PhD Thesis

Study on the antioxidant effect and aggregation properties of carotenoids and their derivatives

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1. Introduction

Carotenoids are one of the most abundant lipophilic pigments in nature, second to chlorophyll. Over 750 carotenoids give the various yellow, orange and red colours to plants, algae, fungi, yeast and higher animals. Plants, some microorganisms, fungi and algae can biosynthesize carotenoids while animals and the human body cannot produce them, hence they must be consumed in the diet. However, there are interesting and functionally significant examples of carotenoid-protein interactions in animals.

Carotenoids have been also known as good antioxidants. In plants and photoautotrophic bacteria, carotenoids being part of the photosynthetic apparatus absorb and convert the sunlight to energy for the cells. Furthermore, they can provide protection of photo-induced macromolecular damage. In food industry, they are used as dyes to improve colours of products after preparation and during storage. The most widely used carotenoids in food and pharmaceutical industry are β-carotene, zeaxanthin, lutein, capsanthin, astaxanthin and canthaxanthin.

The beneficial biological activities of carotenoids are strictly connected with their antioxidant effects and their conjugated polyene chains. They are thought to protect cells from oxidative stress, lipid-peroxidation and they might be effective in prevention of tumour growing, cardiovascular diseases and other inflammation processes.

The hydrophobicity of carotenoids determines their absorption and accumulation. Moreover, carotenoids are extremely sensitive to air, light and traces of acids, causing transformation, isomerisation or decomposition, making them difficult to work with. Coupling of hydrophilic groups or molecules to carotenoids might make their antioxidant effect and stability of the derivatives more favourable. Therefore, they could be utilized in medicine as antioxidants and in food chemistry as colorants more efficiently. We have some recent experience of such behaviour of carotenoid-flavonoid conjugates. Furthermore more hydrophilic carotenoid glycosides and PEGylated carotenoids were synthetized previously by our research group.

In 2004 disodium disuccinate astaxanthin derivative (ZanthoSyn®) was synthesized and since then its cardioprotective and anti-inflammatory effect were examined and proved in clinical trials.

Melatonin proves to be a many-sided molecule. It regulates not only the circadian rhythm, but has a direct and indirect anti-inflammatory effect by reducing the

expression of proinflammatory factors and up-regulating antioxidant enzymes. Moreover it is a potent scavenger of reactive oxygen and nitrogen species. These attributes encouraged us to synthetize carotenoid-melatonin derivatives to create more effective antioxidants.

2. Aims

- Optimization the synthesis of carotenoid succinates and carotenoid-melatonin conjugates to produce compounds with expectedly more powerful antioxidant effect.
- Comparison of the antioxidant capacity of the newly synthetized carotenoid derivatives and their parent carotenoids applying two types of antioxidant assays.
- Investigation of the aggregation properties of the used carotenoids regarding the effect of different polar solvents on size, structure and antioxidant capacity.
- Examination of the antioxidant and anti-inflammatory effect of carotenoids on RAW264.7 mouse macrophage cell line.

3. Experimental procedures

The following instruments were used for the characterization of the synthetized molecules. Melting points were measured on a Stuart SMP30 apparatus and are uncorrected. NMR spectra were recorded with a Bruker Avance III Ascend 500 spectrometer (500/125 MHz or $^{1}H/^{13}C$). Molar masses were obtained by an Autoflex II MALDI instrument (Bruker Daltonics). The elemental analysis measurements were performed on a Fisons EA 1110 CHNS apparatus. The UV-Vis spectrophotometric measurements were implemented on a Jasco spectrophotometer model V-730 UV/Vis (Jasco Corporation, Japan). Electronic circular dichroism (ECD) spectra were recorded at room temperature with a Jasco J-810 spectropolarimeter (Jasco Corporation, Japan). For the particle size analysis Malvern Zetasizer Nano S instrument and for the zeta potential determination Malvern Zetasizer Nano Z instrument were used (Malvern Panalytical Ltd., Great Malvern, Worcestershire, UK). TLC was performed on Kieselgel 60 F_{254} (Merck), and the plates were visualised under UV light. For column chromatography Kieselgel 60 (VWR, particle size 0.063–0.200 mm) was used. The scanning electron microscope (SEM) images were captured with a Jeol JSM-IT500HR (Jeol, Tokyo, Japan) instrument after gold coating (Jeol JFC-1300 auto fine coater, Jeol, Tokyo, Japan).

Two types of antioxidant measurements were performed: ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation) and FRAP (Ferric Reducing Antioxidant Power) assays. In both cases, trolox, a water-soluble vitamin E analogue was used as general standard antioxidant molecule and a TEAC (Trolox equivalent antioxidant capacity) value was calculated for every compound. The solution of ABTS radical cation is produced by reacting ABTS with potassium persulfate in distilled water. This reaction results a deep green-blue solution with absorption maxima at 417, 645, 734 and 815 nm. Of these, 734 nm is most suitable for spectrophotometric monitoring of the reaction between the antioxidant substance and $ABTS^{\bullet+}$. The antioxidant capacity can be measured through the decrease in absorption at 734 nm. The percentage inhibition of absorbance at 734 nm is calculated as $(A_0 - A_{antioxidant})/A_0$, where A_0 is the initial absorbance of the $ABTS^{\bullet+}$ solution and $A_{antioxidant}$ is the absorbance of the reaction mixture after incubation, both corrected for the solvent. The calculated percentage inhibition values are plotted against the final concentration of the antioxidants. The slopes of the curves are compared with that for trolox, and the trolox

equivalent antioxidant capacity (TEAC) value is calculated as the ratio of the slopes for the antioxidant and for trolox. The basis of FRAP assay is that, the antioxidant reduces Fe^{3+} to Fe^{2+} in an acidic medium forming a pink-coloured ferrous-tripyridyltriazine complex. The FRAP reagent consists of $FeCl_3$, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) and acetate buffer with pH 3.0. After mixing and incubating the FRAP reagent and the stock solution of the antioxidants the absorbance was measured at 600nm. During the reaction of Fe^{3+} with the antioxidants the absorbance of the solution increases. The absorbance values were plotted as a function of concentration of the sample compounds and that of trolox. The FRAP values were calculated as in the ABTS assay, i.e., the slope of the concentration-absorbance lines for the carotenoid derivatives was divided by that for the trolox.

In cell culture experiments, RAW264.7 mouse macrophage cell line (ECACC, Salisbury, UK; passage number: 8–15) was used and cultured. MTT and MTS cell viability assays were used to make sure that the applied carotenoids do not exert cytotoxic effect on RAW264.7 cells. To measure the amount of the reactive oxygen species dihydrorhodamine123 (DHR123) fluorescent dye was used and the fluorescent intensity of DHR123 (excitation 490 nm/emission 510–570 nm) was measured with Glomax Multi Detection System.

4. Results

4.1. The antioxidant and aggregation behaviour of natural carotenoids

A systematic investigation on the effect of aggregation on the antioxidant behaviour of carotenoids was performed. Eight carotenoids, the hydrocarbon β-carotene, the monohydroxyl β-cryptoxanthin, lutein, zeaxanthin, 8'-apo-β-carotenal, the keto derivatives astaxanthin and canthaxanthin, as well as the κ-end group-containing keto-carotenoid capsanthin were investigated. The association of carotenoids was studied by UV-vis and circular dichroism spectroscopies, as well as by dynamic light scattering spectroscopy and scanning electron microscopy.

The ABTS assay and TEAC determination were carried out in three different main solvents (96% ethanol, distilled water and PBS). We chose two auxiliary solvents, DMSO and THF, for the preparation of the stock solutions of carotenoids, which were further diluted with the main solvent.

In ethanolic solution, independently of the auxiliary solvent, the UV-vis spectra did not suggest association of any carotenoids, thus the determined TEAC values characterize the individual molecules. In solutions with water or PBS, however, all the spectra showed a definite alteration compared to those in organic solvents, clearly indicating the formation of carotenoid aggregates. The TEAC values for these aqueous systems belong to the colloidal systems formed from carotenoids, whose structure (size) is strongly dependent on the method of production.

The percentage inhibition against ABTS and the corresponding TEAC values seem to be primarily depending on the particle size, which is influenced by both the auxiliary and the main solvent. The different auxiliary solvents have also a strong effect on the type of aggregation (H or J) even in identical main solvents.

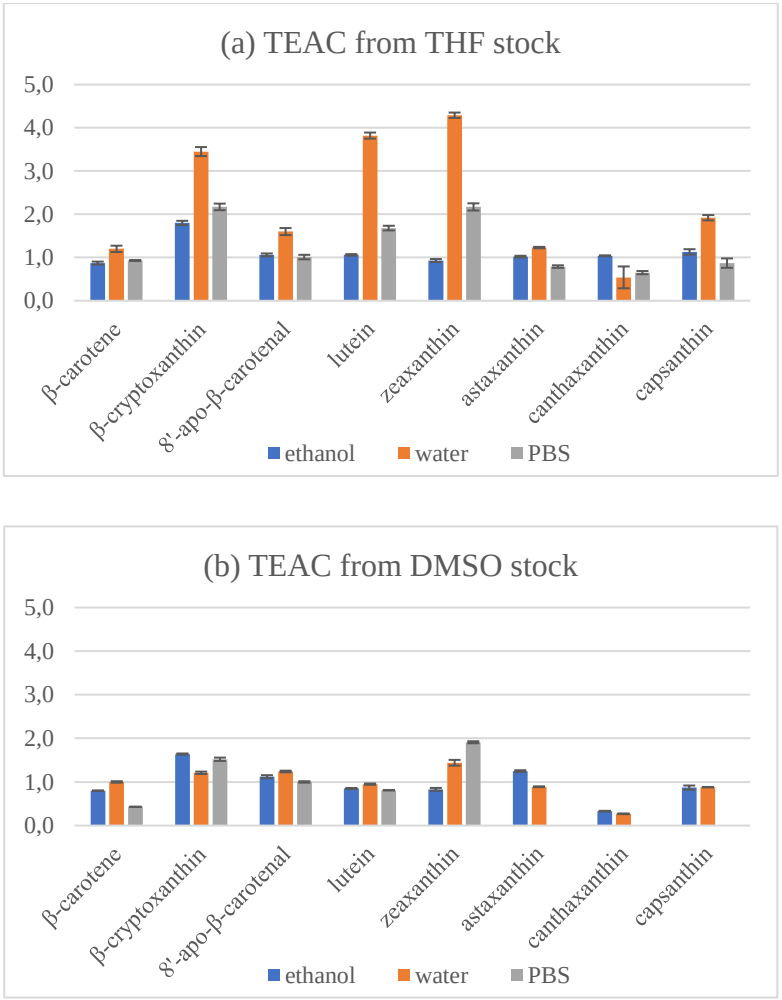

Figure 1. TEAC values of carotenoids (a) from THF (b) from DMSO stock solutions, diluted with ethanol, water, or PBS.

Interestingly, despite of the fact that in all aqueous solutions aggregation of carotenoid molecules was clearly observed, the TEAC values were quite similar to those in ethanol, and in many cases even higher. Especially the 3-hydroxy-β-end group carotenoids from THF stock solution displayed considerably higher TEAC values in PBS than in ethanol, and showed enormous TEAC values in water. (Figure 1.) The DLS measurements for the hydrodynamic diameter of the aggregates revealed a low (typically < 50 nm) and very uniform particle size in water from THF stock, while in PBS or starting from DMSO stock in both main solvents the formed aggregates were considerably larger (> 100 nm).

Considering the above, two opposite effects of aggregation were observed: the higher the aggregate size, the lower the percentage inhibition and the TEAC value, nevertheless, the aggregates exhibited similar TEAC values as individual molecules in

ethanolic solution. The aggregated carotenoids are obviously less accessible to the $ABTS^{\bullet+}$ reagent than the individual molecules, but yet the above findings suggest that the aggregation, to a certain extent, somehow makes the one-electron oxidation more efficient. This finding may initiate further studies on these aggregates to reveal the exact mechanism behind of the unusual antioxidant behaviour and broaden the practical application of carotenoid nanoparticles of similar size.

4.2. Synthesis of carotenoid-melatonin derivatives and their antioxidant behaviour

To prepare the conjugates, carotenoid succinates were synthetized first (Figure 2.), and as starting material, they were coupled with amino-melatonin via amide bond. The prepared succinates are more hydrophilic than the parent carotenoids, and can show enhanced biological activity themselves. Moreover, esters or amides can be synthetized from them relatively easily.

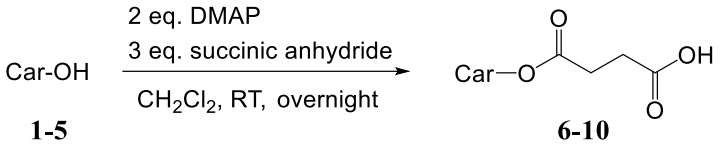

Figure 2. Preparation of succinate esters of hydroxy-carotenoids

The water dispersibility of the newly synthetized carotenoid succinates was determined experimentally, it increased from 0 to 1-2 mg/100 ml water.

For the coupling, instead of Steglich method EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) was chosen in combination with NHS (*N*-hydroxy-succinimide). DIPEA (diisopropylethyl amine) was used as a basic catalyst and also to liberate EDC and the amino-melatonin (**12**) from their corresponding hydrochloride salts (Figure 3.). We planned the preparation of zeaxanthin conjugates both with one and two melatonin moieties.

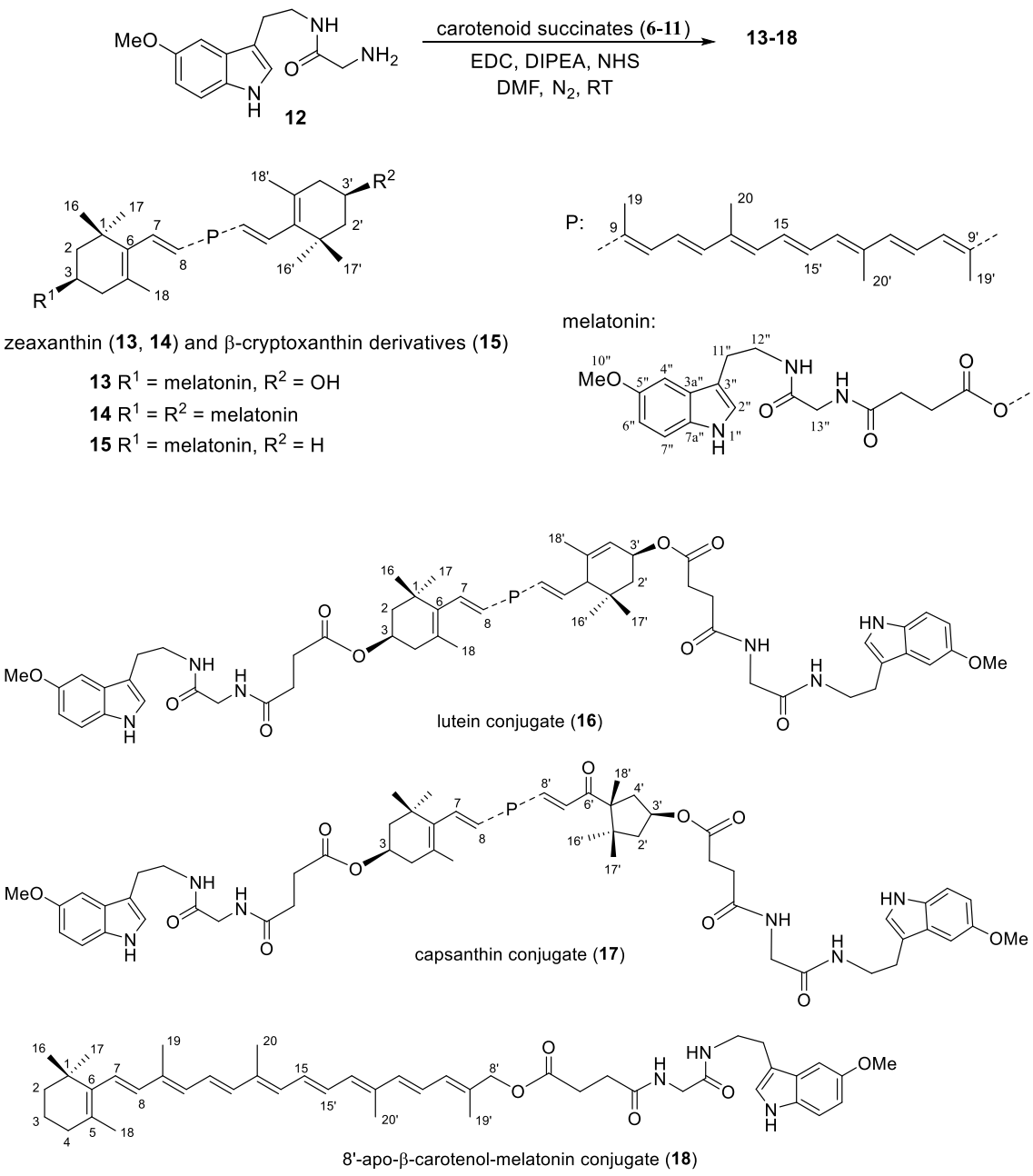

Figure 3. Synthesis of the melatonin conjugates

The aim of the synthesis was to enhance the direct and indirect antioxidant effect of carotenoids and possibly use them in eye drops for prevention against macula degeneration. Nevertheless, the antioxidant capacity showed a solvent dependence in the ABTS assays. In a phosphate-buffered saline (PBS), which ensures an intracellular-like medium, both the hydrophilic succinates and the hydrophobic melatonin conjugates of lutein, capsanthin and 8′-apo-β-carotenol surpassed the TEAC values of the parent

carotenoids, in the case of zeaxanthin and β-cryptoxanthin the conjugates gave similar or lower TEAC as the native carotenoids. (Figure 4.)

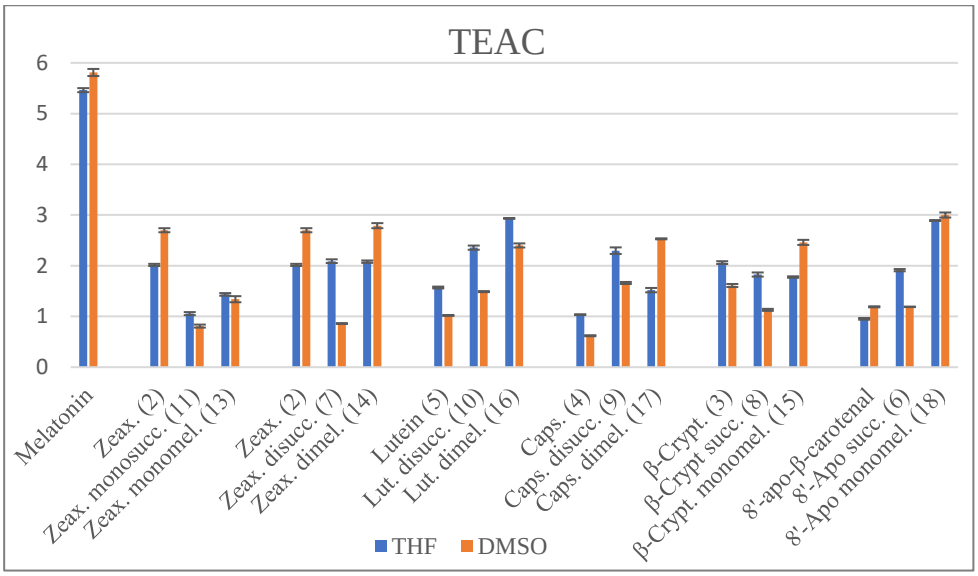

Figure 4. TEAC values determined using $ABTS^{\bullet+}$ radical in PBS ($p < 0.05$).

In aqueous media carotenoids and their derivatives can aggregate. As the aggregation may influence the antioxidant behaviour, UV-VIS spectroscopy and dynamic light scattering photometry was performed to characterize the formed particles. Applying the carotenoid concentrations from ABTS measurements, the UV-VIS spectra showed reduced absorbance and hypsochromic or bathochromic shifts, which suggest special aggregation forms like H-type (card pack) or J-type (head-to-tail) aggregates. The size of the aggregates was measured by dynamic light scattering (DLS) spectroscopy. The hydrodynamic diameter of the succinate aggregates varies typically between 10 and ~400 nm while the melatonin derivatives generally form aggregates between 10–200 nm. The size of the particles mostly depends on the concentration and paradoxically in some cases, larger aggregates were measured at lower concentrations.

According to the FRAP measurements at a lower pH, the best antioxidants were the bismelatonin derivatives of lutein and capsanthin. It is also worth mentioning the considerable differences in the antioxidant behaviour of lutein and zeaxanthin, and their conjugates. The aggregations in the aqueous media most probably influence the antioxidant capacities. To avoid aggregation and on the basis of the determined physicochemical properties, further investigation is needed to find a delivery system for the new conjugates. (Figure 5.). Recently, lutein and zeaxanthin bissuccinates were

successfully used in eye-drop models in the form of RAMEB cyclodextrin complexes as delivery system.

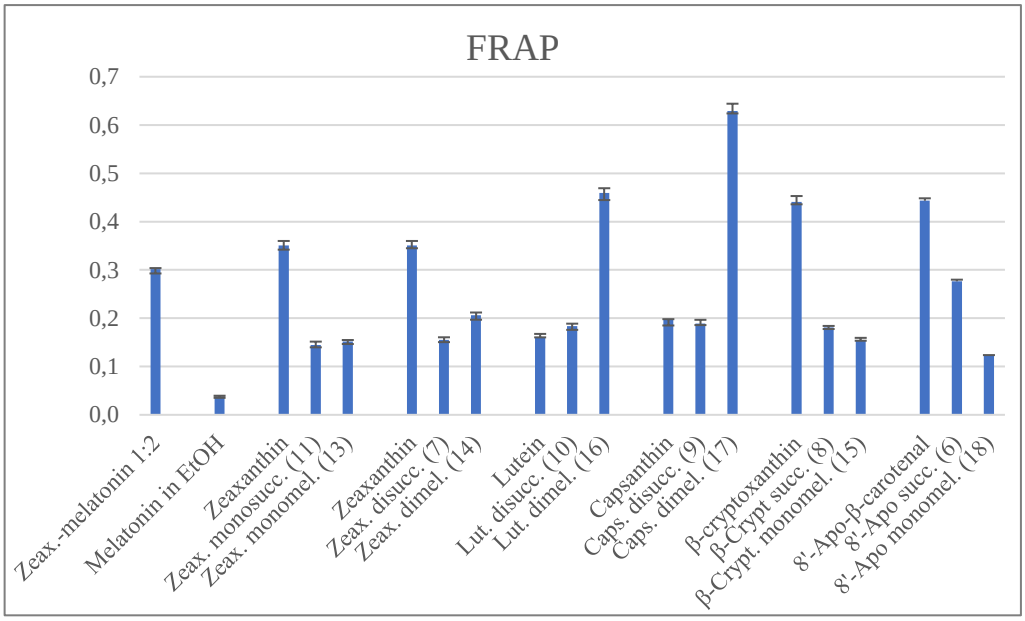

Figure 5. FRAP Values Measured Using Fe^{III}-TPTZ in Acetate Buffer ($p < 0.05$).

5. Cell culture and treatments

The radical scavenger property of carotenoids was detected in RAW264.7 mouse macrophage cell line which being very popular due to its fast proliferation and easy handling is commonly used as an inflammation model. The production of reactive oxygen species (ROS) is one of the most important marks of inflammatory macrophage induction. Therefore, the level of ROS was measured in LPS-induced and carotenoid-treated macrophage cells.

Firstly, MTT assay was carried out to make sure that the applied carotenoids do not have cytotoxic effect on the macrophage cells. 24 hours after LPS and carotenoid treatment, neither of carotenoids exerted cytotoxic effect on the cells. In the case of lutein another purple colour was observed and a bit smaller absorbance was measured implying cell death, although the microscopic checking did not confirm that. Therefore, MTS, another but similar cell viability assay was chosen for lutein. After the same treatment, we made certain that lutein did not have cytotoxic effect on RAW264.7 macrophage cells.

After MTT and MTS assays, the ability of carotenoids to reduce ROS in activated macrophage cells was investigated. All the used carotenoids show promising decrease in ROS level but the ROS concentration reduction strongly depends on the carotenoid concentration. Carotenoid succinates and parent carotenoids were applied in different concentrations and the effect of the chemical modification was investigated, as well. Capsanthin-bissuccinate and lutein-bissuccinate proved to be significantly more effective in decreasing ROS level in LPS-induced macrophage cells at higher concentrations than the parent carotenoids. (Figure 6.)

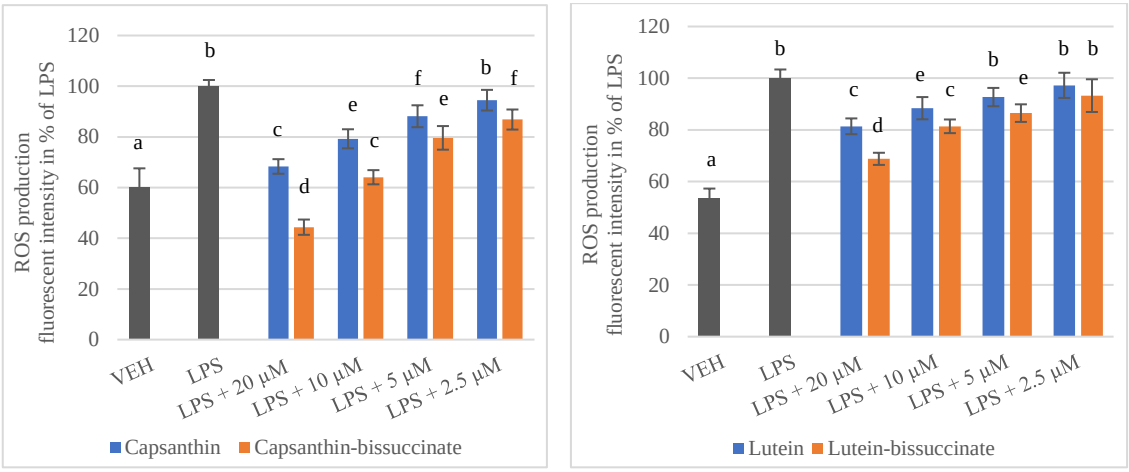

Figure 6. The percentage reduction of ROS production of capsanthin, lutein, and their succinates in different concentrations. Data are presented as means ± SD in percentage of LPS-treated group. Different small letters denote statistically significant difference among groups.

Astaxanthin, capsanthin and their succinates comparing to each other in 10 µM concentration reduced the ROS level the most efficiently. (Figure 7.)

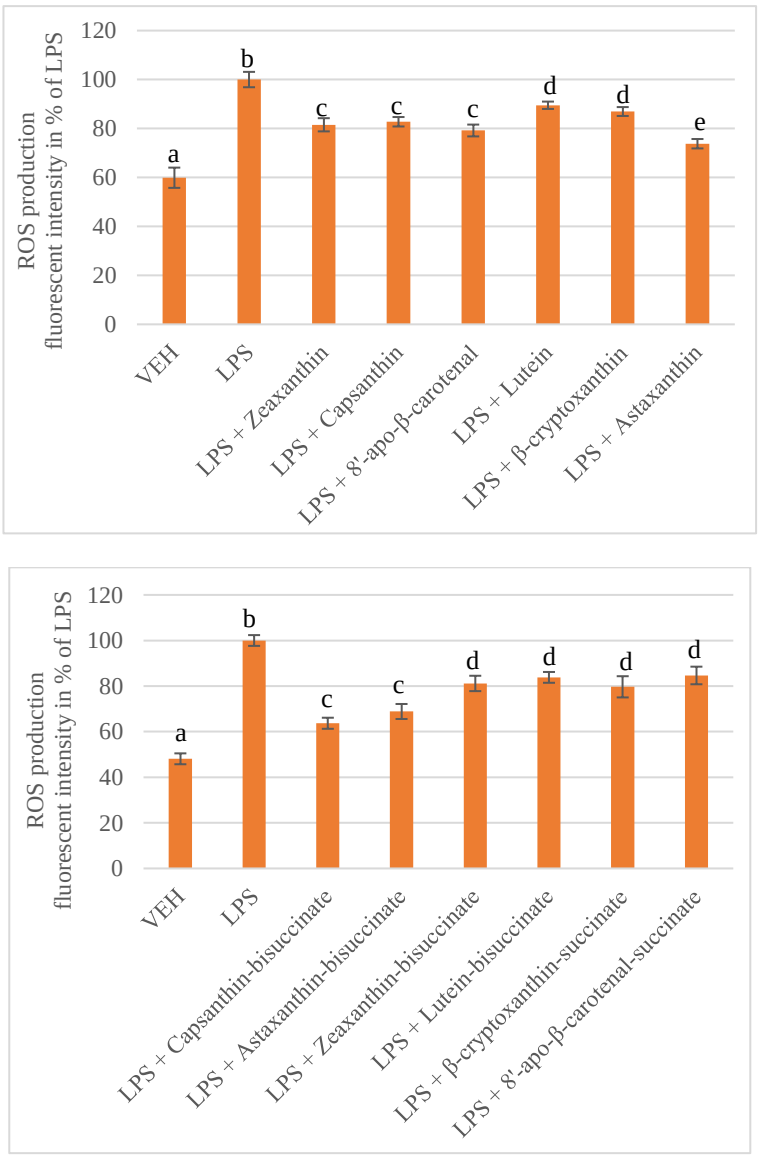

Figure 7. The percentage reduction of ROS production of native carotenoids and carotenoid succinates in 10 µM concentration. Data are presented as means ± SD in percentage of LPS-treated group. Different small letters denote statistically significant difference among groups.

6. Summary

The effect of aggregation of carotenoids on antioxidant capacity is a poorly investigated area. A lot of theories suggest that carotenoid aggregates are less effective than the individual molecules but these hypothesis are not confirmed appropriately and sometimes they are inconsistent. Nevertheless, it is quite difficult to analyze aggregates in liquid phase to gain more information about the physical-chemical properties and the type of the association.

In my thesis I chose to synthetise carotenoid-melatonin derivatives and investigate the antioxidant behaviour of native carotenoids, carotenoid-melatonin derivatives and their aggregates in organic and aqueous systems. THF and DMSO were used as organic auxiliary solvents and ethanol, distilled water and PBS were chosen as main solvents during the ABTS antioxidant measurements. We observed, that both the auxiliary and the main solvents influenced the type of the association, the size of the aggregates, and eventually the antioxidant capacity.

The aim of the synthesis was to enhance the direct and indirect antioxidant effect of carotenoids by coupling carotenoid succinates to amino derivative of melatonin via amide bond. The trolox equivalent antioxidant capacity (TEAC) towards ABTS radical cation of carotenoid succinates and carotenoid-melatonin derivatives was measured in PBS using THF and DMSO as auxiliary solvents. In three cases the carotenoid succinate and the melatonin conjugate surpassed the TEAC value of the parent carotenoid. We also investigated the aggregation properties of carotenoid-melatonin derivatives in aqueous media by UV-vis spectrophotometry, dynamic lights scattering photometry and measuring zeta potential. The melatonin derivatives generally formed aggregates between 10–200 nm and we observed that the size of the particles depends on the concentration and in some cases, larger aggregates were measured at lower concentrations.

The results of the radical scavenging ability of native carotenoids and carotenoid succinates in the cell treatments are promising but further investigations are needed. Stabilizing the size of the aggregates, creating carotenoid nanoparticles and investigating the transport of carotenoids between the blood-brain or blood-retina barrier may be the future task in the research group.

7. List of publications

Publications related to this thesis:

Czett, D.; Böddi, K.; Nagy, V.; Takátsy, A.; Deli, J.; Tone, P.; Balogh, G. T.; Vincze, A.; Agócs, A., Synthesis, Pharmacokinetic Characterization and Antioxidant Capacity of Carotenoid Succinates and Their Melatonin Conjugates. *Molecules* **2022,** *27,* 4822. ***IF.:4,6, Q1, citation: 1***

Czett, D.; Nagy, V.; Kurtán, T.; Király, B.S.; Szabó, P.; Agócs, A.; Deli, J.; Böddi, K., Effect of aggregation behaviour on the antioxidant capacity of carotenoids. *Journal of Molecular Liquids* **2025,** volume 421, 126870, ***IF.: 5.3, Q1***

Other publications:

Linzembold, I.; Czett, D.; Böddi, K.; Kurtán, T.; Király, S. B.; Gulyás-Fekete, G.; Takátsy, A.; Loránd, T.; Deli, J.; Agócs, A.; Nagy, V., Study on the Synthesis, Antioxidant Properties, and Self-Assembly of Carotenoid-Flavonoid Conjugates. *Molecules* **2020,** *25* (3). ***IF.:4,4, citation: 9***

Presentations on national conferences

Czett D., Böddi K., Vörös-Horváth B., Nagy V., Takátsy A., Deli J., Tone P., Vincze A., Balogh Gy. T., Agócs A., Karotinoid-szukcinátok és melatonin-konjugátumaik szintézise és antioxidáns hatásának vizsgálata, Vegyészkonferencia 15-17.06.2022. Eger, Hungary

Poster presentation on international conference

Czett D., Böddi K., Nagy V., Takátsy A., Deli J., Tone P., Balogh Gy. T., Vincze A. and Agócs A., Synthesis and antioxidant capacity of carotenoid succinates and their melatonin conjugates, the 19th International Symposium on Carotenoids 9-14.07.2023. Toyama, Japan.