PhD THESIS

Stability and biological properties of free and encapsulated anthocyanins from berries

(SUMMARY OF THE DOCTORAL THESIS)

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1. Introduction

Anthocyanins, major compounds in the flavonoid group of the polyphenol class, are bioactive compounds responsible for the color and protection of plant tissues exposed to UV radiation (Richmond et al., 2019). Despite their wide range of beneficial health effects, such as antioxidant (Carvalho et al., 2016), antiangiogenic, and antitumor activities (Tarone et al., 2020; Kauppinen et al., 2016), their applicability is limited not only by low stability but also by their low bioavailability (Wu et al., 2020). Due to their instability caused by external factors such as pH, temperature, light, oxygen, enzymes, etc. (Jung et al., 2020; Wu et al., 2020), structural changes occur at the molecular level, leading to a decrease in therapeutic effects (Jiang et al., 2017).In order to avoid the degradation of anthocyanins and thus preserve their beneficial effects when ingested or used in various medical treatments, they can be incorporated into delivery systems or co-pigmented with other molecules (Esfanjani et al., 2018; Fang et al. 2020). As a result, scientists are working to find ways to make anthocyanins more stable, bioavailable, and cellularly internalized, as well as to comprehend the mechanisms behind their biological effects and their intracellular localization (Bunea et al., 2013; Rugina et al., 2015).

Anthocyanins have shown beneficial effects related to the inhibition of angiogenesis both *in vitro* and *in vivo* studies, with implications in several diseases, including diabetic retinopathy (Khan et al., 2018; Tsakiroglou et al., 2019). Anthocyanins have shown an inhibition of tumor development, a reduction in the proliferation of colon cancer cells, blockage of DNA damage in breast cells, and inhibition of HepG2 liver cancer cells (Li et al., 2009). Moreover, anthocyanins such as cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside have shown effective control over the overexpression and abnormal secretion of inflammatory factors by inhibiting the NF-κB transcription factor (Lin et al., 2017). Regulation of oxidative stress and suppression of cellular apoptosis indicate that anthocyanins can protect against UV-B-induced skin injuries and other pathologies involving oxidative damage. Besides inhibiting the expression of inflammatory factors, they also reduce the production of inflammatory substances (Qi et al., 2022). Anthocyanins determined a reduction in amyloid-β1-42 plaques and neuro-apoptotic markers through inhibition of the ρ-JNK/NF-κB/ρ-GSK3β pathway in the brains of mice (Kim et al., 2017).

2. State of the art

Chapter 1 presents the most relevant and novel aspects regarding the chemistry and biochemistry of anthocyanins. The first subchapters include the chemical structure and the most relevant factors affecting the stability of anthocyanins: pH, temperature, light, oxygen, and the presence of other molecules. This is followed by a short overview of the biosynthesis of anthocyanins and a more detailed presentation of anthocyanin's distribution in the most common fruits and vegetable sources. The second subchapter is dedicated to the pharmacological properties of anthocyanins, starting with their bioavailability and absorption. Antioxidant, antiangiogenic, antitumoral, anti-inflammatory, anti-diabetic, and neuroprotective effects are detailed, underlining the most relevant and recent scientific evidence from *in vitro* and *in vivo* studies.

3. Original contributions

Chapter 2. Working hypothesis and the specific objectives

The main goal of this PhD thesis was to incorporate anthocyanins into a carrier system, which could increase the chemical stability of these molecules and their bioavailability. Using pH-sensitive materials to construct this nanosystem will allow us to control the release of anthocyanins inside B16-F10 tumor cells more effectively. All this not before a consistent collection of up-to-date information about anthocyanins as bioactive compounds but also testing their antioxidant activity, bioaccessibility, and stability following exposure to external factors (pH, temperature, light, all-time-dependent), for a much better understanding of the way of action and chemical behavior of these compounds.

In order to achieve this purpose, the doctoral research was directed towards the following objectives:

Objective 1: Extraction, identification, and quantification of anthocyanin-rich extracts from *Amelanchier lamarckii, Aronia melanocarpa, and Vaccinium corymbosum* using high-performance liquid chromatography with photodiode array and mass spectrometry detection (HPLC-PDA, HPLC-MS)

Objective 2: Determining the antioxidant activity and bioaccessibility of phenolic compounds from *Amelanchier lamarckii* and their enzyme inhibitory activity against α-glucosidase, tyrosinase, and acetylcholinesterase

Objective 3: Determining the protective and antioxidant properties of anthocyanin-rich extracts on human retinal pigment epithelial cells (RPE cells, D407 cell line) cultivated in standard or induced oxidative stress conditions;

Objective 4: Testing the stability of anthocyanins from the *Amelanchier lamarckii* extract exposed at different pHs, temperatures, and light, for various time intervals;

Objective 5: Synthesis through layer-by-layer technique and characterization of nanocapsules containing purified anthocyanin fraction from *A. melanocarpa*, charged (+/-) biocompatible polymers and fluorophores;

Objective 6: Monitoring the pH-controlled release of anthocyanins from nanocapsules, their internalization, and cytotoxicity in murine melanoma cells (B16-F10).

Chapter 3. Antioxidant, enzyme inhibitory, and protective effect of *Amelanchier lamarckii* extract

Aim. This study aimed to determine the chemical composition of *A. lamarckii* berries cultivated in Romania, providing original and valuable information for the nutraceutical and health industries regarding the antioxidant activity and enzymatic inhibitory activity of the fruit extract. It also determined the bioaccessibility of major bioactive phenolic compounds.

Methodology. Characterization and quantification of phenolic compounds and anthocyanin fraction were performed using HPLC-PDA and HPLC-ESI$^+$-MS, as well as external calibration. The antioxidant activity of *A. lamarckii* extract (AME) was determined by FRAP and CUPRAC assays. The inhibitory activity of the extract against tyrosinase, α-glucosidase, and acetylcholinesterase was determined by spectrophotometric methods**.** The bioaccessibility of phenolic compounds was determined using the standardized static *in vitro* digestion protocol INFOGEST (Minekus et al, 2014). The protective effect of the extract was investigated using a

cellular model of diabetic retinopathy, where human RPE cells were exposed to high-glucose concentration.

Results and conclusions. The polyphenols of Romanian juneberries *A. lamarckii*, of which phenolic acids, anthocyanins, flavonols, and flavones prove to have a significant contribution to the antioxidant activity potential and the enzymatic inhibitory potential on tyrosinase, α-glucosidase, and acetylcholinesterase. The total concentration of polyphenols in AME decreased six times after the gastric phase compared to the initial AME and ten times after the intestinal phase. The total bioaccessibility of polyphenols indicates that only a small fraction of them is accessible for absorption, maybe due to the chemical transformations under different pH conditions, and the slightly alkaline environment of the small intestine. Flavonols and hydroxycinnamic acids had the highest bioaccessibility, while anthocyanins and flavones could not be detected in the final digesta. In a simulated diabetic retinopathy model, polyphenols of AME exerted a protective effect for the cells cultivated in a high-glucose condition of 30 mM glucose. Instead, in extreme glucose conditions (60 mM glucose), the protective effect of AME on the viability of D407 cells is reduced. Taken together, all these data suggest that *A. lamarckii* might be a valuable source of polyphenolic compounds with antioxidant potential and metabolic disease-protective effects and worth further investigated and exploitation in food and nutraceuticals industries.

The results of this study were published in: Adela Maria DĂESCU, Mădălina NISTOR, Alexandru NICOLESCU, Roxana POP, Andrea BUNEA, Dumitriţa RUGINĂ, Adela PINTEA, 2024, Antioxidant, Enzyme Inhibitory, and Protective Effect of *Amelanchier lamarckii* Extract. Plants 2024, 13, 1347. https://doi.org10.3390/plants13101347. Impact Factor 4.000, Q1

Chapter 4. *In vitro* antioxidant activity of anthocyanidins from cultivated blueberries

Aim. The current study aims to evaluate the capacity of anthocyanidins, antioxidants with a phenolic structure extracted from blueberries, to improve the antioxidant status of RPE cells exposed to high glucose concentrations, a situation that mimics diabetic retinopathy. The specific objectives of the present work were: to extract and characterize the anthocyanidin profile in *Vaccinium corymbosum* L. cv. Bluegold berries cultivated in Romania; to determine the antioxidant activity of the extract by chemical methods and to evaluate the effect of anthocyanidin's extract on the antioxidant status of retinal pigment epithelial cells cultivated in normal and high-glucose conditions.

Methodology. Characterization and quantification of anthocyanidins was performed by HPLC-PDA with external calibration. The antioxidant activity of the extracts was determined by ABTS, FRAP, and CUPRAC assays. The protective effect of the extract was investigated using a cellular model of diabetic retinopathy, where human RPE cells were exposed to high-glucose concentration. The activity of antioxidant enzymes SOD, GPx, CAT, and the level of reduced glutathione GSH were assessed using commercial kits.

***Results and conclusions*.** *Vaccinium corymbosum* Bluegold cultivar is a valuable source of anthocyanidins (95.02 ±2.45 mg/100g FW), especially malvidin (31.68 ± 0.81 mg/100 FW), with remarkable antioxidant activity (10.56 ± 1.87 µmol TE/ g FW - ABTS; 43.31 ± 2.38 µmol Fe2+/g FW - FRAP;104.22 ± 9.44 µmol TE/ g FW - CUPRAC). Malvidin-rich extract did not

display cytotoxicity in RPE cells at concentrations up to 250 μM. Pre-treatment of RPE culture with malvidin for 24 hours improved the viability of cells cultured in a high-glucose medium. Moreover, malvidin determined a decrease in the generation of ROS in both control cells (normal glucose) and cells exposed to oxidative stress induced by high glucose concentration. The administration of malvidin induced an increase in SOD activity, both under normal conditions and under conditions of oxidative stress, but did not have a significant effect on GPx and CAT activities. Additionally, the pre-treatment with malvidin increased the level of GSH in control cells and restored the level of GSH in high-glucose cells. The inhibition of the generation of reactive oxygen species, the increase in GSH concentration, and SOD activity reinforce the hypothesis that malvidin can contribute to the antioxidant defense system of RPE cells. Further studies are necessary to understand the mechanisms behind the antioxidant properties of anthocyanidins in RPE cells.

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Chapter 5. Thermal, photochemical, and pH stability of cyanidin-based anthocyanins in *Amelanchier lamarckii* fruit extract.

Aim. The main goal of this study was to determine the stability of the cyanidin-based anthocyanin extract from *Amelanchier lamarckii* fruit. Our approach involved determining the cumulative effects of glycosylation, pH, temperature, and light on the stability of anthocyanins over time. In this attempt, the extract was exposed simultaneously to different pHs, light intensities, or temperature variations, for different time intervals.

Methodology. In the first experiment anthocyanins (ANTEX) were extracted from berries and exposed to different pHs (1.2, 5.1, 8.0), at room temperature, for 0h, 1h, 4h, 8h, and 24h. In the second experiment, ANTEX was exposed to the same pH conditions and maintained at different temperatures (4°C, 24°C, 37°C, 60°C and 100°C), for 0h, 1h, 4h, 8h, and 24h. In the third experiment, ANTEX was exposed to different light intensities 10% (785 lux), 40% (6773 lux), 60% (10038 lux), and 80% (14296 lux), at different temperatures 10°C, 24°C, 37°C, and 50°C, for the same time intervals. Identification of anthocyanin and their quantification for each experiment was performed by HPLC-PDA with external calibration.

Results and conclusions. Cyanidin-based anthocyanins at pHs of 1.1, 5.1, and 8.0 exhibited good stability for temperatures up to 37°C. However, for temperatures starting at 60°C, regardless of the pH, the retention of total and individual anthocyanins decreased significantly. At room temperature and in the absence of light, the glycosylated derivatives, Cy-3-gal and Cy-3-arab, were more stable toward the variation of pH compared to the aglycon, especially for longer exposure times. At high temperatures, Cy had better retention than the glycosylated form, but this is most probably due to the thermally induced (partial) hydrolysis of the glycosylated derivatives, resulting in the release of the aglycon. Between Cy-3-gal and Cy-3-arab, the first one was generally more stable toward light intensities variation at high temperatures (>37°C) and long duration of exposure (>4h), but with stronger stability for Cy-arab under pH variation at high temperatures (>37°C) and long duration of exposure (>4h). Contrarily, in the conditions of pH and temperature variations, Cy-3-arab had better retention

in almost all experiments. Nevertheless, Cy-3-gal was the major compound in the extract, and, as such, it also had the greatest contribution to the overall stability of the total anthocyanins. It is also important to note the impact of the duration of the treatment. A thermal treatment at 60°C or boiling (100°C) process, prolonged to a maximum of 1 hour at a mildly acidic pH, resulted in a loss of about 23% and, respectively, 46% of total anthocyanins from juneberry extract. Cyanidin-based anthocyanins have great potential as natural colorants in the food industry. Therefore, to preserve more anthocyanins in processed foods with a pH lower than 6, the recommendation should be to minimize thermal damage during food processing prior to lowering the pH of the food. Further studies should focus on elucidating the inter- and intramolecular interaction mechanisms after the exposure of cyanidin derivatives at temperature and/or light. Additionally, testing the stability of anthocyanins in juneberry juice during pasteurization or jam production would provide additional and useful information. However, the importance of the use of dark packaging and alternatives to high-temperature methods in food industry thermal processing in order to maintain the color of anthocyanins when used as natural colorants is evident from the results provided in the present study.

This study was submitted and is under review: **Adela Maria DĂESCU**, Roxana POP, Dumitrita RUGINĂ, Adela PINTEA, Thermal, photochemical, and pH stability of cyanidin-based anthocyanins in *Amelanchier lamarckii* fruit extract, Foods- 3177218

Chapter 6. pH-responsive nanocapsules for anthocyanin delivery, intracellular tracking, and controlled release inside B16-F10 melanoma cells.

***Aim*.** The present article aimed to manufacture a functionalized nanosystem (Nano@AntS) capable of entrapping the anthocyanins (AntS), to carry them inside the melanoma cells, allowing their fluorescence to be easily monitored and releasing AntS for a potential therapeutic effect. In order to fabric Nano@AntS with pH-responsive behavior, for its walls two pH-sensitive weak polyelectrolytes and two fluorophores (pH-sensitive FITC, pKa 6-6.5, and RBITC pH-insensitive) were used. When one of the polyelectrolytes loses electric charge in this pH range, the AntS should be released directly into the melanoma cells to exert their effects.

***Methodology*.** The nanocapsules (Nano@AntS) were synthesized following the layer-by-layer method, based on the polymer electric charge, on a $CaCO_3$(+) core, with PAA(-), followed by AntS(+), PAA(-), fluorophores (RBITC(+); FITC(-), PAA(-) and the upper layer of PEI(+). Nano@AntS were characterized by fluorescence microscopy, SEM, TEM, DLS, and zeta-potential. The viability of the melanoma cells treated with Nano@AntS was determined by the WST-1 assay. The characterization of AntS and the entrapment efficiency was determined by HPLC-PDA.

***Results and conclusions*.** A new nanocarrier system, Nano@AntS, for anthocyanins, can be delivered, monitored, and localized inside B16-F10 melanoma cells. This is possible due to its characteristics: positive-coated surface, spherical shape, nanosize, pH-responsiveness of polymers and fluorophores in the structure, and a loading anthocyanin capacity of 63%. The release of AntS from Nano@AntS exposed to three different solutions (pH 4.5, pH 5.5, pH 6.5) for 24 h was investigated by HPLC-DAD analysis. The release of AntS (155.7 μM Cy-3-gal) observed at a pH of 4.5 can be attributed to the stability of AntS in acidic conditions (Enaru et

al., 2021). At a pH of 5.5, less concentration of AntS was found, maybe because of the carbinol pseudobase form of anthocyanins or a decreased swelling ratio of the polymers. Consequently, because PAA and PEI are sensitive to 6.0 - 6.5 pH quickened the release due to the swelling ratio of the polymers (Lim et al., 2017), leading to a higher concentration of AntS identified (52.0 μM Cy-3-gal). Nano@AntS does not affect the viability of tumor cells, instead, the entrapped AntS (652.70±18.95 μM as Cy-3-gal) exerts a strong cytotoxic effect on B16-F10 melanoma cells. Nano@AntS can localize inside melanoma cells, either in cytoplasm or lysosomes. Nano@AntS might be a potential delivery system for melanoma therapy and can be used in co-therapy if its template is filled with other therapeutic drugs.

This study was submitted and is under review: Mădălina NISTOR#, **Adela Maria DĂESCU#**, Monica FOCSAN, Roxana POP, Adela PINTEA, Maria SUCIU and Dumitrita RUGINĂ, pH-responsive nanocapsules for anthocyanin delivery, intracellular tracking, and controlled release inside B16-F10 melanoma cells, HELIYON-D-24-45376, under review, # equal contributions

4. General conclusions and future perspectives

The studies performed during the doctoral research brought new and valuable information regarding the chemical and biological properties of anthocyanins from berries cultivated in Romania.

A. lamarckii is an important source of phenolic compounds, including anthocyanins, yet not very common in our country. The *A. lamarckii* extract showed significant antioxidant activity potential and enzymatic inhibition potential against tyrosinase, α-glucosidase, and acetylcholinesterase. The simulated *in vitro* digestion indicates that only a small portion of phenolic compounds are available for absorption, among them flavonols and hydroxycinnamic acids were the most bioaccessible, while anthocyanins and flavones had the lowest bioaccessibility. Polyphenols from *A. lamarckii* extract demonstrated a protective effect in a cellular model of diabetic retinopathy when RPE cells were exposed to moderately high glucose concentration (30 mM).

Using chromatography, a chemical antioxidant test, and a retinal cell model of diabetic retinopathy, we evaluated the antioxidant and cytoprotective effect of anthocyanidins from *Vaccinium corymbosum* Bluegold cultivar. Malvidin was identified as the major aglycone in berries, followed by delphinidin and petunidin. Our study proved the lack of toxicity of the malvidin-rich extract on RPE cells cultivated in normal glucose conditions. Moreover, the extract showed a protective effect on the viability of RPE cells cultivated in high-glucose conditions. The cell-based antioxidant activity of the malvidin-rich extract was demonstrated by the inhibition of intracellular ROS production, the increase in the level of reduced glutathione GSH, and the improvement of SOD activity. However, the lack of effect on other antioxidant enzymes and the reduced statistical significance of some results represent the limitations of this study. Further investigations are necessary to understand the mechanisms behind the antioxidant properties of anthocyanidins in RPE cells.

Understanding the color properties and the behavior of anthocyanins in relation to environmental factors is crucial for anticipating changes that may occur in food products during processing. Our study on anthocyanins from *A. lamarckii* improved the knowledge of the effect

of temperature, pH, and light on the stability of these compounds. The fact that all the anthocyanins identified on these berries are based on the same aglycone, cyanidin, allowed us to make a comparison between the stability of free and glycosylated forms, but also between the different glycosylated forms, depending on the sugar moiety. Among the investigated factors, the high temperatures (>60°C) and the basic pH had the most detrimental effect on all anthocyanin, especially when they were applied simultaneously and for a longer duration of exposure (>4 h). For the highest light intensity (14296 lux), at low temperature (10°C), after 24 h, the retention of total and individual anthocyanin remained around 90 %, however as the temperature increased (50°C), the stability of glycosylated forms decreased significantly. In conclusion, to better preserve the anthocyanins in processed foods, the light exposure and the thermal treatment should be minimized (both temperature and time) during food processing before lowering the pH of the food. Further studies should focus on elucidating the mechanisms of inter- and intramolecular interactions following the exposure of anthocyanins to pH, temperature, and/or light.

Anthocyanins from *Aronia* berries, containing cyanidin-monoglycosyl derivatives, were extracted, purified, and characterized by chromatography and further encapsulated with good efficiency in innovative polymeric nanocapsules (Nano@AntS). These constructs, which include biocompatible polymers and fluorophores, are pH-responsive and able to deliver and release anthocyanins at the cellular level in melanoma cells, as revealed by HPLC analysis, fluorescence, and transmission electron microscopy. Having the high loading capacity, the reduced size, the positive surface, and the lack of cytotoxicity, this nanosystem could be used for melanoma therapy or co-therapy.

Future perspectives

In the coming decades, we may witness the development of new therapies for melanoma, considering the cytotoxic potential of anthocyanins encapsulated within fluorescent nanoparticle carriers. This targeted delivery approach could bring new strategies for the treatment of skin cancer, addressing issues related to frequent recurrences and the limited effectiveness of the current treatments. Additionally, the increased stability of cyanidin under various environmental conditions could lead to the formulation of more effective food and pharmaceutical products that preserve the beneficial properties of anthocyanins. These advancements could impact not only human health but also the food industry, improving nutrient preservation and product appearance.

5. Originality and personal contributions

The results of the current doctoral research contribute novel aspects to the chemistry and biochemistry of anthocyanins from berries, representing an original addition to the already existing knowledge in the field of these polyphenolic bioactive compounds.

Original data regarding the chemical composition and antioxidant activity of the Romanian *Amelanchier lamarckii* fruits have been obtained. The anthocyanin-rich polyphenol extracts from *A. lamarckii* exerted enzyme inhibitory activity against tyrosinase, α-glucosidase, and acetylcholinesterase through an *in vitro* approach used to discover potential antidiabetic, anti-skin pigmentation and anti neurodegenerative molecules. Screening for potential antidiabetic effects was extended to a cell culture model of diabetic retinopathy using RPE cells

exposed to high-glucose conditions. To the best of our knowledge, this is the first study that evaluated the antioxidant, enzyme inhibitory and cytoprotective effects of polyphenols from *A. lamarckii* together with the assessment of their bioaccessibility.

Despite the numerous studies that associate blueberries with eye protection and vision improvement, our study provides new information about the antioxidant mechanism of *Vaccinium corymbosum cv.* Bluegold anthocyanidin extract in a cell model of diabetic retinopathy. Beyond the radical scavenging (ABTS) and metal-reducing (Fe^{3+}, Cu^{2+}) activity demonstrated by chemical tests, we showed that malvidin-rich extract effectively neutralized the reactive oxygen species generated in RPE cells in high-glucose conditions and improved the antioxidant status of the cells.

Our research also provided new knowledge on the stability of anthocyanins, compounds susceptible to the detrimental effects of external factors during the storage and processing of food commodities. Using a less investigated European source, *Amelanchier lamarckii* (juneberries), we obtained an extract that was analyzed by HPLC chromatography to track the stability to variations in pH, temperature, and light of individual anthocyanin. This is the first study focused on the *A. lamarckii* juneberry extract stability, revealing the impact of glycosylation on the stability of cyanidin-based anthocyanins in relation to the above-mentioned factors. These data will significantly impact the maintenance of anthocyanin properties in fresh and processed fruits or derived extracts, considering these compounds' color properties and potential pharmacological effects.

Finally, this thesis provided further support for the development of new therapies/or co-therapy for the world's deadliest type of skin cancer, melanoma, which is still a pressing problem due to frequent recurrences and limited responses to conventional therapy. Anthocyanins extracted and purified from *Aronia* berries were successfully encapsulated in the pH-responsive fluorescent nanocarrier (Nano@AntS), which travels through the cellular environment for the delivery of anthocyanins (AntS) and their release at the cellular level. Nano@AntS does not affect the viability of B16-F10 melanoma cells; in contrast, the concentration of AntS entrapped in Nano@AntS was shown to exert a potent cytotoxic effect. Another original aspect was the intracellular monitoring of Nano@AntS by co-encapsulation of the two fluorophores, fluorescein isothiocyanate (FITC) and rhodamine B isothiocyanate (RBITC). According to TEM images and fluorescence microscopy, Nano@AntS were found abundantly in the cytoplasm of B16-F10 melanoma cells, but also inside lysosomes. Nano@AntS can be translated from the laboratory scale to a future medical application for exploitation in novel therapies for melanoma.

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