Ph.D. THESIS

Anthocyanins as phytochemicals with biomedical applications: biochemical characterisation and *in vitro* biological action

(SUMMARY OF THE DOCTORAL THESIS)

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INTRODUCTION

Natural plant-derived polyphenols (resveratrol, flavonoids, phenolic acids, and anthocyanins) have been drawing a lot of interest in the last two decades, due to their effective action in the prevention and amelioration of several chronic health issues, like cardiovascular problems, diabetes-associated illnesses and several forms of cancer [1]. Narrowing the attention down to anthocyanins, they have been the subject of numerous studies conducted on *in vivo* and *in vitro* models, whether as daily consumption or synergized with standard therapies, giving significant results [2, 3].

Anthocyanins are natural pigments responsible for the beautiful color of the leaves' senescence in the autumn and the impressive sight of the forests, acting as protectors of the vegetal tissues exposed to direct light, found in significant amounts in fruits and vegetables [4]. These molecules with remarkable health benefits have been ingested by humans for thousands of years due to their wide distribution in edible foods like roots, fruits, leaves, juices, and extracts, without any problems. Their chemical structure allows them to express anti-oxidative action, which has been directly linked with the amelioration of chronic illnesses like cancer or diabetes [5].

In the last two decades, anthocyanins have become the main focus of study of many scientific types of research and, accordingly, numerous *in vitro* tests revealed antiproliferative, anti-inflammatory, and pro-apoptotic actions. Ever since the beneficial effects of anthocyanins have been described, more and more interest was shown in developing new therapies based on these compounds, and the attempt to understand their molecular mechanisms.

Unfortunately, anthocyanins are very sensitive to environmental conditions, such as pH, light, oxygen, and consequently are easily degraded [4]. The cationic flavylium form of anthocyanins exposed to the above-mentioned conditions can be irreversibly degraded into colorless compounds [4]. So, a comprehensive study of their physico-chemical response to various external factors, as well as their biological behavior when involved in *in vitro* and *in vivo* studies is mandatory for a better understanding of their bioavailability and finding ways to optimize their use at the maximal potential.

Therefore, nowadays scientists are in constant research in order to complete the large puzzle of anthocyanin's properties with the missing pieces as stability, uptake, bioavailability, bioabsorption, mechanisms of action, and tissue distribution. By direct administration of anthocyanins on tumor skin cells, the main disadvantages like their low stability and bioavailability, uncontrolled release, long blood circulation time, non-selective tissue distribution and high dose needed in oral administration could be eliminated [6-8]. It is already known that anthocyanins display skin photo-chemopreventive or anti-cancer effects [9-11]. Protective effects of anthocyanins, such

as cyanidin-3-glucoside and delphinidin, against UVB irradiation, have been reported, in *in vivo* studies on SKH-1 hairless mice skin carcinogenesis model [11, 12]. In *in vitro* studies some anthocyanins, like delphinidin and cyanidin, exerted protective effects on the immortalized keratinocytes cell line HaCaT [12] and on the normal skin cell line JB6 P+ exposed to UVB-induced irradiation [13]. Likewise, the administration of anthocyanin extract from blackberries proved to protect primary keratinocytes against UV-induced oxidative damage by up-regulating the expression of the antioxidant defense enzymes [14]. In our previous study, anthocyanins extracted and purified from blueberries and chokeberries proved to inhibit the proliferation and induced apoptosis of the melanoma murine B16-F10 cell line [8, 15].

Ph.D. thesis aim and objectives

The main directions of this Ph.D. thesis were to gather an updated collection of information regarding the general characteristics of anthocyanins, to describe anthocyanins from different natural sources (raspberries, mulberries, chokeberries, and black carrots), their outstanding *in vitro* effectiveness against the proliferation of different cancers, like human ovarian and cervical cancer, and murine melanoma, and to bring valuable knowledge contribution to their better understanding, by analyzing the most efficient extracting „green solvent" and by proposing a fluorescent approach to monitor the fate of anthocyanins in anti-melanoma treatment. The goals of the thesis were accomplished by meeting the following **objectives**:

Objective 1: The extraction of anthocyanins from natural sources: mulberries, raspberries, chokeberries, and black carrot with different extracting solvents.

Objective 2: The obtaining of purified anthocyanins extracts by solid phase extraction (SPE) method, C18 cartridge.

Objective 3: Individual identification of anthocyanins and concentration measurement by High Performance Liquid Chromatography (HPLC) and HPLC coupled with mass spectroscopy (MS).

Objective 4: The establishment of the best correlation between glycosylated/acylated anthocyanins and extracting solvent.

Objective 5: *In vitro* approach: proliferation and cytotoxicity induced by anthocyanins purified extracts determined by MTT and WST-1 assays.

Objective 6: The enhancement of anthocyanins' fluorescence in solution by complexing them with diphenylboric acid 2-aminoethyl (Naturstoff reagent A).

Objective 7: *In-situ* monitoring of „anthocyanins-diphenylboric acid 2-aminoethyl complex" inside murine melanoma cells.

Ph.D. thesis structure

The thesis is divided into two parts. The first part, the literature review, comprises Chapter 1 and deals with general theoretical aspects of anthocyanins. The

second part, the original contribution, comprises Chapters 2-7, and is dedicated to presenting specific results and experimental measurements on anthocyanins and their extraction, characterization, *in vitro* application on cancer therapies, and intracellular monitoring method:

Literature review

Chapter 1 describes information regarding the physicochemical properties of anthocyanins, their behavior in human digestion as a foundation for possible future therapies and methods of analysis.

Original contribution

Chapter 4 shows the antiproliferative activity of anthocyanins pure extracts from mulberries and raspberries on HeLa and A2780 human cancer cell lines.

Chapter 5 focuses on the comparative efficiency of different solvents (methanol, ethanol, acetone and water) for the anthocyanins extraction from chokeberries, and black carrots, to preserve their antioxidant activity.

Chapter 6 describes real-time fluorescence imaging of anthocyanins complexed with diphenylboric acid 2-aminoethyl for intracellular tracking and monitoring inside B16–F10 melanoma cells.

This Ph.D. thesis is the result of the collaboration between the University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania, and Babes-Bolyai University, Faculty of Physics and Faculty of Chemistry and Chemical Engineering, Cluj-Napoca, Romania.

General conclusions

In **Chapter 4**, relevant data about the chemical anthocyanins composition of two common berries (mulberries and raspberries) were obtained. The major compounds detected in purified anthocyanins extracts were: cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside from mulberries, and cyanidin-3-O-sophoroside and Cyanidin-3-O-sophoroside-5-rhamnoside in raspberries. Both sources exhibited significant antiproliferative effects on HeLa, human cervical cancer cells and A2780, human ovarian cancer cell lines. The anthocyanins were extracted from mulberries and raspberries using acidified methanol and were purified using the solid phase extraction method (SPE), onto Sep-Pak columns. The remaining anthocyanins content was analyzed using high performance liquid chromatography (HPLC) coupled with photodiode array (PDA) and with electron spray ionization mass spectrometry (ESI^+-MS). The *in vitro* experiments were conducted on HeLa and A2789 cell lines and the cell proliferation rate of cells treated with anthocyanins extracts was measured using the MTT assay.

In **Chapter 5**, two different anthocyanins matrices, chokeberry fruits, containing monoglycosylated anthocyanins, and black carrots, containing both acylated and diglycosylated anthocyanins, were used to establish the proper extracting

solvents related to the chemical structure of the anthocyanins. In this light, it was shown that monoglycosylated anthocyanins from chokeberries preferred methanol, while acylated anthocyanins had a better extraction with ethanol. Our results provided new knowledge in the extraction field of anthocyanins and their utilization in the medical and food industries. The anthocyanins fractions were obtained by using the solvent extraction method and were concentrated in a vacuum rotary evaporator. The total anthocyanins content was determined using the differential pH method. The anthocyanins identification and quantification were obtained by HPLC/PDA/ESI$^+$/MS. The antioxidant activity of the extracts were analysed using three different assays: ABTS$^+$, CUPRAC, and FRAP.

In **Chapter 6**, diphenylboric acid 2-aminoethyl (DPBA), a natural dye, was used to form a fluorescent complex with glycosylated anthocyanins extracted from chokeberry fruits, as a promising method to visualize anthocyanins inside B16-F10 tumor cells by modern technique-based fluorescence. Overall, the DPBA proved no toxic effect on the cells and no fluorescence in the absence of anthocyanins, but helped to enhance the fluorescence emitted by anthocyanins, enabling their tracking as fundamental knowledge for a better understanding and exploitation of these bioactive compounds. The chokeberry anthocyanins extract was extracted using acidified methanol and concentrated using a vacuum rotary evaporator, and later purified by the SPE technique, with Sep-Pak C18 cartridges. Anthocyanins were identified and quantified by HPLC coupled with DAD detector and ESI+-MS. The cellular experiments were conducted on B16-F10 murine melanoma cell line and the cytotoxicity analysis were measured with WST-1 assay. The chemical and fluorescence investigation of the anthocyanins-DPBA complex were obtained through steady-state fluorescence spectrometry, nuclear magnetic resonance and high-resolution mass spectrometry analyis, fluorescence microscopy imaging and fluorescence-activated cell sorting (FCM) analysis.

Originality

The findings presented in this Ph.D. thesis might be considered an important addition to the existing knowledge in the field of anthocyanins studies and their implication in medicinal applications. New data were generated for the chemical composition of rapsberries and mulberries, namely, the identification of different anthocyanins in the two sources: cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside (mulberries), cyanidin-3-O-sophoroside, and Cyanidin-3-O-sophoroside-5-rhamnoside (raspberries). The purified fractions of anthocyanins demonstrated antiproliferative potential in HeLa, human cervical cancer cells and A2780, human ovarian cancer cell lines.

Our research could also provide new knowledge in the extraction field of anthocyanins and sustain their further exploration and application in the food industry. Two matrixes were chosen as anthocyanins sources, chokeberries (Aronia melanocarpa) and black carrots (Daucus carota sp.) and four common solvents were

selected for analysis: acetone, methanol, ethanol and water. The best solvent was favored taking into account several criteria: anthocyanins stability, identified amount, chemical structure and anticancer effect on murine melanoma B16-F10 cells. for fruits like chokeberries with exclusive glycosylated anthocyanins composition, methanol proved to be the best extracting option, while for vegetables like black carrots, with acylated anthocyanins content, ethanol was the most suitable way to extract and preserve these antioxidants.

Finally, this thesis provided additional support for a promising strategy for the development of novel monitoring methods of anthocyanins directly into tumor cells, which could be used in a next-generation melanoma therapy. Anthocyanins extracted and purified from natural sources (chokeberry fruits), with low to zero fluorescence, were complexed with diphenylboric acid 2-aminoethyl (DPBA), a non-fluorescent dye. The results showed an enhancement of the emitted fluorescence signal, which could be detected with fluorescence microscopy directly inside melanoma cells, for a better understanding and visualization of their intracellular route and fate. Our results propose DPBA as a promising fluorescent dye to visualize anthocyanins in tumor cells by modern technique-based fluorescence.

Future perspectives

As a result of the above mentioned works and after carrying out a thorough literature review, especially on the most recent studies, we have come to discover the great potential that anthocyanins can provide in the treatment or ammelioration of chronic diseases with high incidence in the modern human population, with supplementray benefits regarding the milder secondary effects compared to the current existing treatments. However, we have also discovered the gaps and limitations in the current knowledge of anthocyanins. The most pressing of all is the endeavor to develop economically sustainable treatments with the capacity to maintain the highly volatile chemical stability of anthocyanins and, accordingly, their beneficial function. For this reason, we would like to proceed further with the anthocyanins studies and to include them in nano-scale polymeric carrying systems as targeted delivery therapy for melanoma, for more precise and rapid action, and for the preservation of their chemical structure and functions.

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