CHARLES UNIVERSITY

FACULTY OF PHARMACY

IN HRADEC KRÁLOVÉ

Department of Pharmacognosy

Phytochemical analysis and biological activity of the alga *Haematococcus pluvialis* and *Chlorella* sp.

Dissertation

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Supervisor: doc. PharmDr. Lenka Tůmová, Csc.

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"Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně, pod vedením své školitelky doc. PharmDr. Lenky Tůmové, CSc. a odborného konzultanta Dr. José Carla Cheel Horny, PhD. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Disertační práce vznikla za podpory GA UK (1134217), SVV (260 550), TAČR (TJ01000013). Práce nebyla využita k získání jiného nebo stejného titulu."

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Abstrakt

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Farmaceutická fakulta v Hradci Králové

Katedra farmakognozie

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Název disertační práce:

Fytochemická analýza a biologická aktivita řasy *Haematococcus pluvialis* a *Chlorella* sp.

V současné době představují mikrořasy nový a slibný zdroj různých biologicky aktivních látek. Tato vědecká práce cílí na dva karotenoidy – astaxantin a lutein, o které je zájem v různých průmyslových odvětvích. Dosud vyvinuté výrobní postupy pro získání těchto dvou hodnotných karotenoidnových pigmentů však vyžadují náročné procesy, jak časově, tak i z hlediska spotřeby organických rozpouštědel.Tato práce se zabývá dvěma aspekty. Prvním je výzkum a vývoj účinných a na průmyslovou úroveň rozšířitelných metod izolace cílových karotenoidů z biomasy mikrořas pomocí vysoce účinné protiproudé chromatografie (HPCCC). Druhým je hodnocení biologické aktivity astaxantinu a jeho esterů, protože tyto dosud byly málo studovány.

V naší práci byl lutein izolován ze zelené řasy *Chlorella vulgaris* pomocí spodní fáze dvoufázového rozpouštědlového systému složeného z *n*-heptanu, etanolu a vody (poměr 5:4:1,5, obj./obj./obj.) (LP4), který sloužil jako rozpouštědlo pro extrakci řasové biomasy a zároveň také jako mobilní fáze pro izolaci luteinu pomocí HPCCC. Ultrazvuková extrakce biomasy s LP4 po dobu 30 minut vedla k získání extraktu bohatého na lutein (3,20 mg/g sušené biomasy). Celkově byly zpracovány 2 g extraktu *Chlorelly vulgaris*, z nichž bylo získáno 60 mg luteinu (čistota 92%). Tento byl dále purifikován pomocí gelové permeační chromatografie, čímž bylo získáno 50 mg luteinu (čistota 97%). Stejná metoda byla také použita pro izolaci luteinu z *Parachlorelly kessleri* kmene HY1, čímž se získalo 150 mg luteinu (95% čistota, 97% výtěžek). Dále bylo z mikrořasy *Haematococcus pluvialis* izolováno pět monoesterů astaxantinu pomocí HPCCC, kde byla jako mobilní fáze použita spodní fáze dvoufázového rozpouštědlového systému (*n*-heptan:acetonitril, poměr 5:5, obj./obj.). Závěrečná purifikace pomocí vysoce účinné kapalinové chromatografie (HPLC) poskytla pět derivátů astaxantinu esterifikovaných kyselinou α-linolenovou (4 mg), kyselinou linolovou (8 mg), kyselinou palmitovou (8 mg), kyselinou olejovou (12 mg) a kyselinou stearovou (1 mg) (98% čistota). K dalšímu zvýšení produktivity byla vyvinuta HPCCC metoda s vícenásobným nástřikem, která umožnila získat větší množství astaxantinových esterů, a to kombinací dvou elučních režimů (reverzní a souběžná eluce). V průběhu souběžné eluce byly mobilní i stacionární fáze čerpány současně, tak, aby se doplňovala stacionární fáze, která se ztrácí během každého separačního cyklu.

Astaxantin je silný přírodní antioxidant s prospěšnými biologickými aktivitami, které však byly primárně prokázány u jeho volné (neesterifikované) formy. Přirozený producent astaxantinu, mikrořasa *Haematococcus pluvialis*, však tento pigment syntetizuje většinou ve formě esterů, které byly dosud málo hodnoceny. Aby se přispělo k možnému komerčnímu použití této látky, bylo testováno několik různých biologických aktivit.

Antioxidační, antiparazitická, cytotoxická, antialergická, imunomodulační, antiagregační avazodilatační aktivita, stejně jako schopnost inhibovat tyrosinázu a produkci melaninu, byly vyhodnoceny u extraktu z *Haematococca pluvialis* a frakcí bohatých na monoestery a diestery astaxantinu izolovaných pomocí HPCCC. Pro stanovení antioxidačních a cytotoxických účinků bylo použito také pět již dříve izolovaných derivátů astaxantinu. Signifikantní antioxidační, anityrosinázová a cytotoxická aktivita byly pozorovány u extraktu *Haematococca pluvialis* společně s frakcí obohacenou o monoestery astaxantinu izolovanou pomocí HPCCC. Slabá antioxidační aktivita byla zaznamenána u monoesterů astaxantinu vázaných s kyselinou *α*-linolenovou (C18:3), palmitovou (C16:0) a stearovou (C18:0). Astaxantin esterifikovaný kyselinou olejovou (C18:1) prokázal cytotoxický účinek proti buněčné linii AGS karcinomu žaludka. Astaxantinové estery částečně prokázaly negativní vliv na antioxidační systémy parazitů. Předběžné hodnocení testovaných látek neprokázalo významné změny motility larev, nicméně byl zaznamenán určitý negativní dopad na jejich morfologii. Všechny testované látky vykázaly pouze inhibici antigenem indukovaného uvolňování *β*-hexosaminidázy při koncentraci 5 μM. Žádná z testovaných látek neprokázala imunomodulační, antiagregační ani vazodilatační aktivitu, ani neinhibovala produkci melaninu.

Abstract

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Title of Doctoral Thesis:

Phytochemical analysis and biological activity of the alga *Haematococcus pluvialis* and *Chlorella* sp.

Nowadays, microalgae represent a novel and promising source of various bio-active compounds. This research work focuses on two carotenoids – astaxanthin and lutein, which are attracting interest from various industrial sectors. However, the production methods so far developed for obtaining these two valuable carotenoid pigments from microalgae imply time- and solvent-consuming operations.

This work deals with two aspects. Firstly, the investigation and development of efficient and scalable isolation methods for producing the target carotenoids from microalgae biomass using high-performance countercurrent chromatography (HPCCC). Secondly, the investigation of the biological activity of astaxanthin and its esters, which have been little studied.

In our study, lutein was isolated from the green microalgae *Chlorella vulgaris* using the lower phase of the biphasic solvent system composed of *n*-heptane–ethanol–water (5:4:1.5, *v/v/v*) (LP4), which served both as a solvent for microalgae biomass extraction and as a mobile phase for lutein isolation by HPCCC. The ultrasound-assisted extraction of biomass with LP4 for 30 min led to an extract enriched in lutein (3.20 mg/g dried biomass). In total, an amount of 2 g of *Chlorella vulgaris* extract was processed through HPCCC affording 60 mg of lutein (92% purity), which was further cleaned up by gel permeation chromatography yielding 50 mg of lutein (97% purity). The same method was also applied for lutein isolation from a chlorophyll-deficient strain of the microalgae *Parachlorella kessleri* HY1, yielding 150 mg of lutein (95% purity, 97% recovery). Next, five astaxanthin monoesters were isolated from *Haematococcus pluvialis* by HPCCC, where the lower phase (LP) of a biphasic solvent system (*n*-heptane:acetonitrile, ratio 5:5, *v/v*) was used as a mobile phase. The isolated astaxanthin monoesters were finally cleaned up using high performance liquid chromatography (HPLC) affording five astaxanthin derivatives esterified with *α*-linolenic acid (4 mg), linoleic acid (8 mg), palmitic acid (8 mg), oleic acid (12 mg) and stearic acid (1 mg) (98% purity). To further increase the processes productivity, a multi-injection HPCCC method was developed to obtain higher amounts of astaxanthin monoesters by combining two elution modes (reverse phase and co-current). In co-current elution mode, both the mobile and stationary phases were pumped simultaneously so that the stationary phase that gets lost during each separation cycle was replenished.

Astaxanthin is a potent natural antioxidant with beneficial bioactivities, which has been demonstrated primarily for its free (non-esterified) form. However, its natural producer, the microalgae *Haematococcus pluvialis* synthesizes astaxanthin mostly in ester forms which have been little valorized so far. To contribute to the possible commercial use of these compounds, several biological activities were tested. The *Haematococcus pluvialis* extract together with the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters were tested for their antioxidant, antiparasitic, cytotoxic, anti-allergic, immunomodulatory, antiaggregant and vasodilatory activity as well as their capacity to inhibit the tyrosinase activity and melanin production. For the allocation of antioxidant and cytotoxic effects, five previously isolated astaxanthin derivatives were also used. A significant antioxidant, tyrosinase-inhibitory and cytotoxic activities were observed in the *Haematococcus pluvialis* extract together with the isolated astaxanthin monoesters. The weak antioxidant activity was noticed when examining the astaxanthin monoesters bonded with the α-linolenic (C18:3), palmitic (C16:0) and stearic (C18:0) acids. The astaxanthin esterified with oleic acid (C18:1) exerted a cytotoxic effect against the AGS human gastric cancer cells. In addition, the potential use of astaxanthin esters as antiparasitic agents as partially demonstrated by their negative effect on the larval antioxidant systems was observed. The preliminary evaluation of the tested substances did not show significant alterations of larval motility, however some negative impact on their morphology was shown. The capacity of astaxanthin esters to act as anti-allergic agents was also tested by demonstrating the inhibition of antigen-induced β-hexosaminidase release at a concentration of 5 μM. Finally, astaxanthin esters and their parent extract showed no immunomodulatory, antiaggregant, and vasodilatory activity; besides, they did not inhibit melanin production on cells.

Content

Introduction

Although macroalgae (seaweeds) have been part of human nutrition for many centuries, as proven by archeological discoveries, the microscopic organisms were only noticed once forming a corpus or a colony.

The first successful microalgae cultivation occurred at the end of 19th century using microalgae *Chlorella* sp. and since that its popularity still arises. During the First World War, the French military used dried seaweeds to feed horses once the grain stocks were depleted. More extensive research of their potential use in nutrition came at its end, when any food source was hopefully considered in Germany. During the Second World War, Europe has widely used the dried and desalinated seaweeds as human stock feed once the grain supplies were over. Similarly, this time was a catalyst for research of valuable substances from algae, but this initiative faded with the discovery of penicillin [**Paddock 2019**]. However, the interest in microscopic algae as a source of protein for the starving world and as a source of valuable substances remained. In the second half of the 20th century, there was the first big boom in the cultivation of microalgae. The *Chlorella* sp. was the first microalgae to be mass produced for food, feed and nutraceuticals, and its commercial potential has been taken into consideration since 1960 [**Ramaraj et al. 2016**]. Since the 1980s, there has been a growing interest in nutritional preparations, food supplements, natural products, and novel food. Along with this, the range of offered products has expanded either in their natural form or as the specific substances of natural origin. The microalgae are generally quite interesting source of natural substances with an almost unlimited range of valuable and bioactive compounds that can be used as innovative functional components of novel food or food supplements.

The microalgae provide a wide range of bioactive molecules with extensive applications in the food and animal feed industries. These can be pigments, proteins, amino acids, polysaccharides, dietary fibers, lipids, fatty acids, polyphenols, probiotics, vitamins, enzymes, and minerals. The algal pigments are divided into three groups: carotenoids, chlorophylls and phycobilins. Out of them, the carotenoids are the most important in terms of practical use and benefits to human health. They serve as a precursor of vitamin A; they have significant antioxidant properties and are also involved in the prevention of various diseases, such as cancer, cardiovascular problems, and degenerative eye changes.

Theoretical framework

1.1 Carotenoids in microalgae

1.1.1 Biosynthesis of carotenoids

Carotenoids are tetraterpenes widely distributed in nature: photosynthetic bacteria, fungi, algae, some species of Archaea, plants, and animals, they all contain these colorful pigments. All of them can synthesize carotenoids *de novo*, except animals. Therefore, animal carotenoids exhibit structural diversity because animals can get them either from the diet or through metabolic reactions. The carotenoid structure is generally composed of a polyene chain together with nine conjugated double bonds. Moreover, there is an end group at each of the ends of this polyene chain. Typical structure shows eight isoprene units together with a 40-C skeleton. [**Britton et al. 2004; Maoka 2020**]. Carotenoids are divided into two groups (i.e., carotenes and xanthophylls) based on the presence of oxygen. Carotenes are purely hydrocarbons occurring in a couple of isomeric forms (i.e., alpha-α, beta-β, gamma-γ, delta-δ, epsilon-ε, and zeta-ζ). Some of their related compounds got the formula $C_{40}H_{56}$ [**Rodriguez-Amaya 1997**]. Xanthophylls contain oxygen and they are perceived as the oxidized derivatives of carotenes. Their general formula is $C_{40}H_{56}O_2$ [**Matsuno et al. 1986**]

Two isoprene isomers, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), serve as the precursors to build up various carotenoid compounds based on their biosynthetic pathways. These pathways vary between plants and animals. Regarding plants, there are two pathways for production of IPP: the cytosolic mevalonic acid (MVA) pathway and the plastidic methylerythritol 4-phosphate (MEP) pathway. In animals, the IPP and DMAPP are generated through the MVA pathway, while these two are determined by the MEP pathway in plants.

The MEP pathway provides a mixture of IPP and DMAPP with a ratio of 5:1. Later, these two isomers undertake several actions, giving rise to the most common carotenoid precursor geranylgeranyl diphosphate (GGPP) that can be transformed by various ways to the carotenes or xanthophyll [**Kuzuyama and Seto 2012; Nisar et al. 2015**]. The phytoene (having a C40 skeleton) is derived from dimethylallyl pyrophosphate (DMAPPP) through geranyl pyrophosphate (GPPP) and geranylgeranyl pyrophosphate (GGPPP) [**Britton 1998**].

1.1.2 Lutein

Lutein [(3R,3'R,6'R)-*β*,*ε*-carotene-3,3'-diol] (**Fig. 1**) is a yellow natural carotenoid. This pigment is synthesized by plants and widely distributed in nature. It is present mainly in fruits, vegetables, and flowers, but also in animal tissues [**Maoka 2011; Bernstein et al. 2016**].

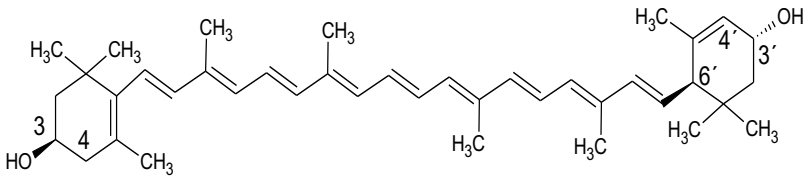


Fig. 1 Chemical structure of lutein [**Bernstein et al. 2016**]

Since animals are not able to produce lutein, this pigment must be absorbed from their diet to accumulate in their tissues [**Sommerburg et al. 1998**]. Therefore, to improve the pigmentation of egg yolks, meat and dairy products, lutein was firstly used as a feed additive in the poultry and livestock industries [**Yaakob et al. 2014**]. Nonetheless, lutein also possesses other beneficial properties, such as antioxidant, light-filtering and bioactive with potential use in the medical treatment of some chronic diseases (e.g., age-related macular degeneration, cataracts, ischemic heart disease, lung cancer, skin cancer, and atherosclerosis) [**Madaan et al. 2017**].

All these abovementioned properties keep lutein in high demand and potentially applicable in pharmaceutical, nutraceutical and cosmetic industry. By 2018, there were reported approximately 850 carotenoids occurring in nature [**Maoka 2020**] and out of all of these, only lutein (together with zeaxanthin) is uniquely distributed in the human macula, retina, and eye lenses [**Mares 2016; Arunkumar et al. 2020**]. Both cataracts and age-related macular degeneration are leading worldwide causes of impaired vision and blindness [**https://www.who.int/news/item/09-10-2003-up-to-45-million-blind-people-globally---and-growing; https://www.who.int/news-room/fact-sheets/detail/blindness-and-visual-impairment**]; therefore, this topic gains a lot of scientific interests. Several epidemiological investigations suggest a correlation between the amount of lutein in the macula and lenses, and the development of these common eye conditions [**Moeller et al. 2000; Gale et al. 2003; Renzi and Johnson 2007; Koo et al. 2014; Ozyrut et al. 2017**], however some clinical and epidemiological studies also demonstrate that lutein supplementation has a preventive effect on the development of these diseases, especially when taken in the diet [**Ma et al. 2012; Ma et al. 2014; Maci et al. 2015; Bernstein et al. 2016; Mares 2016**]. Due to its proven health benefits, lutein is interesting not only from a scientific point of view, but also from a commercial point of view, as evidenced by a study of the global lutein market, which estimates the value of this pigment to reach at US$ 463.16 million in 2027. This is 1.6 times more than it was in 2019, when the global lutein market reached value of US$ 288.41 million. Compound annual growth rate (CAGR) is expected to reach 6.10% over the period of 2020 – 2027 [**https://www.maximizemarketresearch.com/global-lutein-market/**].

Since carotenoids broaden their range of applications, mainly due to the increasing knowledges of consumers about the advantages of their consumption, Europe is expected to maintain its leading position worldwide with a market share of 37.4%. However, the Asia-Pacific market is supposed to reach the highest CAGR of 8.6% over the period of 2020-2027 [**https://www.maximizemarketresearch.com/market-report/lutein-market/661/**].

1.1.3 Astaxanthin

Astaxanthin [3,3′-dihydroxy-*β*,*β*-carotene-4,4′-dione] (**Fig. 2**) is a natural reddish ketocarotenoid occurring in non-esterified (free) and esterified form. The non-esterified (free) astaxanthin has been widely investigated from the point of its health-beneficial properties. Astaxanthin possess various bioactivities beneficial for human health, however these were up to date attributed mainly in its non-esterified form. On the other hand, there are reports claiming that esterified astaxanthin possess better antioxidant, skin-whitening, skin cancer, hepatoprotective and anti-ulcer properties than its free form [**Kamath et al. 2008; Rao et al. 2013b; Rao et al. 2015**].

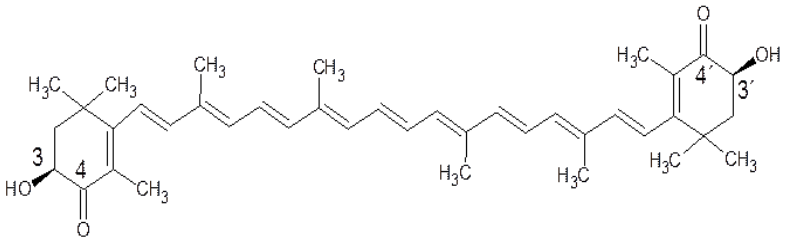


Fig. 2 Chemical structure of astaxanthin [**Lorenz and Cysewski et al. 2000**]

Considering the increasing demand of consumers for the dietary supplements and the necessity to better understand its health-promoting properties, the selection of an appropriate source to obtain this pigment is crucial. The commercial sources of astaxanthin are either the microalgae *Haematococcus pluvialis* or the red yeast fungus *Xanthophyllomyces dendrorhous* (previously known as *Phaffia rhodozyma*). Another commercial way to gain this compound is through the chemical synthesis [**Rao et al. 2014**]. The non-esterified (free) astaxanthin of high purity has been already isolated from the microalgae *Chloroccocum* sp. [**Li and Chen 2001**] and the red yeast *Xanthophyllomyces dendrorhous* [**Du et al. 2016**]. In 2019, a novel protocol for obtaining astaxanthin-containing extract from *Haematococcus pluvialis* by liquid-liquid chromatography was developed [**Bauer and Minceva 2019**]. The astaxanthin esters from *Haematococcus pluvialis* have been recently isolated using the lab scale limited technique called thin layer chromatography (TLC) [**Kamath et al. 2008; Rao et al. 2013b**], nonetheless none of these studies aimed at the scalable isolation of astaxanthin esters.

In 2019, global astaxanthin market reached value of US$ 1.0 billion with North America being the leading country. On the other hand, China has some of the major biotechnology companies worldwide; therefore, the Asia-Pacific market is expected to grow significantly over the period of 2020-2027 [**https://www.grandviewresearch.com/industry-analysis/global-astaxanthin-market**]. Factors such as proven antioxidant properties and increasing interest of customers for the natural carotenoids and healthy lifestyle are supposed to boost the astaxanthin global market up to US$ 3.4 billion in 2027. Compound annual growth rate (CAGR) is expected to reach 16.2% over the period of 2020 – 2027. [**https://www.globenewswire.com/news-release/2020/03/05/1996035/0/en/Astaxanthin-Market-Size-Share-Trends-Analysis-Report-By-Source-By-Product-By-Application-And-Segment-Forecasts-2020-2027.html**].

Moreover, the consumers aim to use nutraceutical and cosmetic products that contain mainly natural pigments, therefore the global market demand for astaxanthin derived from *Haematococcus pluvialis* has increased. [**Shah et al. 2016; https: //www.grandviewresearch.com/industry-analysis/global-astaxanthin-market; http://www.algaeindustrymagazine.com/report-suggests-astaxanthin-market-2-57-billion-2025/**].

1.1.4 Lutein production

Considering the above-mentioned health-promoting properties of lutein and increasing seek for consumers for the organic food products, the identification of an appropriate source of this pigment is pivotal. The global lutein market is expected to reach EUR€ 409 million by 2027 at a Compound Annual Growth Rate (CAGR) of 6.10% over the predicted period 2020–2027 [**Marino et al. 2020**]. So far, only marigold (*Tagetes erecta* L.) has been widely used for lutein production since its flowers contain a high amount of this pigment [**Bernstein et al. 2016; Lin et al. 2015**]. Marigold petals however contain lutein not only in its free form, but also in ester forms [**Sandmann 2015**] reaching the concentration up to 3-6 mg/g [**Piccaglia et al. 1998**]. Lutein is being extracted from the marigold flowers in the form of oleoresin, which contains lutein in a concentration range from 5 to 50% [**Bernstein et al. 2016; Fernández-Sevilla et al. 2010**]. However, marigold cultivation as well as marigold flower harvesting, and processing are influenced by planting area, seasons, climate, and high labor costs [**Sun et al. 2016**]. Moreover, to obtain a pure lutein from oleoresin demands additional purification processes, such as saponification and recrystallization [**Khachik 2007**].

Recently, the solid fractions of grains from *Zea mays* (L.) remaining after the dry-grind process in the fuel corn ethanol production have become the focus of scientific interests as an alternative lutein source [**Li and Engelberth 2018**], while microalgae are already recognized as an attractive source of this compound [**Fernández-Sevilla et al. 2010; Lin et al. 2015**]. Research provided by Lin et al. (2015) demonstrated that under controlled cultivation conditions some microalgal species can have higher lutein productivity rates in comparison with marigold [**Lin et al. 2015**]. Another way how to produce pure lutein is using the chemical synthesis. Nonetheless, its up-scaling is rather expensive for the industrial application and would lead to the co-production of non-naturally occurring and potentially dangerous stereoisomer forms [**Bernstein et al. 2016; Fernández-Sevilla et al. 2010; Hojnik et al. 2008**]. Microalgae have therefore emerged as an attractive source of lutein since they offer several advantages, such as high lutein content and high biomass productivity. Additionally, their cultivation is not limited to seasons and demands lower water consumption in comparison with marigold flowers [**Lin et al. 2015; Fernández-Sevilla et al. 2010**]. Microalgae can be cultivated under several growth conditions: photoautotrophic, heterotrophic and mixotrophic [**Sun et al. 2016**].

When talking about the species of green microalgae *Chlorella*, several strains have achieved high lutein production when heterotrophically cultivated in fermenters [**Shi et al. 1997; Zhang et al. 1999; Shi et al. 2000; Wu et al. 2007**]. That makes them a valuable alternative source for sustainable production of this carotenoid. Nonetheless, no commercial lutein production yet uses microalgae [**Lin et al. 2015**].

There are lab-scale reports demonstrating lutein isolation from microalgae using solid-state chromatography [**Shibata et al. 2004**], countercurrent chromatography [**Li et al. 2001**] and saponification extraction operations [**Li et al. 2002**], all of which required multi-step operations and the use of toxic and environmentally unfavorable organic solvents. Countercurrent chromatography has been shown to be an effective technique for isolating lutein from microalgae [**Li et al. 2001**]; however, this meant a single run-method with a small sample size and the use of toxic solvents. Therefore, to effectively contribute to the sustainable use of microalgae as a potential commercial source of lutein, the development of a high-throughput, efficient and environmentally friendly separation method is needed. In this context, high performance countercurrent chromatography (HPCCC) may play a major role.

1.1.5 Astaxanthin production

Astaxanthin is produced in microalgae when the maternal organism is stressed by various stress stimuli (e.g., lack of nutrients, increased salinity, and excessive sunshine). Some sorts of fish (e.g., salmons, red trout, red sea breams, flamingos) and crustaceans (e.g., krills, shrimps, crabs, crayfish, and lobsters) consume algae and photosynthetic bacteria, therefore subsequently gaining the red-orange pigmentation because of astaxanthin. In addition, these animals are not able to synthesize carotenoids such as astaxanthin *de novo*, therefore their skin color relies on the dietary carotenoids and their food supplementation [**Steven 1948; Goodwin 1984; Torrissen et al. 1990; Lovatelli and Chen 2009**]. Generally, synthetic astaxanthin is not preferred among customers as it contains a mixture of different stereoisomers. In addition, these consumers prefer natural forms to synthetic substitutes [**Boussiba et al. 2000**].

Synthetic astaxanthin or the astaxanthin derived from the red yeast *Phaffia rhodozyma* and *Paracoccus* bacteria is used mainly in the aquaculture sector, while the microalgae *Haematococcus pluvialis* is a leading source of this pigment for human applications (i.e., nutraceuticals, cosmetics, food, and beverages) [**Rodríguez-Saíz et al. 2010**]. Astaxanthin in *Haematococcus pluvialis* is present mainly in the form of esters from which 70% correspond to monoesters, 20% to diesters and only 5% correspond to non-esterified (free) astaxanthin [**Lorenz and Cysewski 2007**]. On the market, astaxanthin from *Haematococcus pluvialis* are sold in the form of crude extract (i.e., oleoresin) that is obtained from microalgae biomass by CO_2 supercritical fluid extraction [**Shah et al. 2016**] or organic solvents. The microalgae *Hameatococcus pluvialis* contains predominantly the esterified forms of this pigment, therefore only the 10–15% (*w/w*) corresponds to total astaxanthin, whereas the rest is composed mostly from acylglycerols and minor carotenoids [**FDA: GRN no. 294; FDA: GRN no. 356**]. Some of the monoesters in *Haematococcus pluvialis* are esterified for example with linoleic (an omega-6 fatty acid) or linolenic acids (an omega-3 fatty acid), both being considered as essential for human nutrition [**Saini and Keum 2018**]. Therefore, the production of individual astaxanthin esters may support the determination of their biological activities and ease the diversification of their market offers.

1.2. High performance countercurrent chromatography (HPCCC)

The HPCCC, a liquid-liquid chromatographic technology, is an effective and flexible preparative scale separation technology with a wide range of use in the isolation of natural products. This technology uses two immiscible liquid phases and is based on partitioning effects [**Ito 2005, Sutherland et al. 2013**].

Liquid chromatography uses one liquid phase to pass through the system and to interact with stationary phase located within the column. The porous matrix such as silica gel, cellulose or diatomaceous earth is typically used as a solid support that allows the retention of the mobile phase [**Conway 1990**]. The HPCCC has no solid support [**Berthod et al. 2009**]; therefore, its liquid-liquid base provides it with several advantages in comparison with the conventional solid support chromatography (e.g., reduced risks of sample denaturation and irreversible sample adsorption; reduced solvent consumption; large sample loading capacity and sample recovery, high predictability; its possible use as a preparative technique) [**Ito 2005; Michel et al. 2014**]. The HPCCC also allows to predict several important parameters, such as the stationary phase retention [**Sutherland 2002**], the retention time of the target compounds [**Sutherland 2002**], retention volume of target compounds [**Kang et al. 2016**]. This information contributes to the isolation process because they help to determine the chromatographic resolution as well as predict solvent consumption and run time.

As stated above, HPCCC uses two immiscible liquid phases. The one retained in the column by centrifugal force is called the stationary phase, while the other one pumped through the column is called the mobile phase. The separation process is therefore based on the difference in partitioning of each target compound between these two phases [**Ito 2005**]. In some cases, an intermediate middle phase can be also included [**Yanagida et al 2007**].

The solvent system used for the separation must exhibit a suitable partition coefficient (*K*) value, a short settling time and a proper density difference between its immiscible phases to assure a good retention of the stationary phase in the chromatographic column that is needed to maintain a good resolution [**Ito 2005; Berthod and Faure 2015**]. The full separation occurs once the separation factor (α) value between each pairs of the target compounds ($\alpha = K2/K1$, $K2 > K1$) is >1.5 [**Ito 2005, Sutherland et al. 2013**].

So far, the HPCCC technology has been used for the isolation of various natural products from different natural sources [**Friesen et al. 2015**]. There are also evidences about the isolation of astaxanthin-containing extract from *Haematococcus pluvialis* [**Bauer and Minceva 2019**], free astaxanthin from the microalgae *Chlorococcum* sp. [**Li et al. 2001**] and from red yeast *Phaffia rhodozyma* [**Du et al. 2016**], as well as the isolation of lutein from *Chlorella vulgaris* [**Li et al. 2001**]. However, none of them aimed to develop efficient and scalable methods for the possible industrial use.

1.3. Biological activity

1.3.1 Immunomodulatory activities

Few years ago, the scientific community has noted the growing demand for the development of novel immunomodulatory approaches to prevent and/or cure various diseases. Several factors such as the increase of antibiotic resistance towards the bacterial infections [**Levy and Marhall 2004; Spellberg et al. 2008**], population ageing [**Nijnik 2013**], bioterrorism threats [**Nijnik 2013**] and pandemic viral infections [**Nijnik 2013**]. The years 2019 and 2020 have brought up a new challenge in the form of the coronavirus pandemic showing to the whole world the real need for discovering novel drugs and methods to cope with infectious diseases that attack human immune system [**Kim et al. 2020a**]. The immune system of all multicellular organisms is classified within two groups: innate (nonspecific) and adaptive (specific) immunity.

The innate immunity plays role in the prevention of infection as well as the activation and participation of already existing defense mechanisms (i.e., physical/anatomical barriers, effector cells, cell receptors, antimicrobial peptides, and soluble mediators), while the adaptive immunity targets the previously identified specific pathogens or antigens. In all multicellular organisms the newly coming pathogens are firstly recognized by the innate immune system ensuring the first defense mechanisms and thorough the time the adaptive immune response is being activated [**Chaplin 2003; Aristizábal and González 2013**]. The immunity evaluation is focused on humoral immune measurements and cellular immune evaluation. Whereas the basic humoral immune tests aim to quantify the level of specific antibody product of an already created immune response *in vivo* (i.e., assessments of the general level of serum immunoglobulins and specific antibodies), its evaluation may also include the examination of B-cell response *in vitro* and the evaluation of an immune response to *de novo* immunization. On the other hand, the cellular immune function is more complex requiring measurements of the current immune response mostly *in vitro*. The *in vivo* cellular immune assay includes the elicitation of a functional response during the process of testing [**Cunningham-Rundles 1999**]. The flow cytometry is a method used for measuring certain cellular processes that occur around the T lymphocyte cell activation-proliferation pathway. The elevated expression of cell surface molecules following the lymphocyte activation can be easily measured using this technique. Once the activation of T lymphocyte cells occurs, the first expressed cell surface marker is the CD69 glycoprotein. It is an early activation antigen that is expressed on induced immune cells. Therefore, in comparison with the activated T lymphocyte cells, the CD69 glycoprotein is not stimulated in the resting T-cells.

After its expression on T lymphocyte cells, CD69 glycoprotein co-stimulates T lymphocyte cells activation and proliferation. Moreover, this glycoprotein is expressed by B lymphocyte cells, natural killer cells, monocytes, neutrophils, and eosinophils [**López-Cabrera et al. 1993; Ziegler et al. 1994; Cunningham-Rundles 1999**]. The immunomodulating effect of various carotenoids (e.g., β-carotene, canthaxanthin and astaxanthin have been already evaluated [**Okai and Higashi-Okai 1996; Hughes 1999**] demonstrating that three abovementioned carotenoids may increase the proliferation of mice immunocompetent cells. Moreover, it was shown that astaxanthin induce the production of the polyclonal antibody (immunoglobulin M and G) of murine spleen cells as well as the highest cytokine-inducing activity [**Okai and Higashi-Okai 1996**]. In our work, the immunomodulatory activity of tested substances was assessed by the measuring of the immune cell activation as expressed by the CD69 glycoprotein. This was evaluated using a flow cytometry as described previously [**Tůmová et al. 2017**].

1.3.2 Antioxidant activity

Antioxidants possess the ability to reactive oxygen species (ROS) and/or free radicals [**Abuajah et al. 2015**]. The free radicals are directly involved in the development of several chronic diseases (e.g., cardiovascular and heart diseases, anemia, inflammation, ageing, cancer) [**Aher et al. 2011**] and the antioxidants protect the organisms against damage caused by these free radicals. These compounds have attracted the attention of the scientific community, mainly in a relation with food and beverages aspects [**Zehiroglu and Ozturk Sarikaya 2019**].

In comparison with recognized antioxidants such as β-carotene, vitamin E, zeaxanthin, lutein and canthaxanthin, astaxanthin appears to possess more potent antioxidant activity [**Miki 1991**]. Moreover, it protects the structure of biological membrane [**McNulty et al. 2007**], lacks pro-oxidant effect [**McNulty et al. 2007**] and increases *in vivo* the activities of antioxidant enzymes [**Kim et al. 2016b**]. The antioxidant activity of astaxanthin can be nonetheless associated with several factors (i.e., the formation of chelate complexes with metals; possible esterification; the inability to aggregate in the ester forms; the formation of neutral radicals under high illuminance in the presence of metal ions; a high oxidation potential) [**Focsan et al. 2017**]. Esterified forms of astaxanthin from *Haematococcus pluvialis* exhibit higher antioxidant activity than non-esterified astaxanthin. Moreover, astaxanthin monoesters possess higher antioxidant activity than astaxanthin diesters in B16F10 mice melanoma cells and HaCaT human keratinocyte cells [**Hwang et al. 2020**].

In our work, the antioxidant activities of tested substances were determined using 2,2-diphenyl-1,picrylhydrazyl (DPPH) as a stable free radical. The DPPH assay was performed as described previously [**Brand-Williams et al. 1995**].

1.3.3 Antiaggregant activity

Nowadays, the cardiovascular diseases (CVDs) are perceived as a major cause of deaths worldwide, out of which the ischemic heart disease, stroke and venous thromboembolism are the most commonly occurring disorders. There are firm opinions that all of these disorders may be induced by thrombosis, during which the aggregation of blood platelets plays a pivotal role in a physiological process of hemostasis [**Fowkes 2017; Lim 2019; Olas 2020**].

It has been also proven that people diagnosed with the CVDs usually suffer from lipid disorders. Consequently, it makes them vulnerable towards the procoagulant state that may arouse the formation of blood clots within the arteries [**Bai, 2006**]. The application of antiaggregant treatment is therefore crucial in reducing the risk of CVDs.

Regarding hemostasis, one of its important stages is the platelet coagulation that can be induced using both different conditions and compounds. Platelet aggregation is a process by which the platelets adhere to each another, and this stage can be induced when adding various compounds in platelet-rich plasma or whole blood. The aggregation itself depends on several factors, such as the concentration of Ca^{2+} ions, fibrinogen, plasma factors and the soluble aggregator compounds such as such as adenosine diphosphate (ADP), arachidonic acid, thrombin, or serotonin. Two sets of aggregation tests are either the optical aggregometry that uses the platelet-rich plasma or the whole blood impedance aggregometry [**Jun 2005; Lauver et al. 2008; Štejnarová 2010; Hendrychová 2015**]. In our work, the whole blood impedance aggregometry was used with ADP serving as the aggregator agonist. The ADP is present in the platelet organelles from which it is released during the formation of the hemostatic plug. This platelet thrombus is being formed around the blood vessel wall rupture in the early stage of hemostasis as a response to its injury and leads to the subsequent platelet adherence [**Štejnarová. 2010; Olas 2020**]. Moreover, ADP is known to induce the platelet aggregation through the activation of P2 receptors for adenine nucleotides and nucleosides, specifically two P2 receptor subtypes: $P2Y_1$ and P_{2T} [**Jin 1998; Jarvis 2000**].

1.3.4 Vasodilatory activity

Globally, the CVDs are the most common cause of human death. In 2016, there is an estimation that 17.9 million of people died from one of these diseases, which represents 31% of all worldwide deaths [**WHO 2017**]. Diseases such as atherosclerosis, hypertension, hyperlipidemia, or diabetes are bonded to various structural and functional changes in arteries, e.g., endothelial dysfunction, vascular remodeling, altered contractility, increased arterial stiffness, inflammation, and calcification [**Hussein et al. 2005; Harvey et al. 2015**].

There is evidence, that free astaxanthin possess the ability to clear the ROS within the cells and increase the nitric oxide (NO) utilization. Moreover, it reduces the oxidative stress, as well as high blood pressure and inflammation [**Guerin et al. 2003; Ohgami et al. 2003; Hussein et al. 2006; Pashkow et al. 2008; Xuan et al. 2016**]. Moreover, the astaxanthin esters also possess the singlet oxygen quenching. The combination of these properties predetermines this compound to effectively increase vasodilator potency. Vasodilatation allows relaxing the smooth muscle within the vessels, therefore enabling their dilatation that leads to the resistance reduction by decreasing the blood pressure [**Chaudhry et al. 2020**]**.**

In our work, the vasorelaxant potency of the astaxanthin and HPCCC isolated fractions enriched in astaxanthin monoesters and diesters were tested using the thoracic aortas of rats. The noradrenaline (NE) was used as the contractor.

1.3.5 Inhibition of tyrosinase activity

Natural light-absorbing pigment melanin is produced in the skin in the organelles called melanosomes (that are synthesized in the melanocyte cells) as a combination of red–yellow phaeomelanin and brown–black eumelanin [**Hearing and Tsukamoto 1991**]. This pigment is derived from the amino acid tyrosine and plays a major role in the skin photoprotection against harmful ultraviolet radiation and can absorb the toxic compounds and chemicals. Skin color depends on the melanin amount, size, and type. As previously stated, the melanosomes are synthesized in the melanocytes that are dendritic cells located closely to the epidermal keratocytes. The process of melanin biosynthesis is called the melanogenesis, where the copper-containing oxidase, tyrosinase, plays a key role. First, the tyrosinase enzyme catalyzes the hydroxylation of tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA) and then the oxidation of L-DOPA to L-dopaquinone. Second, the DOPAchrome tautomerase isomerizes the pigmented intermediate DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA). The tyrosinase related protein 2 (TRP 2) also catalyzes this transformation since it does not possess any other catalytic effect towards the tyrosinase. The tyrosinase related protein 1 (TRP 1) oxidizes the carboxylic acid to indolequinone. To sum up, the tyrosinase enzyme is responsible for the biosynthesis of melanin and its inhibitors are used both in pharmaceutical (e.g., treatment of hyperpigmentation) and cosmetic sectors (e.g., skin lightening agents) [**Tsukamoto et al. 1992; Lim et al. 2009; Moon et al. 2010; Rao et al. 2013b**; **Hendrychová 2015**].

Skin lightening is a practice aiming to enlighten a healthy skin recognized from the antiquity. This practice was given various local names based on the region incidence, mainly in sub-Saharan Africa. The reasons to achieve as white skin as possible vary from the removal of pathological stains to the wish to have different skin color due to the racial issues. The techniques to achieve bleached skin changed across the time (from the natron in Mesopotamia/Egypt from the fourth millennium BCE onwards to the solutions of heavy metals in Europe in 16th century). Nowadays, they include the use of not only certified cosmetics but also cosmetics composed of ingredients/substances that may come from various sources with not always known origin and being potentially harmful or even illegal [**Petit 2019**]. The skin whitening agents approved for human use can be divided based on the depigmentation pathways: tyrosinase inhibition (e.g., hydroquinone; kojic acid; resveratrol), inhibition of tyrosinase activation (e.g., retinol; tretinoin), inhibition of melanosome relocation (linoleic acid), epidermal turnover accelerant (e.g., vitamin C, vitamin E, lactic acid) and free radical trapping agents (e.g., topical steroids) [**Couteau and Coiffard 2016**]. Several plant species have been already examined for the presence of these agents, such as *Aloe ferrox* [**Lynch et al. 2011**], *Morus alba* [**Yang et al. 2012**], *Phyla nodiflora* [**Ko et al. 2014**] or *Populus nigra* [**Maack et al. 2016**]. The enzyme tyrosinase catalyzes the rate-limiting step of the process of pigmentation; therefore, its inhibition is applied as the most conventional method to get the skin hypopigmentation/depigmentation [**Solano et al. 2006**]. Literature reports also studies focused on the examination of the tyrosinase inhibitory activity of the marine algae. Fucoxanthin isolated from *Laminaria japonica* showed the ability to repress the activity of tyrosinase in UVB-irradiated guinea pigs as well as melanogenesis in UVB-irradiated mice [**Shimoda et al. 2010**].

Also, the extracts from *Sargassum polycystum* and *Padina tenuis* significantly decreased the activity of fungal tyrosinase as well as the content of melanin in human epidermal melanocytes [**Quah et al. 2014**]. Two of bromophenols that are present in *Symphyocladia latiuscula* exhibited a significant competitive inhibition of tyrosinase activity against L-tyrosine substrates and a moderate activity against L-DOPA [**Paudel et al. 2019**]. Fucofuroeckol-A isolated from *Eisenia bicyclis* served as a non-competitive tyrosinase inhibitor as well as it demonstrated a significant inhibitory effect on the 3-isobutyl-1-methylxanthine-induced synthesis of melanin in the murine melanoma B16F10 cells [**Bo Shim and Young Yoon 2018**]. Regarding the freshwater algae, there is an evidence that astaxanthin monoesters and diesters obtained from *Haematococcus pluvialis* possess a potent, dose-dependent tyrosinase inhibitory effect in serum and skin homogenates of UV−7,12-dimethylbenz(a)anthracene (DMBA)-induced skin cancer in rats. Regarding the mechanism of action, both astaxanthin monoesters and diesteres demonstrated the inhibition on L-DOPA oxidase activity of the tyrosinase enzyme. The efficacy of anti-tyrosinase activity is represented as astaxanthin diesters > astaxanthin monoesters > free astaxanthin [**Rao et al. 2013b**]. Also, the astaxanthin extract from the shells of shrimp *Litopenaeus vannamei* manifested a strong tyrosinase inhibition in a dose-dependent manner (3–50 μg/mL) in human dermal fibroblast cell [**Chintong et al. 2019**]. In our work, the influence of astaxanthin extract from *Haematococcus pluvialis* plus fractions enriched in astaxanthin monoesters and diesters was evaluated in a relation with tyrosinase activity. The activity of fungal tyrosinase was measured spectrophotometrically using L-DOPA as a substrate according to Jiménez et al. (2001) [**Jiménez et al. 2001**]. Later, the cell viability test was assessed on B16-F10 mouse melanoma cells using a resazurin dye as described earlier [**Präbst et al. 2017**]. Finally, melanin content was measured colorimetrically as described previously [**Gruber and Holtz 2013**].

1.3.6 Antiparasitic activity

Mesocestoides vogae are cestode tapeworms belonging to the family Mesocestoididae. Metacestode stage –tetrathyridia reproduce asexually, in the liver and peritoneal (body) cavity of various hosts (i.e., rodents, reptiles, carnivores), possessing the ability to further migrate into the lungs and kidneys [**Specht and Voge 1965; Etges 1991; Padgett and Boyce 2004**]. Larvae undergo encapsulation by the host's effector systems in the parenchymal organs (e.g., liver). Larval tetrathyridia migrate from peritoneal cavity and damage the blood supply in organs, therefore the early stages of infection cause the parenchymal destruction of the host's liver. The encapsulation and parenchymal damage results in developing of fibrosis, what is hallmark of this disease. In the late stages of infection, the massive proliferation of tetrathyridia occurs in the peritoneal cavity of mice [**Specht and Widmer 1972**]. The most important nutrients for the metabolism of cestode tapeworms are glucose and amino acids [**Pappas and Read 1975**].

Regarding the anatomy of tetrathyridia, they are covered by soft ciliated tegument, parenchyma, haustorium, muscles, nervous and excretory systems. The mechanism of the larvae movement is assured by the muscles composed of three layers, allowing them to use the circular, diagonal, and longitudinal motion [**Terenina et al. 1999**].

The World's Health Organization (WHO) claims that overall, more than 1.5 billion people (24% of the whole world's population) are being infected with soil-transmitted helminth infections and millions of people harbor flatworm infections caused by cestode of the genus *Echinococcus*, *Schistosoma*, and other medically important species. These helminths are responsible for worsening the human health, which can lead to death.

The WHO recommends the chemical treatment involving the use of albendazole (400 mg), mebendazole (500 mg) and ivermectin [**www.who.int/news-room/fact-sheets/detail/soil-transmitted-helminth-infections**]. However, there is risk of anthelminthic resistance [**Geerts et al. 1997; Geerts and Gryseels 2002**] as demonstrated by the possibility of drug resistance against the commonly used drugs [**Mondragón-Ancelmo et al. 2019**]. Therefore, the identification of the novel sources of anthelmintic agents is crucial.

Secondary metabolites of higher plants are well-known source of molecules that possess the anthelmintic activity [**Athanasiadou et al. 2007**], some of them being well-known antioxidants [**Abdel-Mageed et al. 2012; Graziose et al. 2012**]. The methanolic extract from *Abutilon indicum* leaf demonstrated the anthelmintic activity *in vitro* against the sheep tapeworm *Moniezia expansa*. The GC-MS analysis revealed its composition, presenting several bio-active compounds, including astaxanthin among others [**Thooyavan et al. 2018**]. In the literature, there are reports demonstrating that astaxanthin may have antiparasitic effect. The *in vitro* study [**Contreras-Ortiz et al. 2017**] showed that astaxanthin can decrease the viability of *Trypanosoma cruzi* trypomastigote up to death in a dose-dependent manner [**Contreras-Ortiz et al. 2017**]. The anthelmintic properties of algae, as well as of their derivatives, have been also widely investigated [**Davyt et al. 1998; Davyt et al. 2001; Gnana Selvi et al. 2016; Taki et al. 2020**].

In our work the effect of *Haematococcus pluvialis* crude extract together with the fractions enriched in astaxanthin monoesters and diesters was determined over the natural parasite of mice – the tapeworm *Mesocestoides vogae*. The larvae have been obtained aseptically from the abdominal cavity of mice following lavage with saline and further processed. The following tests were performed: MTT reduction assay, Neutral red assay, superoxide dismutase (SOD) inhibition assay and the evaluation of glutathione-S-transferase (GST) activity.

1.3.7 Cytotoxic activity

According to the WHO, cancer is a large variety of illnesses characterized by irregular proliferation of abnormal cells that can occur almost in any organ or body tissue. Later, the cell growth expands into the circumjacent organs; cancer cells metastasize and form secondary tumors most commonly causing death. In 2018, cancer was responsible for the death of about 9.6 million people, becoming the second leading cause of death globally [**The World Health Organization 2021**]. In 2018, Global Cancer Observatory (GLOBOCAN) reported approximately 18.1 million of new cases of cancer, while they are reported approximately 19.3 million of its new cases in 2020 [**Bray et al. 2018; Global Cancer Observatory 2020**]. Several factors are involved in the spreading of cancer, including aging, population growth, changes of prevalence or distribution of the main risk factors [**Omran 1971; Gersten and Wilmoth 2002**]. Some studies conducted in the high-income countries have shown that expulsion/diminution of lifestyle and environmental risk factors may be crucial in reducing the risk of cancer development [**Brown et al. 2018; Islami et al. 2018; Wilson et al. 2018**]. Nowadays, cancer mortality as well as its incidence are growing rapidly. The cumulative risk of incidence presumes that 1 in 8 men and 1 in 10 women will develop this disease during their lifetime [**Bray et al. 2018**].

To sum up, cancer is one of the most life-threatening illnesses that requires an appropriate treatment. Standard therapy involves the use of chemotherapy or radiotherapy that has a wide range of side effects (e.g., cardiac, neurological, renal, or pulmonary toxicity) [**Roy et al. 2017**]. Therefore, the scientific community focuses on searching for less toxic and more potent approaches to deal with this disease. Natural products represent one of the possibilities since three quarters of antitumor compounds used in medicine are associated with them [**Demain and Vaishnav 2011**].

Some of the microalgae compounds have been already associated with the cytotoxic response against several cancer cell lines [**Shanab et al. 2012; Lin et al. 2017**]. One of them, astaxanthin, may play a key role in cancer therapy because of its several pathways how to cope with this disease [**Zhang and Wang 2015; Faraone et al. 2020**]. Various studies have suggested that astaxanthin possess the anti-cancer effect in several types of cancer, e.g., leukemia [**Zhang et al. 2011**], hepatocellular carcinoma [**Song et al. 2011; Song et al. 2012**], oral cancer [**Kavitha et al. 2013**], bladder carcinogenesis [**Tanaka et al. 1994**], lung cancer [**Ramamoorthy et al. 2020**], colon carcinogenesis [**Prabhu et al. 2009; Nagendraprabhu and Sudhandiran 2011; Yasui et al. 2011**], mammary tumors/breast cancer [**Nakao et al. 2010; Ahn et al. 2020**] or skin cancer [**Rao et al. 2013b; Davinelli et al. 2018; Catanzaro et al. 2020**]. Astaxanthin dietary supplementation has significant impact on reduction of cancer development in comparison with astaxanthin supplementation after the tumor induction suggesting that the adequate astaxanthin/antioxidant status before the disease initiation may play role in its development [**Prabhu et al. 2009; Nakao et al. 2010**]. However, only the free form of astaxanthin was evaluated in these studies. Astaxanthin present in microalgae *Haematococcus pluvialis* is present mainly in the form of esters, esterified with different fatty acids [**Holtin et al. 2009; Régnier et al. 2015**]. One of the fatty acids that esterifies astaxanthin monoesters from microalgae *Haematococcus pluvialis* is oleic acid, which is believed to play an important role in cancer prevention [**Carrillo et al. 2012**]. In our work, the cytotoxic activity of astaxanthin and its esters were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.

1.3.8 Anti-allergic activity

The number of humans suffering from allergic diseases continues increasing with estimation of 10 – 20% of worldwide population being already affected. Therefore, these inflammatory disorders present a clinical health issue requiring new approaches to cope with them [**Ring et al. 2014; Do et al. 2017; Appel et al. 2018**]. Nowadays, the most prescribed therapy against the inflammatory allergy is the use of non-steroidal anti-inflammatory drugs (NSAIDs). It is estimated that 30% of all hospital admissions due to the preventable adverse drug reactions are due to the NSAIDs administration, which got an impact on several systems (cardiovascular, respiratory, gastrointestinal, and renal). These drugs increase the incidence possibility of health threats such as a risk of stroke, heart attack, increased bleeding, gastrointestinal bleeding, bronchospasms, or renal damage [**Pirmohamed et al. 2004; Lemanske and Busse 2006; Bhala et al. 2013; Davis and Robson 2016**]. Therefore, to discover les harmful novel agents with a possible use in the medical treatment is very important.

The rat basophilic leukemia (RBL) 2H3 histamine-releasing mast cell line is commonly used to study the allergy. On their surface, the mast cells typically express the high-affinity receptor for Immunoglobulin E (IgE) (FceRI) that plays a key role in the development of the allergic reaction. To develop this reaction, several procedures must occur. In the beginning, the allergen must interact with specific IgE antibodies that are bonded to FceRI. The receptor is activated after the IgE antigen is bonded with FceRI. Then, the complex biological reactions arise leading to the allergic reactions (e.g., inflammatory disorder) [**Bochner and Schleimer 2001; Appel et al. 2018; Guo et al. 2018**].

Therefore, the allergic inflammation can be identified by IgE-dependent activation of mucosal mast cells. Moreover, it can be also defined by the infiltration of eosinophils arranged by elevated numbers of activated CD4+ T helper type 2 (Th2) lymphocytes [**Kubo et al. 2017**]. Mast cells seem to have a key role in the immediate response to antigen, because tryptase, histamine and prostaglandin 2 (PGD2) belong amongst their product and they were immediately released after the antigen bronchoprovocation [**Schwartz 1985; Holgate et al. 1986**].

In literature, there is evidence that some plant species may possess anti-allergic properties. Tewtrakul and Subhadhirasakul (2007) demonstrated the anti-allergic effect of *Kaempferia parviflora* and *Zingiber cassumunar* against RBL-2H3 cell line [**Tewtrakul and Subhadhirasakul 2007**]. Also, *Boesenbergia thorelii* appeared to have a potential use in the allergy treatment [**Madaka and Tewtrakul 2011**]. Bak et al. (2011) examined 164 plant species against RBL-2H3 cell line. The highest inhibitory activity of degranulation was found in *Lactuca indica* var. laciniata. Several others showed the inhibitory activity of degranulation higher than 50% at 125 μg/mL [**Bak et al. 2011**]. Recently, algae have gained more attention from the scientific community. Kim et al. (2020**b**) demonstrated that mojabanchromanol isolated from *Sargassum horneri* possess some anti-allergic properties against the IgE/BSA-stimulated bone marrow-derived cultured mast cells (BMCMCs) [**Kim et al. 2020b**]. It was discovered that the *Scytosiphon* sp. extract, possessing high concentration of polyphenols, has higher anti-allergic activity than commonly used drug disodium cromoglycate (DSCG) (IC_{50} = 1.13 mg/mL) [**Chen et al. 2015**].

Marine algae are known for possessing various active agents from the group of phlorotannins (e.g., phlorofucofuroeckol A; phlorofucofuroeckol B; dieckol; 6,6 -bieckol; 6,8 -bieckol; 8,8 -bieckol), carotenoids (e.g., α-carotene, β-carotene, astaxanthin, zeaxanthin, fucoxanthin, α-linolenic acid, β-linolenic acid, and docosahexaenoic acid), polysaccharides (e.g., alginic acid, porphyran, fucoidan), polyunsaturated fatty acids and phycocyanins [**Vo et al. 2012**]. Astaxanthin was later confirmed to possess the anti-allergic properties in dinitrofluorobenzene-induced contact dermatitis mice model and RBL-2H3 cell lines [**Kim et al. 2015**]. Esterified forms of astaxanthin from freshwater microalgae *Haematococcus pluvialis* exhibit higher anti-inflammatory activity than non-esterified astaxanthin. Moreover, astaxanthin monoesters possess higher anti-inflammatory activity than astaxanthin diesters in B16F10 mice melanoma cells and HaCaT human keratinocyte cells [**Hwang et al. 2020**].

In our work, the effect of *Haematococcus pluvialis* extract and the fractions enriched in astaxanthin monoesters and diesters were tested for their ability to inhibit the mast cell degranulation, which was assessed by A23187-induced or antigen-induced β-hexosaminidase release in RBL-2H3 cells.

Objectives

- To develop and optimize an efficient lutein extraction method using non-toxic solvents.
- To develop, optimize and apply an efficient and scalable high performance countercurrent chromatography method to isolate lutein from the microalgae *Chlorella vulgaris* and *Parachlorella kessleri*.
- To develop, optimize and apply an efficient and scalable high performance countercurrent chromatography method to isolate astaxanthin esters from the microalgae *Haematococcus pluvialis*.
- To evaluate the immunomodulatory, antioxidant, antiaggregant, vasodilatory, antiparasitic, cytotoxic and anti-allergic activity as well as the capacity to inhibit the tyrosinase activity and melanin production of the *Haematococcus pluvialis* extract and the fractions enriched in astaxanthin monoesters and diesters isolated using HPCCC.
- To evaluate the antioxidant and cytotoxic activity of the five astaxanthin monoesters isolated by HPCCC.

Comments on the publications

1.4 Purification of lutein from the green microalgae *Chlorella vulgaris* by integrated use of a new extraction protocol and a multi-injection high performance counter-current chromatography (HPCCC)

Fábryová, T.; Cheel, J.; Kubáč, D.; Hrouzek, P.; Vu, D.L.; Tůmová, L.; Kopecký, J.: Purification of lutein from the green microalgae *Chlorella vulgaris* by integrated use of a new extraction protocol and a multi-injection high performance counter-current chromatography (HPCCC). *Algal Research*, **2019**, vol. 41, 101574.

Lutein is a yellow xanthophyll dye belonging to the class of carotenoids with a high potency in the treatment of eye diseases as well as several chronic diseases such as ischemic heart disease or lung cancer. Up to date, its major source is the marigold flowers, whose cultivation cope with several inconveniences (e.g., planting area, seasonal changes, high costs, purification); therefore, it is challenging to find an alternative source. In these circumstances, microalgae appear to be a good option and *Chlorella* sp. has previously demonstrated high lutein production.

In this work, the HPCCC was used to isolate this pigment from the microalgae *Chlorella vulgaris*. This microalgae species was heterotrophically cultivated in a fermenter under stable conditions for 5 days. An amount of 24 Kg of dried disintegrated biomass of *Chlorella vulgaris* was then obtained.

Selected based on its physico-chemical properties, the biphasic solvent system composed of *n*-heptane-ethanol-water (5:4:1.5, *v/v/v*) was used for both the microalgal biomass extraction and further lutein isolation. Several extraction methods and solvents were compared and finally, the ultrasound assisted extraction of microalgae biomass with the LP of the selected biphasic solvent system for 30 min was selected for the biomass extraction prior to the lutein HPCCC isolation. An amount of 30 g of *Chlorella vulgaris* biomass was extracted with 600 mL (ratio of solvent volume to biomass was 20 mL/g) of the LP of the selected biphasic system yielding 2.7 g of dried extract, which was subsequently used for the HPCCC isolation of lutein. To maximize the productivity of the process, a multi-injection HPCCC isolation method was developed and applied. LP4 (mobile phase) was pumped at a flow rate of 8 mL/min. Finally, 2 g of *Chlorella vulgaris* extract were processed affording 60 mg of lutein (92% purity). The resulting fraction was further cleaned up by gel permeation chromatography with a yield of 50 mg of lutein (97% purity). The chemical identity of the purified compound was confirmed by mass spectrometry and ultraviolet and visible (UV-Vis) absorption spectroscopy in comparison with an authentic standard.

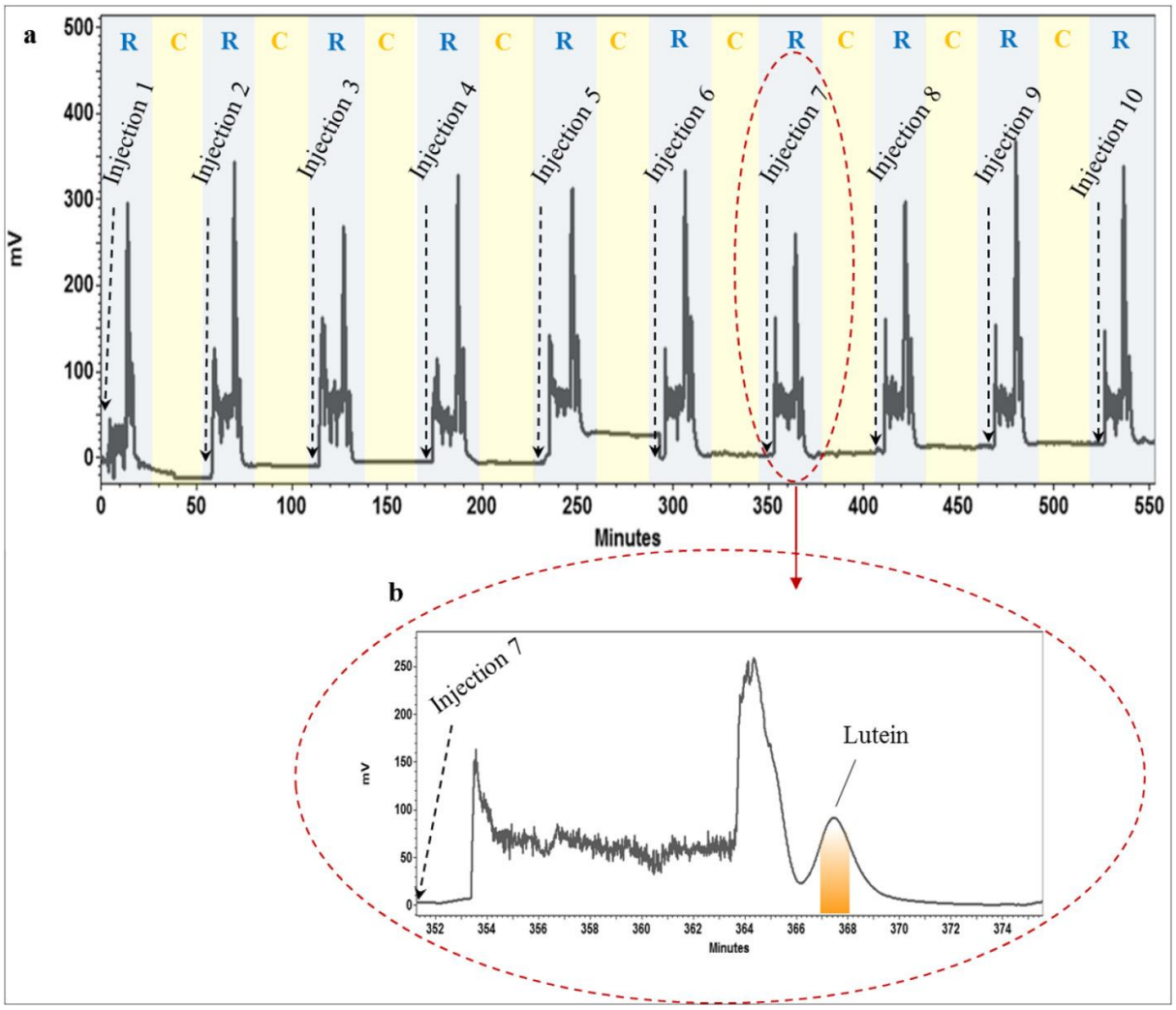


Fig. 3. Isolation of lutein by a multi-injection HPCCC method (a). Elution modes: Reverse (R) and co-current (C) elution modes. Loading per injection: 200 mg of *Chlorella* extract. Runs: 10 consecutive injections. Rotational speed: 1200 rpm. Column temperature: 28 °C. Detection: 440 nm. Representative extension of the chromatographic cycle 7 (**b**).

1.5 Isolation of astaxanthin monoesters from the microalgae *Haematococcus pluvialis* by high performance countercurrent chromatography (HPCCC) combined with high performance liquid chromatography (HPLC)

Fábryová, T.; Tůmová, L.; Correia da Silva, D.; Pereira, D.M.; Andrade, P.B.; Valentão, P.; Hrouzek, P.; Kopecký, J.; Cheel J.: Isolation of astaxanthin monoesters from the microalgae *Haematococcus pluvialis* by high performance countercurrent chromatography (HPCCC) combined with high performance liquid chromatography (HPLC). *Algal Research*, **2020**, vol. 49, 101947.

Astaxanthin is the most powerful antioxidant found in nature and possess several other beneficial bioactivities. Its major natural producer is the microalgae *Haematococcus pluvialis*, in which this compound is being found mainly in the form of esters. However, these esters have not been yet extensively valorized. Also, to diversify the commercial offer of these valuable astaxanthins, a scalable and efficient isolation technology is required.

Five astaxanthin monoesters were isolated from *Haematococcus pluvialis* using the HPCCC technology. Several biphasic solvent systems were developed and their physicochemical properties of were evaluated to select the more suitable system for HPCCC isolation of astaxanthins. The LP and upper phase (UP) of a selected solvent system (*n*-heptane:acetonitrile, ratio 5:5, *v/v*) served as a mobile and stationary phases, respectively. Commercial biomass of *Haematococcus pluvialis* (4 g) was extracted with 0.250 L of the mixture of ethanol and ethyl acetate (ratio 1:1, *v/v*) yielding 1.2 g of crude extract.

An amount of 200 mg of the biomass extract was subjected to the HPCCC allowing a separation of the target astaxanthin esters. A multi-injection HPCCC method was subsequently developed by combining two elution modes (reverse phase and co-current) to increase the process productivity. In co-current elution mode, both mobile and stationary phases were pumped at the same time using a quaternary pump at flow rates of 3 and 1 mL/min, respectively. Therefore, the stationary phase that got normally lost during each separation cycle was refilled. In total, five injections of samples (200 mg of extract, each) were achieved. Final purification of the HPCCC fractions was performed with HPLC affording five astaxanthin derivatives esterified with α-linolenic acid (4 mg), linoleic acid (8 mg), palmitic acid (8 mg), oleic acid (12 mg) and stearic acid (1 mg) with purities of 98%, as determined by HPLC analysis. Only astaxanthin esterified with oleic acid exhibited a cytotoxic effect against human gastric cancer cells (AGS cell line). The results demonstrated a useful approach for obtaining individual astaxanthin esters from *Haematococcus pluvialis*, confirming the high separation efficiency of the HPCCC. Since astaxanthin esters appear to differ in their effect over cancer cells, the concept of diversification of the astaxanthin market remains open.

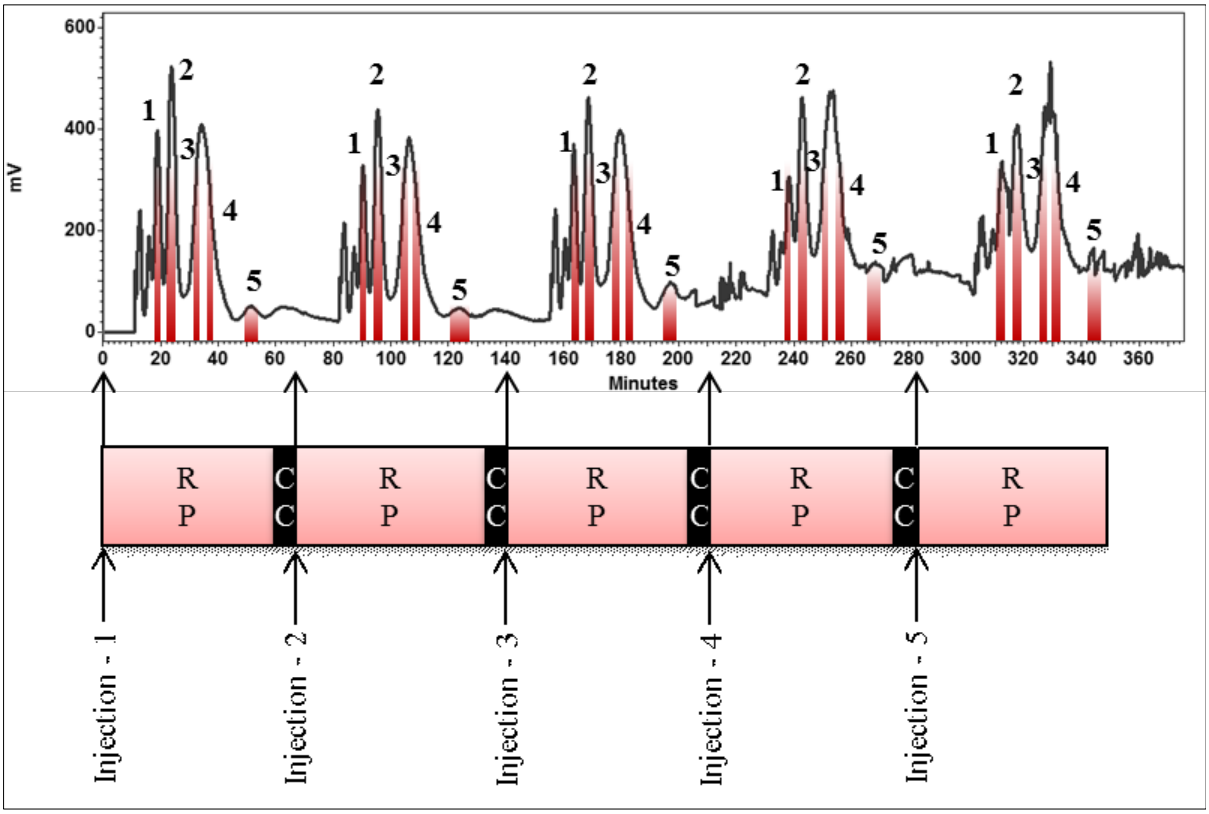


Fig. **4**: Multi-injection HPCCC method for obtaining astaxanthin monoesters from *Haematococcus pluvialis* biomass. **1**: astaxanthin-C18:3 (*α*-linolenic acid). **2**: astaxanthin-C18:2 (linoleic acid). **3**: astaxanthin-C16:0 (palmitic acid). **4**: astaxanthin-C18:1 (oleic acid). **5**: astaxanthin-C18:0 (stearic acid). System 1: mixture of *n*-heptane and acetonitrile (ratio 1:1, *v/v*). Elution modes: Reverse (R) and co-current (C). Loading per injection: 200 mg of biomass extract. Runs: 5 consecutive injections. Flow rate: 4 mL/min. Rotational speed: 1200 rpm. Column temperature: 28 °C. Detection: 480 nm. Elution modes: RP, reverse phase; CC, co-current.

1.6 Separation of the glycosylated carotenoid myxoxanthophyll from *Synechocystis salina* by HPCCC and evaluation of its antioxidant, tyrosinase inhibitory and immune-stimulating properties

Nováková, M.; Fábryová, T.; Vokurková, D.; Dolečková, I.; Kopecký, J.; Hrouzek, P.; Tůmová, L.; Cheel J.: Separation of the glycosylated carotenoid myxoxanthophyll from *Synechocystis salina* by HPCCC and evaluation of its antioxidant, tyrosinase inhibitory and immune-stimulating properties. *Separations*, **2020**, vol. 7, 73.

Global demand for pigments and raising awareness about the potential risk of consuming their synthetic versions contribute to the search and preferences for novel promising natural pigments. Myxoxanthophyll is a natural carotenoid rarely found in nature and barely examined. Although it was proven to possess antioxidant and anti-hyperglycemic properties, no scalable isolation technology for obtaining this compound has not been yet developed.

Several biphasic solvent systems were developed, and their physicochemical properties were evaluated to determine their capacity to be used in the HPCCC isolation of myxoxanthophyll. The LP and UP of a selected solvent system (*n*-heptane:ethanol:water, ratio 2:4:4, *v/v/v*) served as a mobile and stationary phases, respectively. An amount of 40 g of *Synechocystis salina* biomass was extracted with LP using the ultrasound-assisted extraction for 30 min yielding 4.736 g of the crude extract. Some of 70 mg of the biomass extract was injected to the HPCCC allowing a separation of myxoxanthophyll.

To increase the productivity of the HPCCC separation process, a multi-injection HPCCC method was developed by combining two elution modes (reverse phase and co-current). In co-current elution mode, both mobile and stationary phases were pumped at the same time using a quaternary pump at flow rates of 2.5 and 1.5 mL/min respectively. Therefore, the stationary phase that got normally lost during each separation cycle was refilled. In total, four injections of samples (70 mg of extract, each) were achieved, yielding 20 mg of myxoxanthophyll, which was finally purified with HPLC. A final myxoxanthophyll yield obtained was of 15 mg (98% purity).

The antioxidant and tyrosinase activity of myxoxanthophyll were tested at the concentration range from 0 to 200 μM. Regarding its capacity to scavenge free radicals, myxoxanthophyll showed moderate dose dependent effect on the free radical DPPH. It also showed a mild dose non-dependent effect on the tyrosinase inhibition. Its immunomodulatory activity was tested at the concentration range from 20 to 60 μM. The potential activation of immune cells (CD69+) was measured by flow cytometry, however except granulocytes, no immune cells were activated. The response of granulocytes increased demonstrating a stimulation index of 2.37 (20 μM) and 2.77 (60 μM).

To conclude, these results will serve as a keystone for a myxoxanthophyll large-scale production model. Although this compound showed only weak antioxidant and tyrosinase activities, it demonstrated immune-stimulating properties by the activation of human granulocytes. Therefore, myxoxanthophyll shows potential benefits to human health, and more research should be performed on this topic.

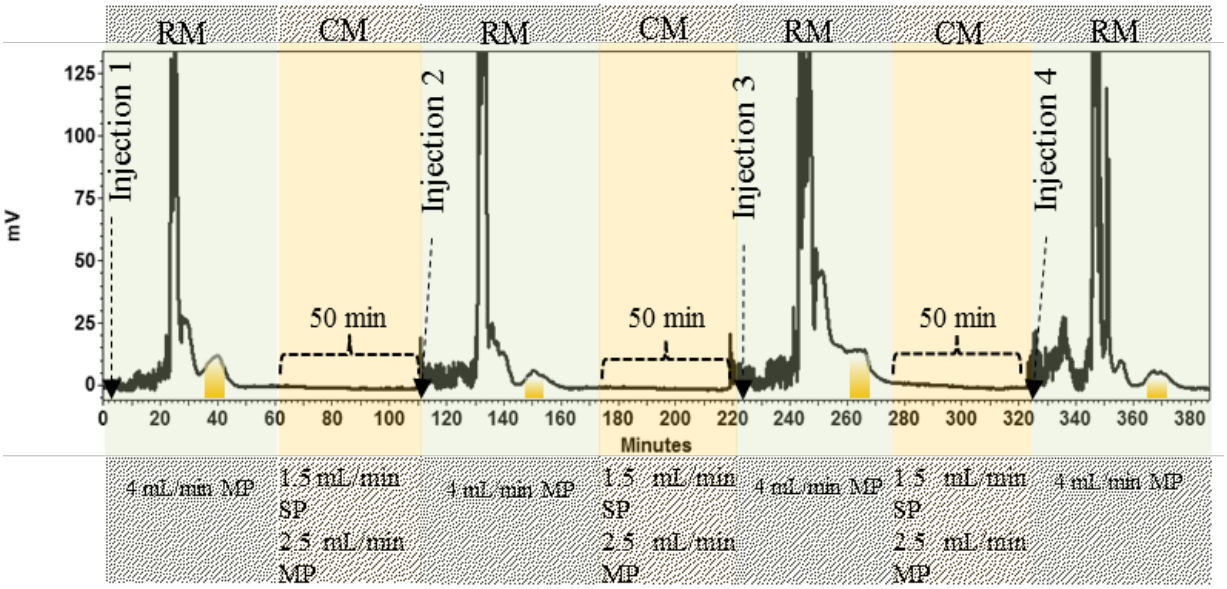


Fig. 5. Isolation of myxoxanthophyll by a multi-injection HPCCC method. Biphasic solvent system: System 7 (*n*-Hep:EtOH:H_2O, 2:4:4, *v/v/v*). Elution modes: Reverse (RM) and co-current (CM) elution modes. Loading per injection: 70 mg of *Synechocystis salina* extract. Runs: 4 consecutive injections. MP: mobile phase (LP of system 7). SP: stationary phase (UP of system 7). Rotational speed: 1400 rpm. Column temperature: 28 °C. Detection 478 nm.

1.7 High-performance countercurrent chromatography for lutein production from a chlorophyll-deficient strain of the microalgae *Parachlorella kessleri* HY1

Fábryová, T.; Kubáč, D.; Kuzma, M.; Hrouzek, P.; Kopecký, J.; Tůmová, L.; Cheel, J.: High-performance countercurrent chromatography for lutein production from a chlorophyll-deficient strain of the microalgae *Parachlorella kessleri* HY1. *Journal of Applied Phycology*, **2021**.

In this study, we presented a novelty in the field of lutein isolation using HPCCC. It is well-known that green microalgae are a recognized lutein source; however, their use requires additional processing (e.g., saponification, purification). To avoid these steps, the chlorophyll deficient *Parachlorella kessleri* HY1 strain was used in this work.

A variety of biphasic solvent systems were developed, and their physicochemical properties were evaluated to select the best system based on their capacity to be employed in the HPCCC isolation of lutein. The LP and UP of a selected solvent system (*n*-heptane:ethanol:water, 5:4:1.5, *v/v/v*) served as a mobile and stationary phases, respectively. Moreover, the LP was used both as a solvent for *Parachlorella kessleri* HY1 strain biomass extraction and as a mobile phase for lutein isolation using the HPCCC technology. Several extraction methods and solvents were compared. The maceration extraction of microalgae biomass with the LP of the selected biphasic solvent system for 30 min (lutein yield of 6.71 mg/g) was selected for the biomass extraction prior to the lutein HPCCC isolation.

Some 36 g of *Parachlorella kessleri* HY1 biomass was extracted with 720 mL of LP of the selected biphasic solvent system using the maceration extraction process for 30 min yielding 3.96 g of the crude extract. An amount of 300 mg of the biomass extract was injected to the HPCCC allowing the separation of lutein. To increase the productivity of the separation process, a multi-injection HPCCC method was developed by combining two elution modes (reverse phase and co-current). In the co-current elution mode, both mobile and stationary phases were pumped at the same time using a quaternary pump at flow rates of 6 and 2 mL/min, respectively. Therefore, the stationary phase that got normally lost during each separation cycle was replenished. Additionally, to make this whole process economically feasible and environmentally friendly, the HPCCC mobile and stationary phases were independently formulated based on nuclear magnetic resonance analyses. This approach allowed us to prepare both phases separately, which helped us to reduce the overall consumption of organic solvents and the isolation run time. In total, ten injections of samples (300 mg of extract, each) were achieved, yielding 150 mg of lutein (98% purity, 97% recovery).

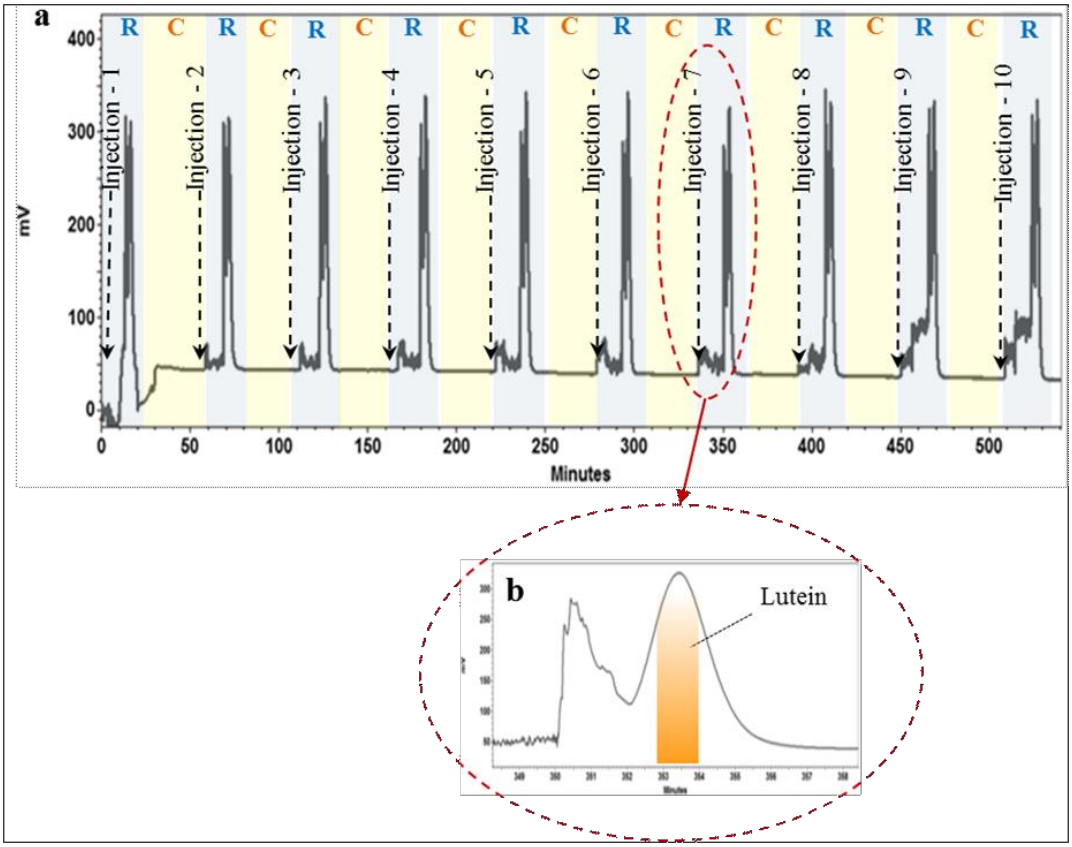


Fig. 6. Isolation of lutein by a multi-injection HPCCC method (**a**). Elution modes: Reverse (R) and co-current (C) elution modes. Loading per injection: 300 mg of *Chlorella* extract. Runs: 10 consecutive injections. Rotational speed: 1200 rpm. Column temperature: 28 °C. Detection 440 nm. Representative extension of the chromatographic cycle 7 (**b**).

1.8 Biological evaluation of *Haematococcus pluvialis* esters isolated using the high-performance counter-current chromatography (HPCCC)

Fábryová, T.; Vokurková, D.; Hrčková, G.; Dolečková, I.; Kořinek, M.; Mladěnka, P.; Applová, L.; Migkos, T.; Correia da Silva, D.; Pereira, D.M.; Andrade, P.B.; Valentão, P.; Hrouzek, P.; Kopecký, J.; Tůmová, L.; Cheel, J.: Manuscript in preparation.

The reddish ketocarotenoid astaxanthin is being recognized as the most powerful antioxidant found in nature. This compound is also responsible for fish and crustacean pigmentation and possesses a high potential to be used against a wide range of human diseases. Major natural producer of astaxanthin is the green microalgae *Haematococcus pluvialis*, in which this compound is found mainly in the form of esters. This article focuses on the biological evaluation of astaxanthin extract from *Haematococcus pluvialis* and fractions enriched in astaxanthin monoesters and diesters. The article will disclose the influence of the esterification type either as mono- or di- esters on the biological properties. This information will also contribute to the commercial diversification of these compounds.

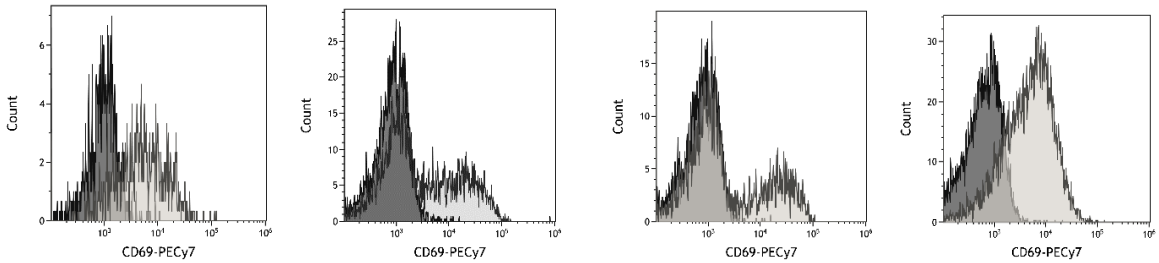
Antioxidant and immunomodulatory activities

Astaxanthin is known for its immune-stimulating properties. There are several investigations proving its immunomodulatory effects. This carotenoid enhances the physiological interferon (IFN) γ and T helper cytokins (i.e., interleukin 2) secretion in primary cultured lymphocytes both *in vitro* and *ex vivo* [**Lin et al. 2016**]. Recently, the astaxanthin was proved to immunomodulate several apoptotic approaches in hormonal receptor positive MCF-7 breast cancer cells [**Fouad et al. 2021**].

The immune cell activation was determined by the expression of cell surface glycoprotein CD69 (cluster of differentiation 69), as measured by flow cytometry. The response to tested substances was shown as a shift to the right in the representative histograms (**Fig. 7**), however no immune cells tested were activated.

Positive control-phytohemagglutinin (PHA)

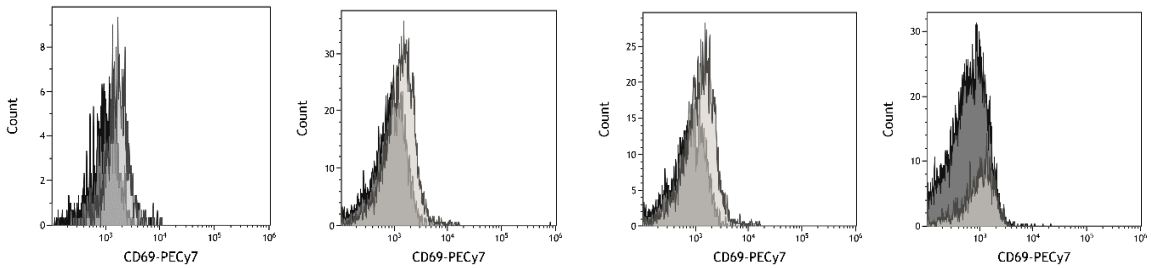
NK cells Lymphocytes T-lymphocytes Granulocytes



(a)

Negative control

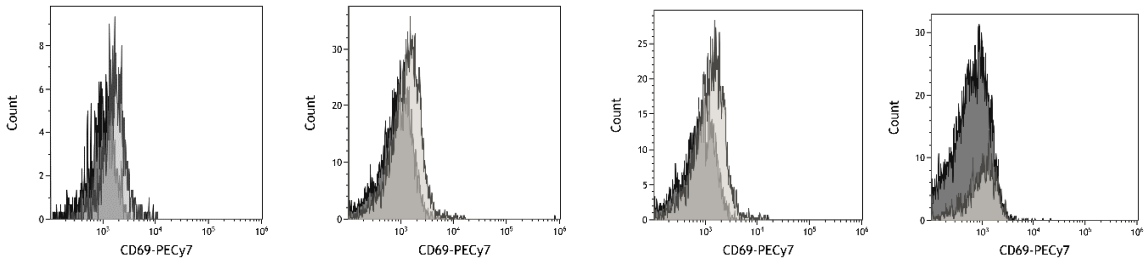
NK cells Lymphocytes T-lymphocytes Granulocytes



(b)

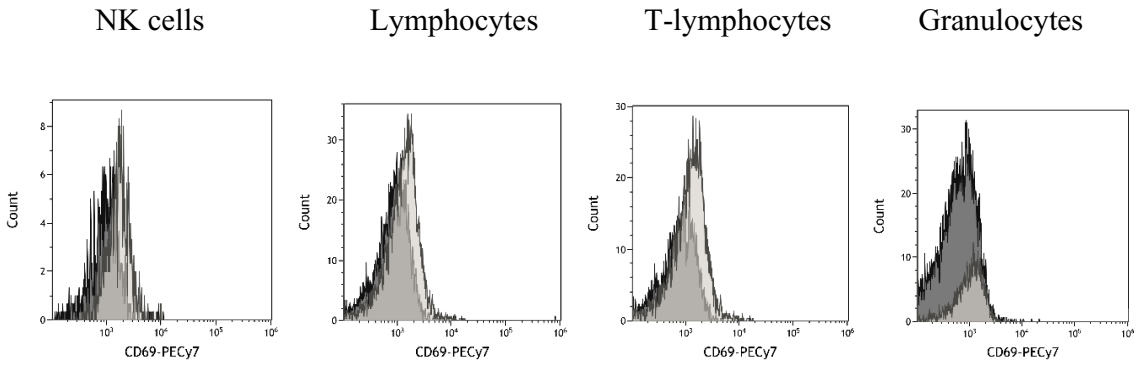
DMSO (4%)

NK cells Lymphocytes T-lymphocytes Granulocytes



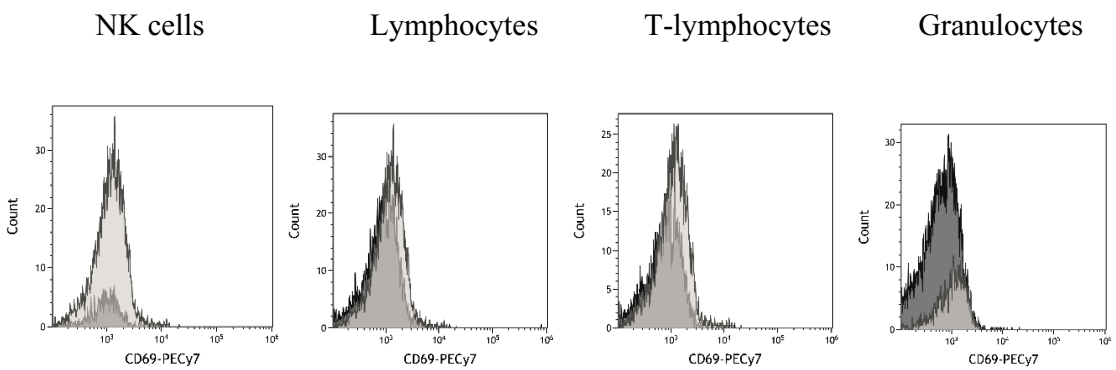
(c)

***Haematococcus pluvialis* extract (200 µg/mL)**



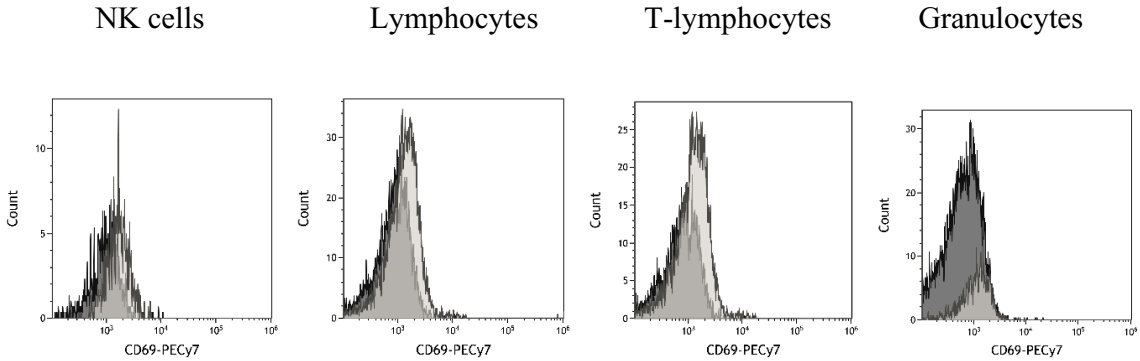
(d)

ME (200 µg/mL)



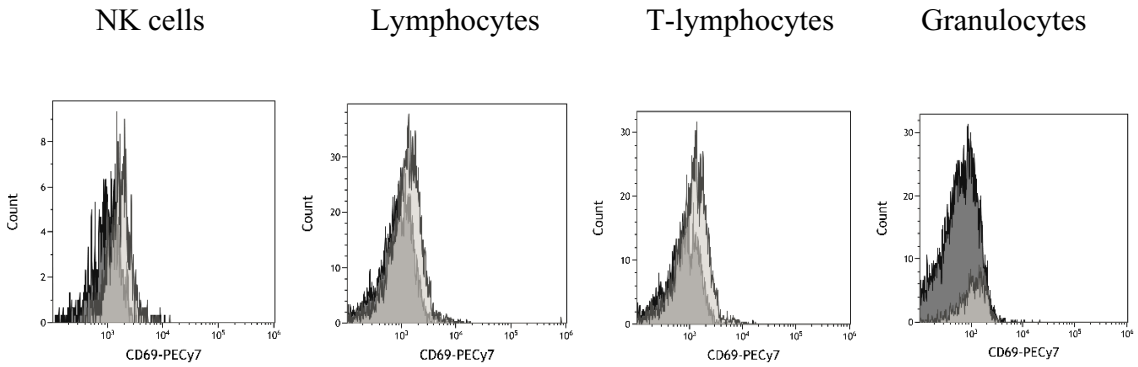
(e)

DE (200 µg/mL)



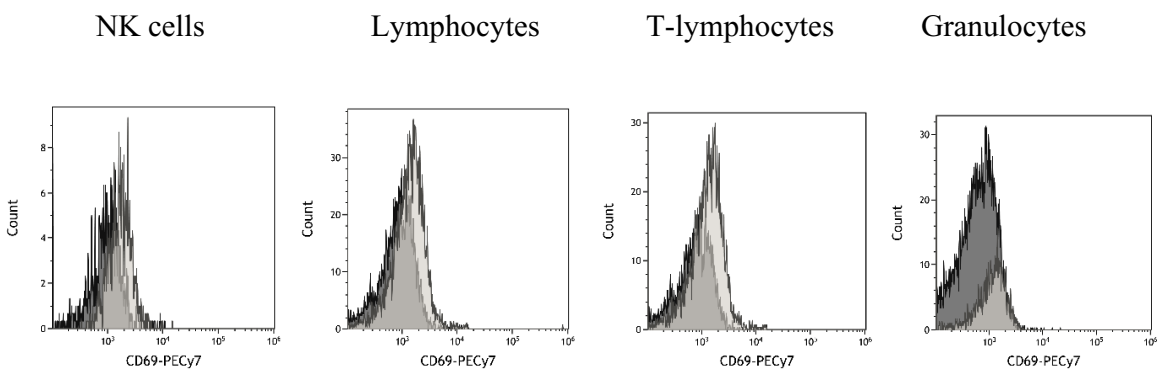
(f)

Astaxanthin-C18:3 (*α*-linolenic acid) (80 µM)



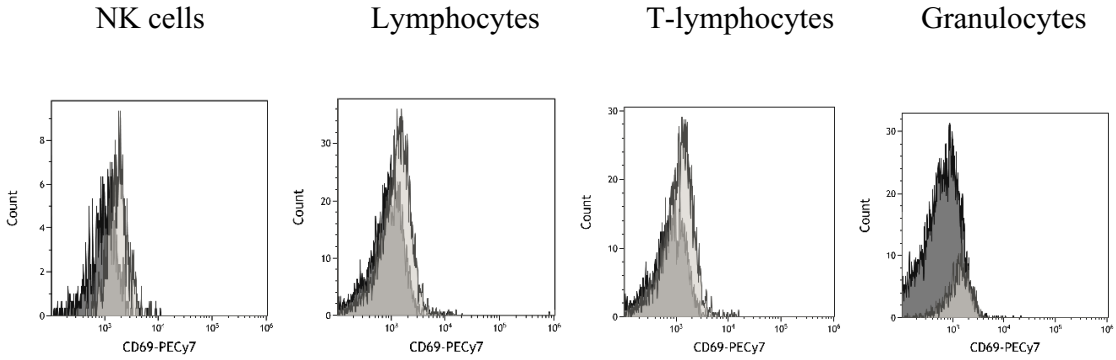
(g)

Astaxanthin-C18:2 (linoleic acid) (80 µM)



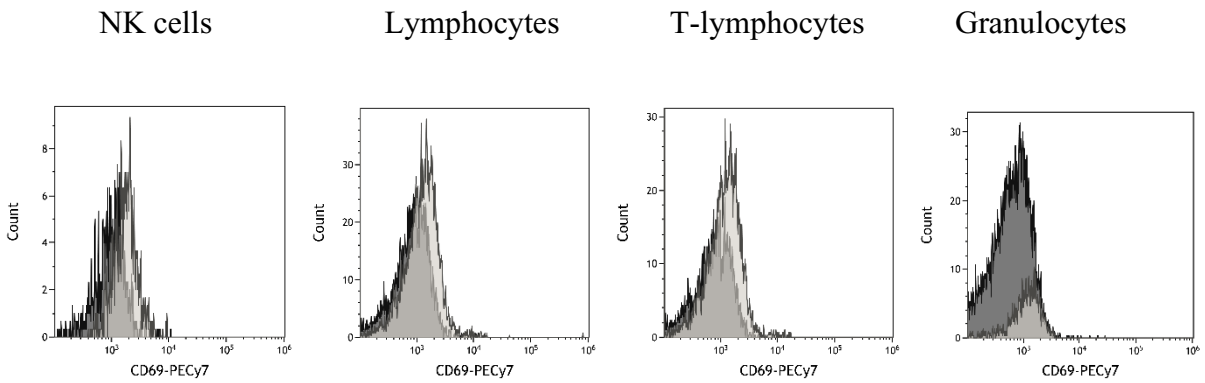
(h)

Astaxanthin-C16:0 (palmitic acid) (80 µM)



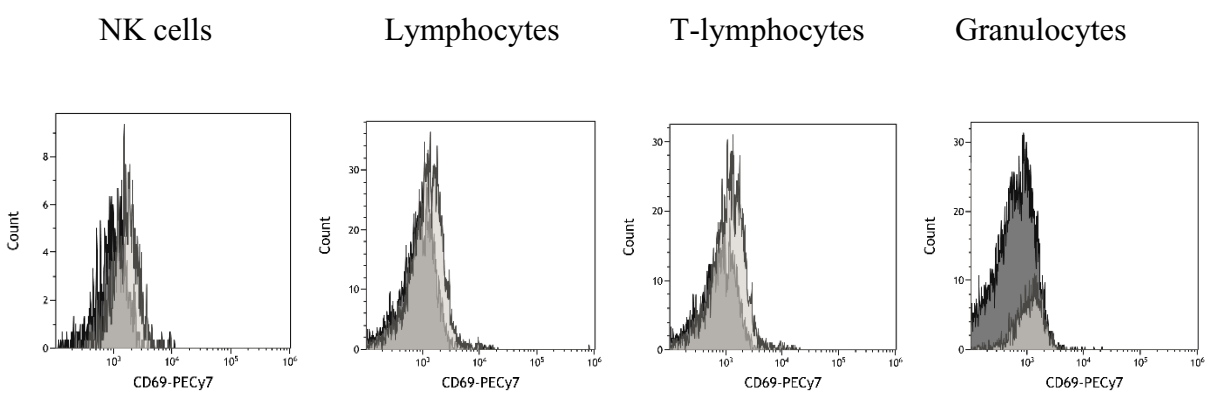
(i)

Astaxanthin-C18:1 (oleic acid) (80 µM)



(j)

Astaxanthin-C18:0 (stearic acid) (80 µM)



(k)

Fig. 7. Mean fluorescent intensity of CD69 expression on activated immune cells from human peripheral blood in response to treatment with *Haematococcus pluvialis* extract, fractions enriched in astaxanthin monoesters and diesters, and five individually isolated astaxanthin monoesters for 24 h is shown as histograms. The filled histograms (dark grey) represent the group control (untreated) and the other histograms (light grey) the stimulated (pretreated) group. The mitogen PHA was used as a positive control, showing the activation of immune cells (**a**). In negative control, the cells were treated neither with tested substances nor PHA, and no immune cell activation was observed (**b**). To achieve a proper solubility, 4% DMSO was used, and its impact was evaluated (**c**). No immune cells were activated in *Haematococcus pluvialis* extract nor fractions enriched in astaxanthin monoesters and diesters at 200 µg/mL (**d-f**). no immune cells were activated by individual astaxanthin monoesters at 80 µM (**g-k**). The histograms are representative of three separate experiments using cells from three different healthy donors.

The antioxidant activity was evaluated using the DPPH free radical scavenging method. The significant, dose-dependent antioxidant activity was observed in the case of astaxanthin as previously demonstrated [**Chintong et al. 2019**], the extract from *Haematococcus pluvialis* and the fraction enriched in astaxanthin monoesters (**Fig. 8**). The weak antioxidant activity occurred when examining the astaxanthin monoesters bonded with the α-linolenic (C18:3), palmitic (C16:0) and stearic (C18:0) acids at the concentration of 100 µg/mL (**Fig. 9**).

Several works reported the antioxidant activity of astaxanthin [**McNulty; 2007; Kamath et al. 2008; Park et al. 2010; Wolf et al. 2010; Kim and Kim 2018**] and it was demonstrated also for astaxanthin esters from *Haematococcus pluvialis* at oral administration of 250 μg/kg for two weeks, once they helped to restore the levels of antioxidant enzymes (i.e., catalase, superoxide dismutase, glutathione, and lipid peroxidase) in carbon tetrachloride treated rats [**Rao et al. 2015**].

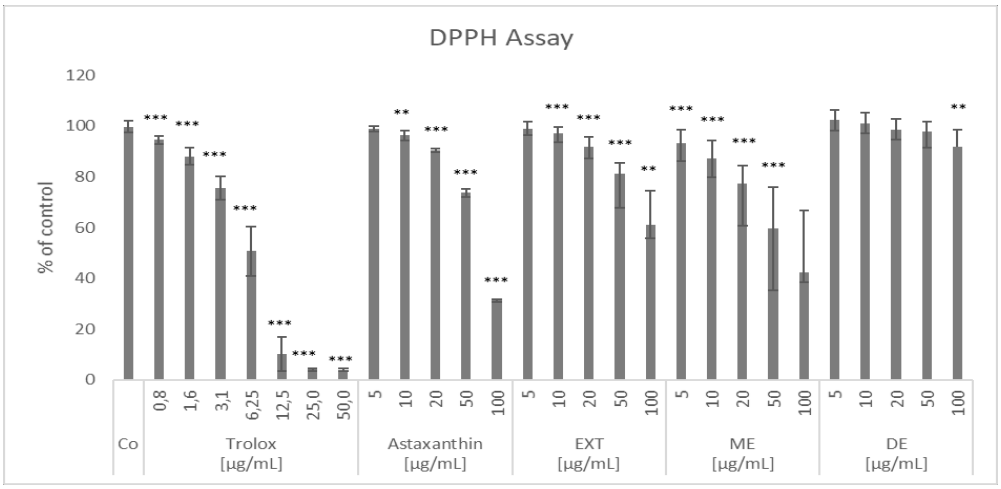


Fig. 8: The free radical scavenging activity of the tested substances determined by DPPH assay. Trolox and astaxanthin were used as positive controls. **EXT**: Extract of *Haematococcus pluvialis*. **ME**: fraction enriched in astaxanthin monoesters. **DE**: fraction enriched in astaxanthin diesters. The data represent mean±SD from 3 experiments. Student's T-test was used for the statistical analysis. *p<0.05, ** p<0.01, ***p<0.001.

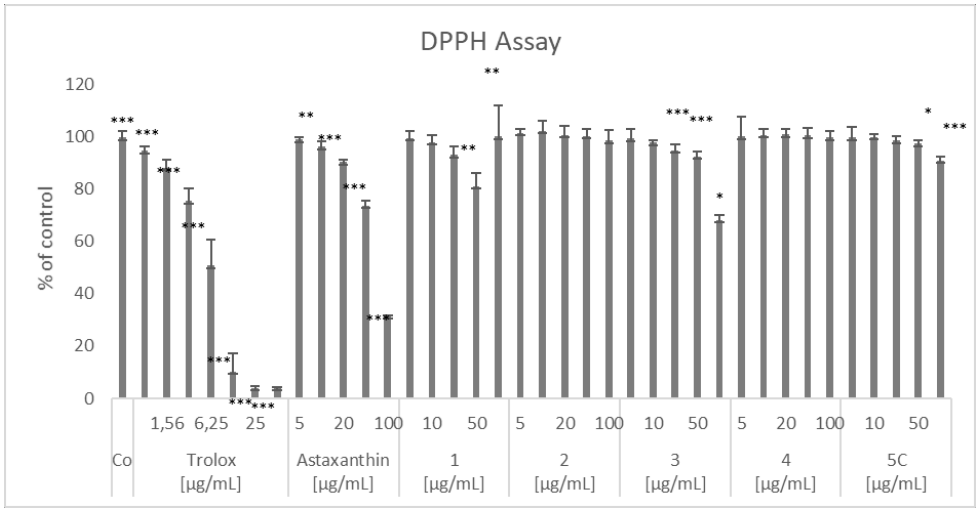


Fig. **9**: The free radical scavenging activity of the tested substances determined by DPPH assay. Trolox and astaxanthin were used as positive controls. **1**: astaxanthin-C18:3 (*α*-linolenic acid). **2**: astaxanthin-C18:2 (linoleic acid). **3**: astaxanthin-C16:0 (palmitic acid). **4**: astaxanthin-C18:1 (oleic acid). **5**: astaxanthin-C18:0 (stearic acid). The data represent mean±SD from 3 experiments. Student's T-test was used for the statistical analysis. *$p<0.05$, ** $p<0.01$, ***$p<0.001$.

Antiaggregant and vasodilatory activity

The whole blood impedance aggregometry was applied to evaluate the antiaggregant properties of tested substances. The **Fig. 10** shows the maximal inhibition of human platelet aggregation of the tested samples at the concentration of 8 µg/mL in comparison with the standard at the concentration of 2.29 µM. Neither the extract, nor the isolated fractions showed the platelet inhibition. Surprisingly, the fraction enriched in astaxanthin diesters even increased a little bit their aggregation, but this result was not statistically significant. However, the literature provides reports confirming the antiplatelet activity of astaxanthin [**Deng 2017; Satti 2020**].

There is a significant evidence that even the disodium disuccinate derivative of astaxanthin can reduce an *ex vivo* platelet aggregation, thus reduce the secondary thrombosis while maintaining normal homeostasis, probably due to scavenging of ROS [**Lauver 2008**]. Another study reports the antithrombotic and antihypertensive properties of astaxanthin on hyperlipidemic rats [**Sasaki 2011**]. On the other hand, there is also a study denying the influence of astaxanthin derived from a proprietary prodrug Xancor on the platelet, coagulation, or fibrinolytic factors in both aspirin-naïve and aspirin-treated subjects [**Serebruany 2010**]. The preliminary results suggest that none of the tested substances have significant effect on the platelet aggregation of healthy human individuals.

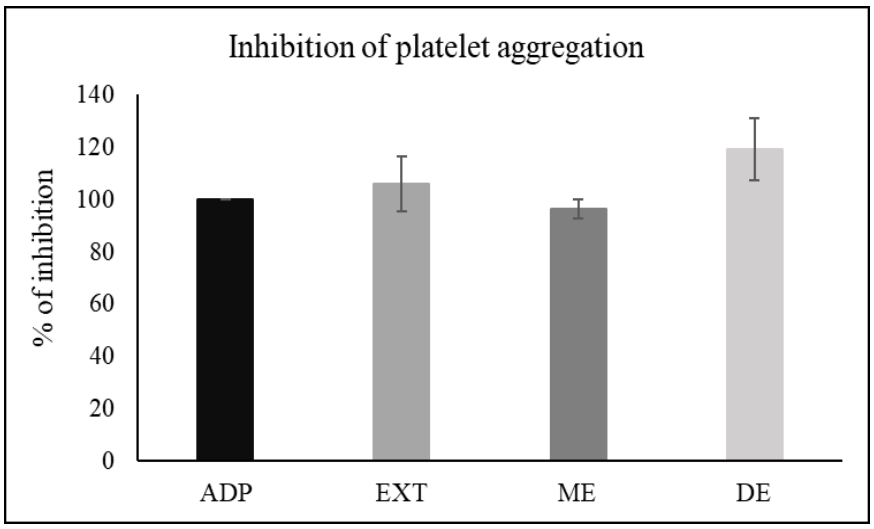


Fig. **10**: Effect of the *Haematococcus pluvialis* extract and the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters on the aggregation of human platelets. **ADP**: Adenosine-5´ diphosphate. **EXT**: Extract of *Haematococcus pluvialis*. **ME**: fraction enriched in astaxanthin monoesters. **DE**: fraction enriched in astaxanthin diesters. The concentration of the *Haematococcus pluvialis* fractions was 8 µg/mL, the concentration of ADP was 2.29 µM.

The thoracic aortas of male Wistar:Han rats were used to determine the vasodilatory activity of tested substances, however none of them significantly demonstrated the vasorelaxant potency. Astaxanthin is known to possess vasodilatory and antihypertensive properties [**Hussein 2005**]. However, this compound possesses only the vasorelaxant response towards sodium nitroprusside. It does not affect the endothelium-dependent acetylcholine-induced vasodilation [**Yanai 2008**]. On the concentration-dependent curve (**Fig. 11**), the responses were plotted as the increase in rat aorta relaxation expressed as a percentage of the maximum contraction to DMSO (2%). This concentration-response curve for NE before and after adding the tested fractions was not significantly different by 2-way ANOVA. The 95% confidence intervals are represented by dashed lines above and below the solid lines, demonstrating that the dashed lines of the tested fractions overlap with the control sample.

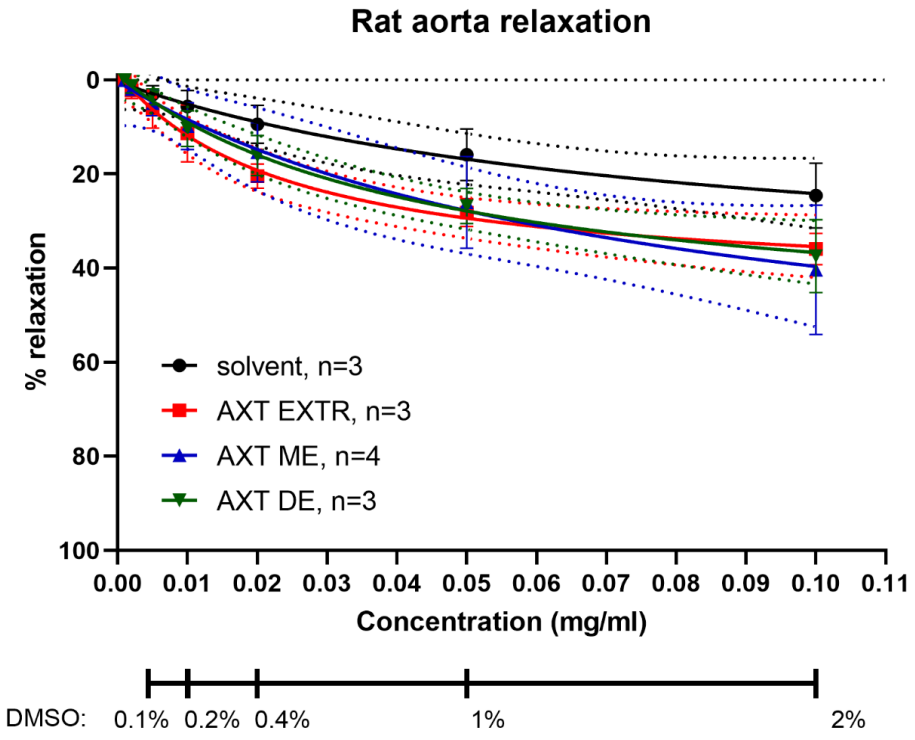


Fig. **11**: Effect of the *Haematococcus pluvialis* extract and the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters on aortic rings precontracted with NE. **EXT**: Extract of *Haematococcus pluvialis*. **ME**: fraction enriched in astaxanthin monoesters. **DE**: fraction enriched in astaxanthin diesters. The results are means from the experiments with at least 3 aortic rings. The 2-way ANOVA test was used to determine if there is a significant difference between the control and treated aortic rings. The 95% confidence intervals are represented by dashed lines above and below the solid lines.

Inhibition of tyrosinase activity

The activity of fungal tyrosinase was measured spectrophotometrically using L-DOPA as a substrate. From the tested substances, astaxanthin monoesters were shown to be the most potent inhibitor of the fungal tyrosinase which probably also significantly contributed to the overall inhibitory effect of astaxanthin extract. Melanin content in the B16-F10 melanoma cells was measured colorimetrically after 48h treatment of tested substances. Slight decrease in melanin production into the culture medium was observed only in the case of α-MSH-stimulated B16-F10 melanocytes treated with free astaxanthin (**Fig. 12**).

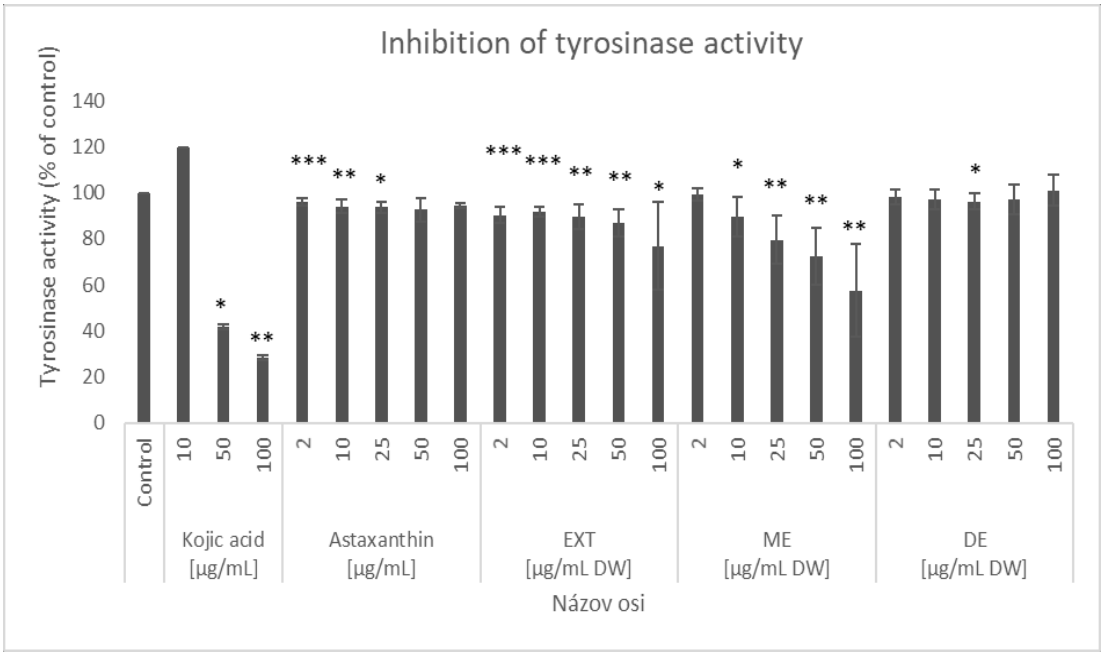


Fig. **12**: Inhibition of the fungal tyrosinase by the tested substances using L-DOPA as a substrate. **EXT**: Extract of *Haematococcus pluvialis*. **ME**: fraction enriched in astaxanthin monoesters. **DE**: fraction enriched in astaxanthin diesters. The data represent mean±SD from 3 experiments. Student's T-test was used for the statistical analysis. *p<0.05, ** p<0.01, ***p<0.001.

Antiparasitic activity

The MTT – (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay is used for determination of cytotoxic effect on cells, but it plays a role in the evaluation of respiration (i.e., viability) of the parasites.

This test enables the determination of the effect of the *Haematococcus pluvialis* extract and the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters on the basal metabolism (influence on the respiratory chain and the enzymes, mitochondrial ATP production) of the tapeworm *Mesocestoides vogae*. This assay is based on the spectrophotometric determination of the activity of those enzymes that reduce the MTT to the blue formazan crystals. Our results showed that none of tested compounds at 10 µM concentration was cytotoxic to larvae and on contrary, they elevated the enzymatic activity (succinate dehydrogenase and NADP dehydrogenase), mostly for the fraction enriched in astaxanthin monoesters. The results are expressed as the ratio of the absorbance corresponding to the amount of extracted formazan in treated larvae versus the untreated control. As seen on the **Fig. 13**, the fractions enriched in astaxanthin monoesters and diesters significantly increase the metabolic activity of the *Mesocestoides vogae* larvae probably due to their effect on the enzymes involved in the respiratory chain. The T-test ($P<0.05$) was used to determine if there is a significant difference between the control and treated larvae. Values were presented as mean ± standard error of three independent experiments, each performed in triplicate.

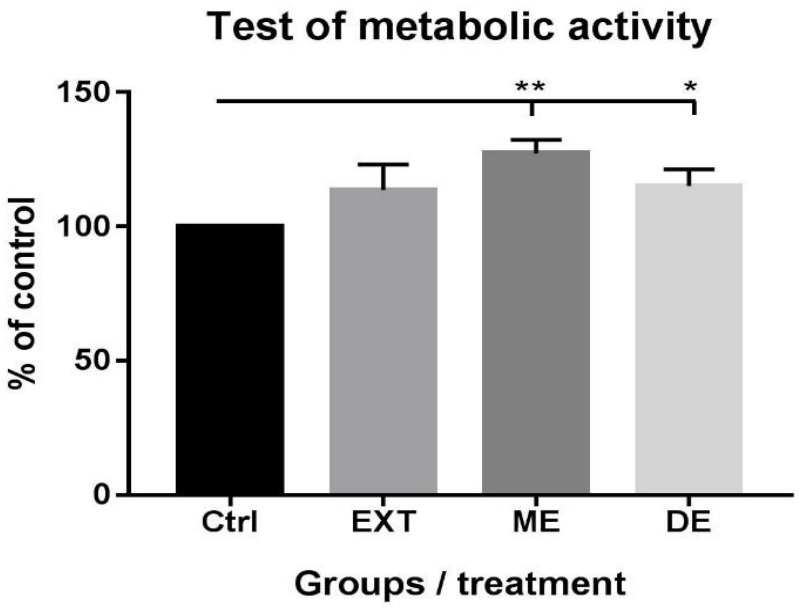


Fig. **13**: Effect of the *Haematococcus pluvialis* extract and the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters on the basal metabolism of the *Mesocestoides vogae* larvae. **Ctrl**: control. **EXT**: Extract of *Haematococcus pluvialis*. **ME**: fraction enriched in astaxanthin monoesters. **DE**: fraction enriched in astaxanthin diesters. The concentration of the *Haematococcus pluvialis* fractions was 10 µg/mL. Statistical significance: *$p<0.05$, **$p<0.01$. Results presented as mean±standard error of three measurements. T-test (GraphPad Prism 7 Software, Inc, San Diego, CA, USA) was used to determine if there is a significant difference between the control and treated cells.

The neutral red assay, used to determine the cell viability in fresh larvae, was modified as stated previously [**Hrčková et al. 2018**]. The neutral red dye can penetrate the cell membrane of living eukaryotic cells and be incorporated into them, therefore one of the ways how to evaluate the viability of these cells is to examine their active transport.

In a living state, the eukaryotic cells transport the neutral red dye using the active transport and store it in their lysosomes. The neutral red assay was used to determine the effect of the extract from *Haematococcus pluvialis* and the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters on the active transport in the *Mesocestoides vogae* larvae. The results are expressed as the percentage of the absorbance of untreated control corresponding to the treated larvae. The accumulation of natural red dye was significantly higher in larvae incubated with the microalgal extract and the fraction of astaxanthin monoesters (**Fig. 14**), demonstrating that out of the esters present in the extract, the astaxanthin monoesters are those that influence the active transport in the *Mesocestoides vogae* larvae. Data showed that all three compounds were not harmful to cytoplasmatic membrane and tegument of larvae indicating the lack of cytotoxicity. The T-test ($P<0.05$) was used to determine if there is a significant difference between the control and treated larvae. Values were presented as mean ± standard error of three independent experiments, each performed in triplicate.

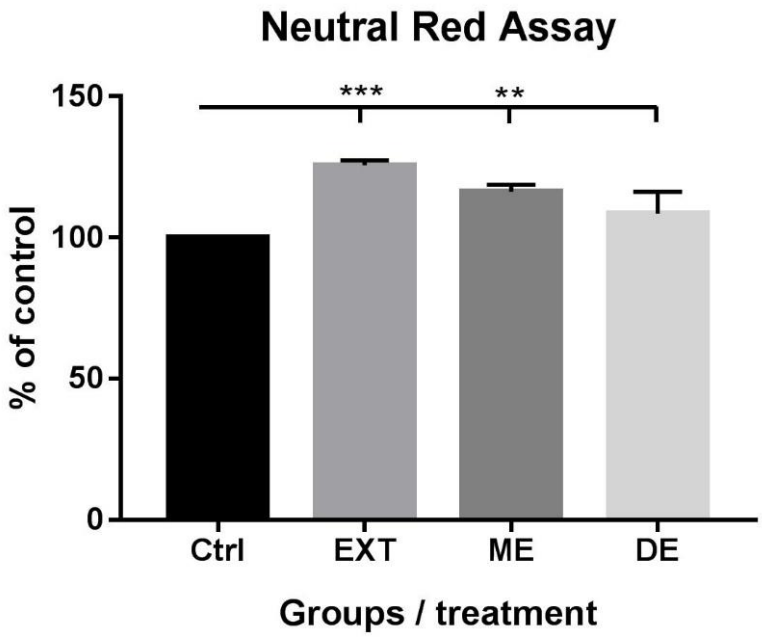


Fig. **14**: Effect of the *Haematococcus pluvialis* extract and the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters on the cell viability of the *Mesocestoides vogae* larvae. **Ctrl**: control. **EXT**: Extract of *Haematococcus pluvialis*. **ME**: fraction enriched in astaxanthin monoesters. **DE**: fraction enriched in astaxanthin diesters. The concentration of the *Haematococcus pluvialis* fractions was 10 µg/mL. Statistical significance: **p<0.01, ***p<0.001. Results presented as mean±standard error of three measurements. T-test (GraphPad Prism 7 Software, Inc, San Diego, CA, USA) was used to determine if there is a significant difference between the control and treated cells.

The preliminary results of anthelmintic activity suggest that astaxanthin esters do not disrupt the metabolism of the *Mesocestoides vogae* larvae, therefore they are not cytotoxic against these larvae. On the other hand, we found out that both sets of astaxanthin esters have partially a negative effect on the larval antioxidant systems at the concentration of 10 µg/mL as was demonstrated by the results of the experiments with the SOD inhibition (60% inhibition) and GST activity (**Fig. 15**). Especially astaxanthin diesters significantly decreased the GST activity in the *Mesocestoides vogae* larvae. In summary, the preliminary evaluation of the tested substances did not show significant alterations of larval motility; however, some negative impact on their morphology was noted.

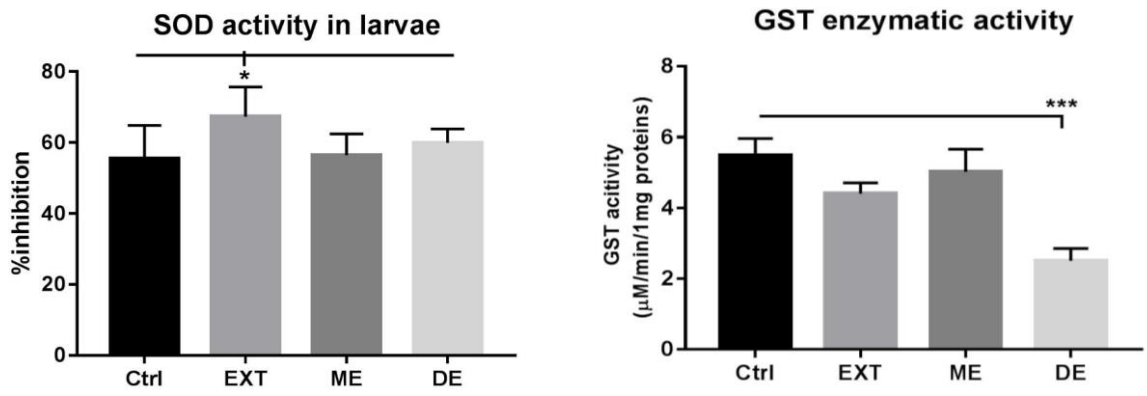


Fig. 15: Effect of the *Haematococcus pluvialis* extract and the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters on the SOD and GST activity in the *Mesocestoides vogae* larvae. **Ctrl**: control. **EXT**: Extract of *H. pluvialis*. **ME**: fraction enriched in astaxanthin monoesters. **DE**: fraction enriched in astaxanthin diesters. The concentration of the *Haematococcus pluvialis* fractions was 10 µg/mL. Statistical significance: *$p<0.05$, ***$p<0.001$. Results presented as mean±standard error of three measurements. T-test (GraphPad Prism 7 Software, Inc, San Diego, CA, USA) was used to determine if there is a significant difference between the control and treated cells.

Cytotoxic activity

The cytotoxic effect was assessed against AGS human gastric cancer cells. The impact of the tested substance upon cell viability was inferred by the MTT reduction assay. The significant effect was observed in the astaxanthin extract (**Fig. 16**) at the concentration of 250 µg/mL. Therefore, the fractions enriched in astaxanthin monoesters and diesters were also evaluated (**Fig. 17**) demonstrating the cytotoxic activity of the fraction enriched in astaxanthin monoesters at the concentration of 250 µg/mL.

Based on these results, five individually isolated astaxanthin monoesters have been tested. The HPCCC isolated the astaxanthin esterified with oleic acid (C18:1) exerted a cytotoxic effect at 50 μM (**Fig. 18**), however the similar effect at the same concentration has been previously already proved by the non-esterified (free) astaxanthin [**Kim et al. 2016a**]. Nevertheless, when comparing the free astaxanthin with its derivatives esterified with fatty acids, these derivatives show better bioavailability [**Rao et al. 2013a; Rao et al. 2013b**].

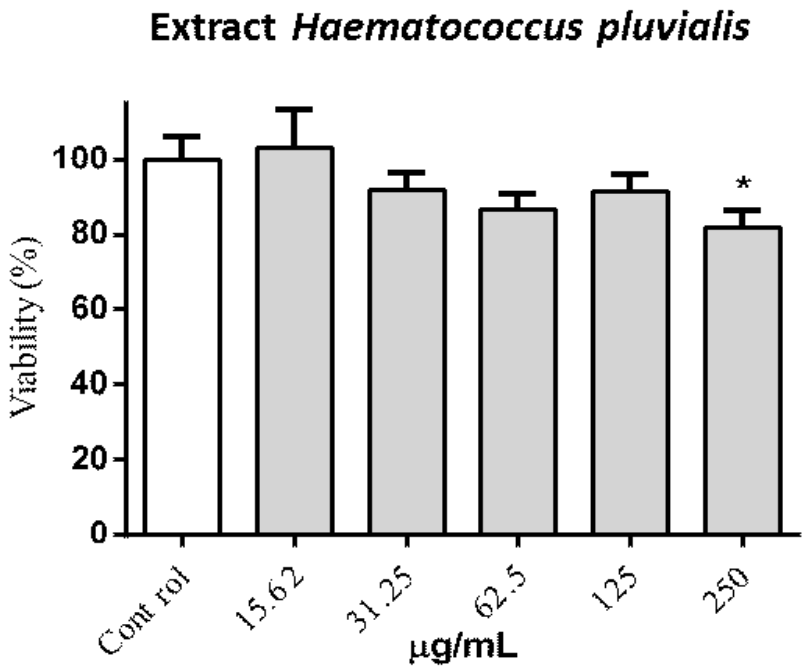


Fig. **16**: Effect of the *Haematococcus pluvialis* extract on the viability of human gastric cancer cells (AGS cell line) after 24 h treatment. Statistical significance: *p<0.05. Results presented as mean±standard error of three measurements. Student's T-test (GraphPad Prism 6 Software) was used to determine if there is a significant difference between the control and treated cells. Free astaxanthin (non-esterified astaxanthin) was used for comparison purposes.

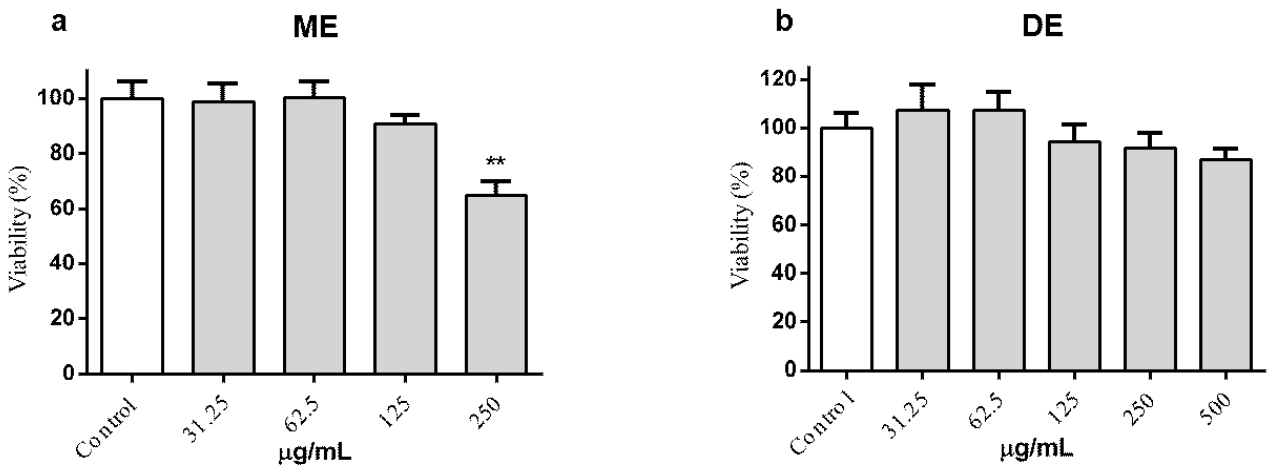


Fig. **17**: Effect of the HPCCC isolated fractions enriched in astaxanthin monoesters (**a**) and diesters (**b**) on the viability of human gastric cancer cells (AGS cell line) after 24 h treatment. Statistical significance: **p<0.01. Results presented as mean±standard error of three measurements. Student's T-test (GraphPad Prism 6 Software) was used to determine if there is a significant difference between the control and treated cells. Free astaxanthin (non-esterified astaxanthin) was used for comparison purposes. **ME**: fraction enriched in astaxanthin monoesters. **DE**: fraction enriched in astaxanthin diesters.

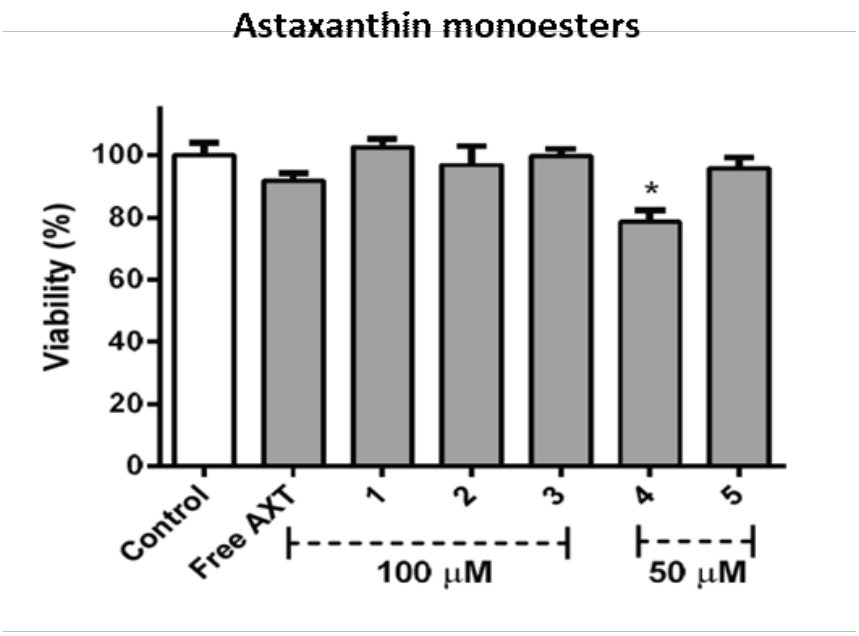


Fig. **18**: Effect of the isolated astaxanthin monoesters on the viability of human gastric cancer cells (AGS cell line) after 24 h treatment. Statistical significance: *p<0.05. Results presented as mean±standard error of three measurements. Student's T-test (GraphPad Prism 6 Software) was used to determine if there is a significant difference between the control and treated cells. Free astaxanthin (non-esterified astaxanthin) was used for comparison purposes. **1**: astaxanthin-C18:3 (*α*-linolenic acid). **2**: astaxanthin-C18:2 (linoleic acid). **3**: astaxanthin-C16:0 (palmitic acid). **4**: astaxanthin-C18:1 (oleic acid). **5**: astaxanthin-C18:0 (stearic acid). Compounds **4** and **5** were tested at a lower concentration (50 μM) due to their limited solubility in the culture medium. **AXT**: astaxanthin.

Anti-allergic activity

Astaxanthin is known to exert the anti-allergic properties. Topical use of astaxanthin alleviated the allergic reaction in a case of DNFB-induced allergic contact dermatitis in mice [**Kim et al. 2015**] bringing a hope that the astaxanthin may be used in a treatment of patients suffering from steroid-induced adverse reactions [**Aziz et al. 2020**].

The anti-allergic activity was evaluated through the inhibition of degranulation that was assessed by A23187-induced or antigen-induced *β*-hexosaminidase release in RBL-2H3 cells. Azelastin (10 µM) was used as a positive control and inhibited 51.3 ± 4.8% of A23187-induced degranulation. All tested substances demonstrated only the inhibition of antigen-induced *β*-hexosaminidase release at the concentration of 5 µM (**Fig. 19**). These results are in accordance with the study performed by Sakai et al. (2009) who demonstrated that astaxanthin remarkably limits the antigen-induced *β*-hexosaminidase release in RBL- 2H3 cells as well as the bone marrow-derived mast cells [**Sakai et al. 2009**].

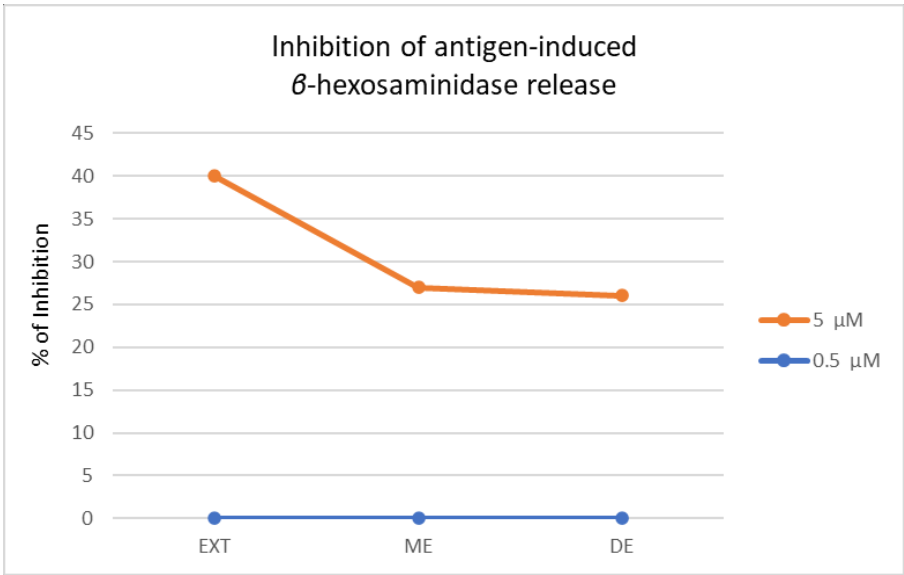


Fig. **19**: Effect of the *Haematococcus pluvialis* extract and the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters on inhibition of antigen-induced *β*-hexosaminidase. Results are presented as mean±SEM of three measurements; compared with the control value (A23187 or antigen only). Azelastin (10 μM) was used as a positive control and inhibited 51.3 ± 4.8% of A23187-induced degranulation.

Further research on this topic is crucial to bring the better understanding of the astaxanthin actions**.** More effort must be devoted to ascertaining the full biological profile of these substances using different bioassays.

Conclusion

My dissertation aimed to the investigation and development of efficient liquid-liquid isolation methods using HPCCC to obtain microalgal carotenoids and the evaluation of their biological properties to determine their health promoting properties. This information may contribute to the utilization of microalgae as a sustainable natural source for obtaining valuable compounds for the nutraceutical, food, animal feed, cosmetic and pharmaceutical sectors.

Based on the previously determined objectives, I can summarize the obtained results as follows:

- An efficient method for biomass extraction was developed, optimized, and applied to produce an extract enriched in lutein from the microalgae *Chlorella vulgaris* and *Parachlorella kessleri*. In the case of *Chlorella vulgaris*, the ultrasound-assisted extraction of microalgae biomass with the LP of the biphasic solvent system composed of *n*-heptane–ethanol–water (5:4:1.5, *v/v/v*) was selected leading to a biomass extract with a lutein yield of 3.20 mg/g dried biomass. In the case of *Parachlorella kessleri*, the maceration extraction of microalgae biomass with the LP of the selected biphasic solvent system composed of *n*-heptane–ethanol–water (5:4:1.5, *v/v/v*) for 30 min was chosen leading to a biomass extract with a lutein yield of 6.71 mg/g dried biomass.

- An efficient and scalable isolation method to obtain lutein from *Chlorella vulgaris* and *Parachlorella kessleri* HY1 strain using HPCCC was developed and optimized. The LP of the selected biphasic solvent system (*n*-heptane–ethanol–water, 5:4:1.5, *v/v/v*) was used as mobile phase. Given that the mobile phase served both as an extraction solvent and a mobile phase during the HPCCC separation process, it improved the isolation process in two aspects. First, it avoided the presence of highly lipophilic impurities in the prepared extract that could contaminate the produced lutein. Second, it allowed us to develop the continuous isolation process without the need to stop the whole system for starting a next separation run. In the case of *Chlorella vulgaris*, we processed 2 g of the microalgae extract yielding 60 mg of lutein (92% purity) that were further purified by gel permeation chromatography. Finally, we obtained 50 mg of lutein with 97% purity. In the case of *Parachlorella kessleri* HY1, the nuclear magnetic resonance (NMR) was applied to determine the molar proportions of the individual solvents contained in the UP and LP of the selected biphasic solvent system (system 4) composed of *n*-heptane–ethanol–water (5:4:1.5, *v/v/v*), so that we can evaluate the possibility of performing the preparation of each liquid phase separately. We successfully demonstrated that the phases prepared independently based on the NMR measurements have the same capacity as the phases taken directly from parent biphasic solvent system to be used in the HPCCC isolation. Therefore, a low solvent consumption was required for the isolation HPCCC operation. Overall, we processed 3 g of the microalgae extract yielding 150 mg of lutein (95% purity, 97% recovery) without the need of additional purification processes.

- Five astaxanthin monoesters were isolated from *Haematococcus pluvialis* by HPCCC, where the LP of a selected biphasic solvent system (*n*-heptane:acetonitrile, ratio 5:5, *v/v*) was used as mobile phase. A final purification step of the resulting five astaxanthin monoesters was performed with HPLC affording five astaxanthin derivatives esterified with *α*-linolenic acid (4 mg), linoleic acid (8 mg), palmitic acid (8 mg), oleic acid (12 mg) and stearic acid (1 mg) (98% purity). To further increase the processes productivity, a multi-injection HPCCC method was developed, optimized, and applied.

- *Haematococcus pluvialis* extract and the isolated fractions enriched in astaxanthin monoesters and diesters were tested for their immunomodulatory, antioxidant, antiaggregant, vasodilatory, antiparasitic, cytotoxic and anti-allergic activity. The ability to inhibit the tyrosinase activity and melanin production was also tested. The tested substances showed no significant immune-stimulating, anti-platelet aggregation, vasorelaxant potency, and melanin production inhibitory effects. Despite of not being cytotoxic against the *Mesocestoides vogae* larvae, the astaxanthin esters (10 μg/mL) partially exerted a negative effect on the larval antioxidant systems as was demonstrated by observing the SOD inhibition and the decrease of GST enzymatic activity. All tested substances exerted an anti-allergic effect by inhibiting the antigen-induced *β*-hexosaminidase release at the concentration of 5 μM. The crude extract from *Haematococcus pluvialis* exhibited a significant antioxidant, anti-tyrosinase and cytotoxic activity, observing that the fraction enriched in astaxanthin monoesters significantly contribute to these effects.

- Since the fraction enriched in astaxanthin monoesters proved to be responsible for the antioxidant as well as the cytotoxic activity against the AGS cell line, we also evaluated the isolated astaxanthin monoesters to ascertain which of them are responsible for these activities. We observed antioxidant activity of the astaxanthin esterified with the α-linolenic (C18:3), palmitic (C16:0) and stearic (C18:0) acids at 100 μg/mL. The astaxanthin esterified with oleic acid (C18:1) exerted a cytotoxic effect at 50 μM.

Overall, this work presents an efficient and scalable separation method for the isolation of various valuable pigments from microalgae. Several optimization works were introduced to improve the economics and environmental issues of the whole isolation process such as the use of non-toxic solvents, the use of a chlorophyll-deficient strain, inclusion of nuclear magnetic resonance analysis and consecutive isolation approaches.

The results betoken the differences within the biological properties of individual astaxanthin esters, which may support a new perspective for diversifying the market offer of these compounds.

Contributor's share of publications included in the dissertation

Fábryová, T.; Cheel, J.; Kubáč, D.; Hrouzek, P.; Vu, D.L.; Tůmová, L.; Kopecký, J.: Purification of lutein from the green microalgae *Chlorella vulgaris* by integrated use of a new extraction protocol and a multi-injection high performance counter-current chromatography (HPCCC). *Algal Research*, **2019**, vol. 41, 101574.

- investigation, formal analysis

Fábryová, T.; Tůmová, L.; Correia da Silva, D.; Pereira, D.M.; Andrade, P.B.; Valentão, P.; Hrouzek, P.; Kopecký, J.; Cheel J.: Isolation of astaxanthin monoesters from the microalgae *Haematococcus pluvialis* by high performance countercurrent chromatography (HPCCC) combined with high performance liquid chromatography (HPLC). *Algal Research*, **2020**, vol. 49, 101947.

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Nováková, M.; **Fábryová, T**.; Vokurková, D.; Dolečková, I.; Kopecký, J.; Hrouzek, P.; Tůmová, L.; Cheel J.: Separation of the glycosylated carotenoid myxoxanthophyll from *Synechocystis salina* by HPCCC and evaluation of its antioxidant, tyrosinase inhibitory and immune-stimulating properties. *Separations*, **2020**, vol. 7, 73.

- methodology, investigation
- writing-review and editing

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- extraction and isolation experiments
- data analysis
- manuscript revision

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Supplementary materials

1.9 List of figures

Fig. 11 Effect of the *Haematococcus pluvialis* extract and the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters on aortic rings precontracted with NE.

Fig. 12 Inhibition of the fungal tyrosinase by the tested substances using L-DOPA as a substrate.

Fig. 13 Effect of the *Haematococcus pluvialis* extract and the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters on the basal metabolism of the *Mesocestoides vogae* larvae.

Fig. 14 Effect of the *Haematococcus pluvialis* extract and the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters on the cell viability of the *Mesocestoides vogae* larvae.

Fig. 15 Effect of the *Haematococcus pluvialis* extract and the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters on the SOD and GST activity in the *Mesocestoides vogae* larvae.

Fig. 16 Effect of the *Haematococcus pluvialis* extract on the viability of human gastric cancer cells (AGS cell line) after 24 h treatment.

Fig. 17 Effect of the HPCCC isolated fractions enriched in astaxanthin monoesters (**a**) and diesters (**b**) on the viability of human gastric cancer cells (AGS cell line) after 24 h treatment.

Fig. 18 Effect of the isolated astaxanthin monoesters on the viability of human gastric cancer cells (AGS cell line) after 24 h treatment.

Fig. 19 Effect of the *Haematococcus pluvialis* extract and the HPCCC isolated fractions enriched in astaxanthin monoesters and diesters on inhibition of antigen-induced *β*-hexosaminidase.

1.10 List of abbreviations

α	Separation factor
ADP	Adenosine diphosphate
AXT	Astaxanthin
CVDs	Cardiovascular diesases
DE	Fraction enriched in astaxanthin diesters
DHICA	5,6-dihydroxyindole-2-carboxylic acid
DMAPP	Dimethylallyl diphosphate
DMAPPP	Dimethylallyl pyrophosphate
DMBA	7,12-dimethylbenz(a)anthracene
DPPH	2,2-diphenyl-1,picrylhydrazyl
DSCG	Disodium cromoglycate
FceRI	High-affinity receptor for Immunoglobulin E
GGPP	Geranylgeranyl diphosphate
GGPPP	Geranylgeranyl pyrophosphate
GLOBOCAN	Global Cancer Observatory
GPPP	Geranyl pyrophosphate
GST	Glutathione-S-transferase
HPCCC	High-performance countercurrent chromatography
HPLC	High performance liquid chromatography
IgE	Immunoglobulin E
IPP	Isopentenyl diphosphate
K	Partition coefficient
L-DOPA	L-3,4-dihydroxyphenylalanine

LP4	Lower phase of the biphasic solvent system composed of *n*-heptane–ethanol–water (5:4:1.5, *v/v/v*)
LP	Lower phase
ME	Fraction enriched in astaxanthin monoesters
MEP	Methylerythritol 4-phosphate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVA	Mevalonic acid
NE	Noradrenaline
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
PGD2	Prostaglandin 2
PHA	Phytohemagglutinin
RBL	Rat basophilic leukemia
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Th2	T helper type 2
TRP 1	Tyrosinase related protein 2
TRP 2	Tyrosinase related protein 2
UP	Upper phase
WHO	World's Health Organization

1.11 List of all publications

Fábryová, T.; Cheel, J.; Kubáč, D.; Hrouzek, P.; Vu, D.L.; Tůmová, L.; Kopecký, J.: Purification of lutein from the green microalgae *Chlorella vulgaris* by integrated use of a new extraction protocol and a multi-injection high performance counter-current chromatography (HPCCC). *Algal Research*, **2019**, vol. 41, 101574.

IF 4.008

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IF 1.900

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IF 3.016

1.12 List of publications related to the dissertation

Fábryová, T.; Cheel, J.; Kubáč, D.; Hrouzek, P.; Vu, D.L.; Tůmová, L.; Kopecký, J.: Purification of lutein from the green microalgae *Chlorella vulgaris* by integrated use of a new extraction protocol and a multi-injection high performance counter-current chromatography (HPCCC). *Algal Research*, **2019**, vol. 41, 101574.

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1.13 List of attended conferences

Fábryová, T.; Cheel, J.; Tůmová, L. Extraction, isolation, identification and biological assessment of astaxanthin esters, The 7th Postgraduate and 5th Postdoc Conference, FaF UK, Hradec Králové, Czech Republic, **2017**

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Fábryová, T.; Nováková, M.; Vokurková, D.; Kopecký, J.; Hrouzek, P.; Cheel, J.; Tůmová, L. Separation of the carotenoid myxoxanthophyll from *Synechocystis salina* by high performance countercurrent chromatography and evaluation of its immune-stimulating properties, The 11th Postgraduate and Postdoc conference, FaF UK, Hradec Králové, Czech Republic, **2021**

1.14 List of awarded grants

Development and application of countercurrent chromatografy separation techniques for the isolation of astaxanthin esters from microalgal biomass, Technological Agency of Czech Republic (TA ČR), program Zéta (TJ01000013), **2018–2019** role: principal investigator.

Phytochemical analysis and biological activity of the algae *Haematococcus pluvialis*, Grant Agency of Charles University (GA UK) (1134217), **2017–2019** role: principal investigator.

1.15 List of awarded patents

Způsob získání fytokomplexu na bázi astaxantinu z biomasy mikrořas, spisová značka: PV 2019-799, patent číslo **308677**