wt), followed by Legacy, while the lowest amount was obtained in Bluegold cultivar (101.88 mg/ 100g fr. wt). Delphinidin-3-O-galactoside (peak 1) was identified as one of the major compounds in Toro cultivar and one of the minor compounds in Bluegold cultivar, while malvidin-3-O-galactoside (peak 10) was identified as one of the major compound in Toro, Bluegold, Legacy, 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 all the anthocyanins.
Retention times, UV-Vis and mass spectral data of anthocyanins in analyzed ARF samples obtained from blueberry cultivars

Timpii de renție și caracteristicile spectofotometrice ale antocianilor obținuți din afine

<table>
<thead>
<tr>
<th>Peak</th>
<th>( t_R )</th>
<th>( \lambda_{max} )</th>
<th>Molecular ( \text{(m/z)} )</th>
<th>Fragment ( \text{(m/z)} )</th>
<th>MW</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>An 1</td>
<td>8.1</td>
<td>276, 526</td>
<td>465</td>
<td>303</td>
<td>465</td>
<td>Delphinidin-3-O-galactoside</td>
</tr>
<tr>
<td>An 2</td>
<td>9.3</td>
<td>276, 524</td>
<td>465</td>
<td>303</td>
<td>465</td>
<td>Delphinidin-3-O-glucoside</td>
</tr>
<tr>
<td>An 3</td>
<td>11.2</td>
<td>279, 517</td>
<td>449</td>
<td>287</td>
<td>449</td>
<td>Cyanidin-3-O-galactoside</td>
</tr>
<tr>
<td>An 4</td>
<td>11.5</td>
<td>276, 524</td>
<td>435</td>
<td>303</td>
<td>435</td>
<td>Delphinidin-3-O-arabinoside</td>
</tr>
<tr>
<td>An 5</td>
<td>12.9</td>
<td>280, 517</td>
<td>449</td>
<td>287</td>
<td>449</td>
<td>Cyanidin-3-O-glucoside</td>
</tr>
<tr>
<td>An 6</td>
<td>13.6</td>
<td>276, 526</td>
<td>479</td>
<td>317</td>
<td>479</td>
<td>Petunidin-3-O-galactoside</td>
</tr>
<tr>
<td>An 7</td>
<td>15.2</td>
<td>279, 517</td>
<td>419</td>
<td>287</td>
<td>419</td>
<td>Cyanidin-3-O-arabinoside</td>
</tr>
<tr>
<td>An 8</td>
<td>18.0</td>
<td>276, 526</td>
<td>463</td>
<td>301</td>
<td>463</td>
<td>Paeonidin-3-O-galactoside</td>
</tr>
<tr>
<td>An 9</td>
<td>18.2</td>
<td>276, 526</td>
<td>449</td>
<td>317</td>
<td>449</td>
<td>Petunidin-3-O-arabinoside</td>
</tr>
<tr>
<td>An 10</td>
<td>20.5</td>
<td>276, 527</td>
<td>493</td>
<td>331</td>
<td>493</td>
<td>Malvidin-3-O-galactoside</td>
</tr>
<tr>
<td>An 11</td>
<td>23.4</td>
<td>276, 526</td>
<td>493</td>
<td>331</td>
<td>493</td>
<td>Malvidin-3-O-glucoside</td>
</tr>
<tr>
<td>An 12</td>
<td>26.3</td>
<td>276, 528</td>
<td>463</td>
<td>331</td>
<td>463</td>
<td>Malvidin-3-O-arabinoside</td>
</tr>
</tbody>
</table>
The anthocyanin content in blueberries determined by HPLC-DAD.

Antocianii din fructele de afine determinați prin metoda HPLC-DAD.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>Vaccinium corymbosum L. (mg per 100 g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bluegold</td>
</tr>
<tr>
<td></td>
<td>Anthocyanins: Total</td>
<td>101.88 ± 2.36</td>
</tr>
<tr>
<td>An 1</td>
<td>Delphinidin-3-O-galactoside</td>
<td>9.51 ± 3.29c</td>
</tr>
<tr>
<td>An 2</td>
<td>Delphinidin-3-O-glucoside</td>
<td>3.64 ± 0.15de</td>
</tr>
<tr>
<td>An 3</td>
<td>Cyanidin-3-O-galactoside</td>
<td>6.17 ± 0.01c</td>
</tr>
<tr>
<td>An 4</td>
<td>Delphinidin-3-O-arabinoside</td>
<td>5.61 ± 1.46b</td>
</tr>
<tr>
<td>An 5</td>
<td>Cyanidin-3-O-glucoside</td>
<td>3.33 ± 0.18b</td>
</tr>
<tr>
<td>An 6</td>
<td>Petunidin-3-O-galactoside</td>
<td>10.78 ± 3.84d</td>
</tr>
<tr>
<td>An 7</td>
<td>Cyanidin-3-O-arabinoside</td>
<td>4.68 ± 0.05a</td>
</tr>
<tr>
<td>An 8</td>
<td>Paeonidin-3-O-galactoside</td>
<td>4.92 ± 0.28bc</td>
</tr>
<tr>
<td>An 9</td>
<td>Petunidin-3-O-arabinoside</td>
<td>5.97 ± 1.33c</td>
</tr>
<tr>
<td>An 10</td>
<td>Malvidin-3-O-galactoside</td>
<td>29.33 ± 12.69b</td>
</tr>
<tr>
<td>An 11</td>
<td>Malvidin-3-O-glucoside</td>
<td>4.43 ± 0.56d</td>
</tr>
<tr>
<td>An 12</td>
<td>Malvidin-3-O-arabinoside</td>
<td>13.53 ± 4.43b</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD, n=3. Different letters between columns denote statistically difference at p < 0.05. nd - not detected.
Determination of antioxidant activity

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). The ability of blueberry ARF to reduce cupric ion (Cu²⁺) is shown in Table 8.

CUPRAC values for the blueberry varieties were within the range of 134.76-185.78 μmol TE/ g fr. wt and the highest antioxidant activity was obtained for the Toro cultivar. Currently, there are no published data on the antioxidant activity of blueberries extracts using the CUPRAC method.

The ABTS assay uses K₂S₂O₈ as oxidant and measures the antioxidants ability to scavenge the radical ABTS⁺⁺ compared with Trolox, a vitamin E analogue. The blueberry radical scavenging activity shown in Table 14 ranged from 6.05 to 11.96 μmol TE/ g fr. Wt.

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 received the highest antioxidant ORAC value.

Table 8

Antioxidant activity results obtained by complementary assays ABTS, CUPRAC and ORAC on selected cultivar of blueberry.

<table>
<thead>
<tr>
<th>Blueberry cultivar</th>
<th>Antioxidant activity (μM TE/ g fr.wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CUPRAC</td>
</tr>
<tr>
<td>Bluegold</td>
<td>134.76 ± 53.03ᵃ</td>
</tr>
<tr>
<td>Nui</td>
<td>163.04 ± 23.45ᵇ</td>
</tr>
<tr>
<td>Darrow</td>
<td>168.65 ± 30.33ᵇ</td>
</tr>
<tr>
<td>Legacy</td>
<td>171.15 ± 46.49ᵇ</td>
</tr>
<tr>
<td>Nelson</td>
<td>175.25 ± 69.06ᵇ</td>
</tr>
<tr>
<td>Hanna’s Choice</td>
<td>153.89 ± 57.12ᵇ</td>
</tr>
<tr>
<td>Toro</td>
<td>185.78 ± 92.06ᵇ</td>
</tr>
</tbody>
</table>

The data expressed as Mean ± SD. Different letters in each column denote statistical difference at p < 0.05.

Inhibition of tumor cell proliferation

Blueberry anthocyanins inhibited the proliferation of B16-F10 cells in a dose dependent manner. Treatment with 200 and 400 μg/ ml for 24 h stimulated the B16-F10 cell proliferation with 20 %. Treatment with 550, 600 and 650 μg/ ml ARF-T for 24 h decreased with 16.5 %, 46.9 %, and 59.0 % respectively B16-F10 cell proliferation. Higher concentrations than 650 μg/ ml decreased cell proliferation by more than 70 %. The calculated IC₅₀ value was 615.2 μg/ ml and was obtained from the dose–response curve shown in Fig.13.
Fig. 13. 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 proliferation 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

Cellular membrane integrity assessment

To assess cellular membrane integrity after 24 h of incubation of B16-F10 cells with or without ARF-T treatment, the secreted lactate dehydrogenase (LDH) was measured. ARF-T doses of 550, 600, 650 μg/ml increased the leakage lactate dehydrogenase (LDH) in B16-F10 cells by 9 %, 10 % and 12 % compared to control (Fig. 14).
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

Acridine orange/ Ethidium bromide staining

The cell membranes blebbing and the formation of the apoptotic bodies in B16-F10 cells exposed to ARF-T treatment could be observed in Fig. 15A. About 20% of cells ARF-T treated from total viable cells counted were in apoptosis (Fig. 58B). The decrease in observed cell proliferation is due to induction of apoptosis, being the major mode of anthocyanin-induced cell death. About 18% of cells counted were colored red, which could be due to necrosis appearing after a time of sustained apoptosis. Our results suggest that blueberry anthocyanins (600 µg/ml) can induce apoptosis in B16-F10 melanoma murine cells after 24 h treatment (Fig. 15B).

Fig. 15. Effects of blueberry anthocyanins on B16-F10 murine melanoma cells (A). Phase contrast microscopy on B16-F10 cells treated by 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 ARF-T. Cells were observed under inverted fluorescence microscope. The white arrows indicate apoptotic cells (original magnification: ×40)

Fig. 15. Efectele antocianilor din afine asupra celulelor tumorale B16-F10 (A). Microscopie în contrast de fază pentru celulele B16-F10 tratate cu ARF-T pentru 24 h. (B) Colorarea EB/AO după tratament și înaintea tratamentului pentru 24 h a celulelor B16-F10 cells. Celulele au fost observate cu microscopul cu florescență inversată. Săgetile albe indică celule apoptotice (magnificația: ×40)

**Apoptotic cell death**

Apoptosis leads to nuclear DNA breakdown into multiples of 200–500 bp oligonucleosomal size fragments. In Fig. 16 a high number of ARF-T treated B16-F10 murine melanoma cells were found TUNEL positive compared to untreated cells using immunocytochemical staining. Treatment with 600 μg/ ml ARF-T for 24 h increased the number of TUNEL positive cells with 14 % compared to their corresponding control cells with percent TUNEL positive cells of 2 % (Fig. 17). Data obtained in this study prove that ARF-T induce apoptosis in B16-F10 treated melanoma.

Fig. 16. Confocal microscopy of B16-F10 cells TUNEL staining with or without ARF-T treatment for 24 h. TUNEL-positive cells are shown as green fluorescence. Numerous normal nuclei stained in red with Draq5
Fig. 16. Microscopia confocală pentru celulele B16-F10 cu și fără tratamentul cu ARF-T pentru 24h. Celulele TUNEL pozitive sunt marcate cu florescență. Nucleii normali sunt colozați în roșu cu Draq5.

![Graph showing apoptosis index](image)

**Fig. 17.** Quantification of apoptosis induction in B16-F10 cells treated with 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.

Data regarding the effects of blueberry anthocyanins on cell proliferation murine melanoma cells have not been reported previously and could therefore be recorded as a novel biological activity. Unfortunately, their mechanism of action remains unknown. Recently, it was suggested that mulberry anthocyanins could mediate B16-F1 cell metastasis by reduction of MMP-2 and MMP-9 activities involving the suppression of the Ras/PI3K signaling pathway (Huang et al., 2008).

**CONCLUSIONS**

- Five varieties of blueberry were purchased from local farmers and analysed by HPLC-DAD-ESI-MS for the purification, identification and quantification of anthocyanin content.
- The purification of the extracts was done on Ambertite XAD-7&Sephadex LH-20 in order to obtain anthocyanin-rich fraction and to test it for its ability to inhibit the proliferation and to stimulate apoptosis in B16-F10 metastatic murine melanoma cell line.
After the chromatographic analysis 12 individual anthocyanins were identified and quantified. Malvidin-3-O-galactoside, petunidin-3-O-galactoside and delphinidin-3-O-galactoside being the majors anthocyanins in all varieties studied.

The highest anthocyanin content was found in Toro cultivar (195.01 mg/ 100g fr. wt), followed by Legacy, while the lowest amount was obtained in Bluegold cultivar (101.88 mg/ 100g fr. wt).

Regarding antioxidiantactivity the highest scores were found for Toro cultivar, consistent with the higher anthocyanins concentration.

Blueberry anthocyanins inhibited the proliferation of B16-F10 cells in a dose dependent manner. Treatment with 200 and 400 μg/ ml for 24 h stimulated the B16-F10 cell proliferation with 20 %.

Treatment with ARF in doses of 550, 600, 650 μg/ml increased the leakage lactate dehydrogenase (LDH) in B16-F10 cells by 9 %, 10 % and 12 % compared to control.

The percent of apoptotic cells determined by Acidine orange/ Ethidium bromide staining. About 20 % of cells ARF-T treated from total viable cells counted were in apoptosis.

Treatment with 600 μg/ ml ARF-T for 24 h increased the number of TUNEL positive cells with 14 % compared to their corresponding control cells with percent TUNEL positive cells of 2 %

This study found that anthocyanin-rich fraction obtained from Vaccinium corymbosum cv. Toro could inhibit melanoma tumor cell proliferation and induce apoptosis. Before it can be declared that blueberry anthocyanins intake can reduce melanoma cancer risk further studies are required in order to clarify their mechanisms and to evaluate their bioavailability.
GENERAL CONCLUSIONS

Considering the main objectives of the research and the results obtained by different experimental investigations, we can point out the main achievements:

1. **It was performed comparative** evaluation of quantity and antioxidant activity of anthocyanins from four varieties of chokeberry, three varieties of cultivated highbush blueberries and two types of wild blueberries.

2. **There were compared** different solid phase extraction methods in order to obtain high purity extracts.

3. The chemopreventive effects of treatment with chokeberry anthocyanin-rich fraction two cancer cell lines (B16-F10 melanoma murine and HeLa tumor cervical cell lines) were demonstrated.

4. **It was realized** a complete study of various blueberry cultivars regarding anthocyanins identification and their antioxidant activity and the antitumor and proapoptotic effects of anthocyanin-rich fraction on B16-F10 melanoma murine cells.

Considering the main results of the research, presented in chapters 3-6 we can conclude that:

**Chapter 3 summarize** the antioxidant activity and composition of anthocyanins using HPLC chromatography revealed the fingerprint of each extract and the quantitative distribution of different anthocyanin each extract. The chapter conclusions are:

a) The HPLC analysis showed that *Aronia melanocarpa* ‘Nero’ cultivar extract was found to be the richest in phenolics, flavonoids and anthocyanins, among the four varieties tested. Cyanidin glycosides were the major anthocyanin derivatives identified in chokeberry especially the 3-O-galactoside and 3-O-arabinoside.

b) For blueberry extracts the highest values regarding total phenolics, flavonoids and anthocyanins were obtained for Wild 1 sample. The most representative anthocyanidins in *Vaccinium myrtillus* were delphinidin and petunidin while in *Vaccinium corymbosum*, delphinidin and malvidin.

c) The total phenolics concentration found in chokeberry extracts varied from 1586.5 to 2059.5 mg/g fresh weight (expressed in equivalents of gallic acid). For blueberry the lowest phenolic content was found in Duke variety, while highest phenolic content in Wild 1.

d) The antioxidant capacity of analyzed extracts (as determined by five, complementary assays (ABTS, HPS, ORAC, FRAP, CUPRAC) was positively correlated with the anthocyanin content.

e) As a final conclusion, this study demonstrates that the fruits of chokeberry and blueberry (cultivated and wild) commonly consumed in Romania are a good...
source of anthocyanins. The wild blueberries contain a higher amount of anthocyanins than cultivated ones.

As presented in Chapter 4, the results revealed differences among the three solid phase extraction techniques to obtain high purity anthocyanin fractions. Accordingly:

a) The Oasis MCX was found to have the higher efficacy than the others SPE methods used for extracts purity determination.

b) For the evaluation of ARFs purity by HPLC and molar absorptivity assay, the values obtained were different depending on the method used. The values obtained by HPLC for chokeberry extracts were range between 97.7%±2.0 (Oasis MCX) and 82.6%±4.3 (Amberlite XAD-7& Sephadex LH-20) while for blueberry extracts the obtained values were the range of 99.9% ± 1.9 -75.90% ±4.8 (Oasis MCX, Amberlite XAD-7& Sephadex LH-20 respectively). However the purity calculated by molar absorptivity were found to be lower.

c) The highest purity achieved using molar absorptivity analysis for chokeberry ARF was through MCX cartidges for about 79.18 %, comparing to Amberlite XAD-7 & Sephadex LH-20 (63.39 %). For blueberry extracts the results were similar, the highest purity being obtained for Oasis MCX sorbent. Accurate balances clean and dry containers, properly functioning desiccators, large enough sample, sample evaporated to complete dryness, contamination by compounds that do not absorb in the visible range are only few fact which can affect all those calculation.

d) The use of HPLC method was found to give higher values for anthocyanin rich-fraction than molar absorptivity method.

As presented in Chapter 5, the chemopreventive effects of chokeberry anthocyanin-rich fraction upon two cancer cell lines (B16-F10 melanoma murine and HeLa tumor cervical cell lines) were proven. We noticed that:

a) The cyanidin glycosides purified fraction from *Aronia prunifolia* fruits inhibited gradually tumor cell proliferation after 24h or 48 h of treatment, proving to be cytotoxic for HeLa and B16-F10 cells, respectively.

b) The tumor cells used in this experiment change their morphology according to the concentration of C-ARF and treatment period.

c) ARF inhibited growth of the of human cervical cancer HeLa with an IC50 value of about 171 μg/ml whilst for B16-F10 (B) metastatic murine melanoma cells -treated cells by C-ARF the IC 50 was 313 μg/ml.

d) The intracellular reactive oxygen species level as determined by the fluorescence test with 2′,7′-dichlorofluorescein (DCF) shows that the cells treated with chokeberry extract had increased fluorescence compared with untreated cells, indicating their proapoptotic effect by ROS accumulation in both tumor cells.
e) The effects were significantly higher when 150 or 300 \( \mu g/ml \) anthocyanin fraction was applied on HeLa and B16-F10 cells, respectively.

According to the results obtained in Chapter 6, the chemopreventive effects of anthocyanin rich-fractions obtained from blueberry on B16-F10 cells. The chapter conclusions are

a) Five varieties of blueberry were purchased from local farmers and analysed by HPLC-DAD-ESI-MS for the purification, identification and quantification of anthocyanin content.

b) The purification of the extracts was done on Ambertite XAD-7&Sephadex LH-20 in order to obtain anthocyanin-rich fraction and to test it for its ability to inhibit the proliferation and to stimulate apoptosis in B16-F10 metastatic murine melanoma cell line.

c) After the chromatographic analysis 12 individual anthocyanins were identified and quantified. Malvidin-3-\( O \)-galactoside, petunidin-3-\( O \)-galactoside and delphinidin-3-\( O \)-galactoside being the majors anthocyanins in all varieties studied.

d) The highest anthocyanin content was found in Toro cultivar (195.01 mg/ 100g fr. wt), followed by Legacy, while the lowest amount was obtained in Bluegold cultivar (101.88 mg/ 100g fr. wt).

e) Regarding antioxididant activity the highest scores were found for Toro cultivar, consistent with the higher anthocyanins concentration.

f) Blueberry anthocyanins inhibited the proliferation of B16-F10 cells in a dose dependent manner. Treatment with 200 and 400 \( \mu g/ ml \) for 24 h stimulated the B16-F10 cell proliferation with 20 %.

g) Treatment with ARF in doses of 550, 600, 650 \( \mu g/ml \) increased the leakage lactate dehydrogenase (LDH) in B16-F10 cells by 9 %, 10 % and 12 % compared to control.

h) The percent of apoptotic cells determined by Acridine orange/ Ethidium bromide staining. About 20 % of cells ARF-T treated from total viable cells counted were in apoptosis.

i) Treatment with 600 \( \mu g/ ml \) ARF-T for 24 h increased the number of TUNEL positive cells with 14 % compared to their corresponding control cells with percent TUNEL positive cells of 2 %

This is the first study regarding chemical composition and chemoprotective effects of anthocyanin-rich fractions obtained from Aronia melanocarpa cultivated in Romania also the effects of blueberry anthocyanins on cell proliferation murine melanoma cells have not been reported previously and could therefore be recorded as a novel biological activity. Experiments presented in this study are original contributions and bring more valuable results for the research regarding chemical and biological properties of
anthocyanins. We demonstrated that the anthocyanins from chokeberries and blueberries could have a health benefits, acting as antitumor agents, these fruits being highly recommended for daily consumption.
REFERENCES


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