การศึกษาฤทธิ์การยับยั้งเอนไซม์ไทโรซิเนส ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์คีเลชัน ของโลหะของแอสตาแซนธินใน *Haematococcus pluvialis*

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โครงการปริญญานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
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Evaluation of the anti-tyrosinase, antioxidant, and metal chelating activities of astaxanthin in *Haematococcus pluvialis*

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หัวข้อโครงการปริญญานิพนธ์	การศึกษาฤทธิ์การยับยั้งเอนไซม์ไทโรซิเนส ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์คีเลชันของโลหะของแอสตาแซนธินใน *Haematococcus pluvialis*
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คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย อนุมัติให้โครงการปริญญานิพนธ์ฉบับนี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรบัณฑิต

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ชื่อโครงการ : การศึกษาฤทธิ์การยับยั้งเอนไซม์ไทโรซิเนส ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์คีเลชันของโลหะของแอสตาแซนธินใน *Haematococcus pluvialis*

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สารแอสตาแซนธิน คือ สารในกลุ่มแคโรทีนอยด์ มีสีแดง ละลายได้ในไขมัน แหล่งจากธรรมชาติที่พบสารแอสตาแซนธินได้มากที่สุดคือ *Haematococcus pluvialis* โดยสารแอสตาแซนธินนี้ได้รับความสนใจจากนักวิจัยทั้งทางอุตสาหกรรมยาและเครื่องสำอางเนื่องจากประโยชน์ที่หลากหลายของแอสตาแซนธิน อาทิ ฤทธิ์ต้านอนุมูลอิสระ อย่างไรก็ตามยังไม่พบงานวิจัยที่ศึกษาผลของรูปแบบที่ต่างกันของสารสกัดแอสตาแซนธิน เช่น ในรูปน้ำมันและเรซินต่อฤทธิ์ทางชีวภาพที่ได้ งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาเปรียบเทียบฤทธิ์ทางชีวภาพ ได้แก่ ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์คีเลชันของโลหะและการยับยั้งเอนไซม์ไทโรซิเนสของสารสกัดแอสตาแซนธินในรูปของน้ำมัน (oil) และโอลีโอเรซิน (oleoresin) ที่มีจำหน่ายในท้องตลาด โดยมีการพิสูจน์เอกลักษณ์ของสารสกัดแอสตาแซนธินทั้ง 2 ชนิด โดยใช้เครื่อง UV spectrophotometer ที่ความยาวคลื่น 300–700 nm ในการศึกษาฤทธิ์ต้านอนุมูลอิสระทำโดยวิธี DPPH และใช้อัลฟาโทโคฟีรอลเป็นกลุ่มควบคุมผลบวก ส่วนการศึกษาฤทธิ์คีเลชันของโลหะทำโดยวิธีการคีเลชันของเฟอร์รัสไอออนและใช้ EDTA เป็นกลุ่มควบคุมผลบวก ในการศึกษาฤทธิ์การยับยั้งเอนไซม์ไทโรซิเนสจะใช้แอลไทโรซีนเป็นสารตั้งต้นทำปฏิกิริยากับเอนไซม์ไทโรซิเนสจากเห็ดเพื่อให้เกิดเม็ดสีเมลานิน โดยใช้กรดโคจิกเป็นกลุ่มควบคุมผลบวก จากผลการศึกษาที่ได้ พบว่าสารสกัดแอสตาแซนธินในรูปของ oil และ oleoresin มีลักษณะการดูดกลืนแสงที่คล้ายกัน โดยความยาวคลื่นที่สารสกัดดูดกลืนแสงได้มากที่สุด คือ 476 nm สารสกัดแอสตาแซนธินในรูปของ oil มีประสิทธิภาพต้านอนุมูลอิสระโดยมีค่า IC_{50} เท่ากับ 0.4981 ± 0.17 mg/mL นอกจากนี้ สารสกัดแอสตาแซนธินในรูปของ oil ที่ความเข้มข้น 500 µg/mL มีฤทธิ์ต้านอนุมูลอิสระมากกว่าในรูปของ oleoresin ถึง 3 เท่า ส่วนอัลฟาโทโคฟีรอลแสดงฤทธิ์ต้านอนุมูลอิสระได้โดยมีค่า IC_{50} เท่ากับ 19.25 ± 0.08 µg/mL ในส่วนของฤทธิ์คีเลชันของโลหะพบว่าสารแอสตาแซนธินทั้งในรูป oil และ oleoresin ไม่มีฤทธิ์คีเลชันของโลหะ อย่างไรก็ตามสารแอสตาแซนธินในรูปของ oleoresin ที่ความเข้มข้นต่ำ ๆ พบเกิดการคีเลชันของโลหะเพิ่มขึ้น ส่วน EDTA แสดงฤทธิ์คีเลชันของโลหะได้โดยมีค่า IC_{50} เท่ากับ 20.65 ± 0.98 µg/mL ในส่วนของฤทธิ์ยับยั้งเอนไซม์ไทโรซิเนสไม่สามารถทดสอบได้เนื่องจากพบปัญหาเกี่ยวกับเอนไซม์ไทโรซิเนส การศึกษานี้แสดงให้เห็นว่าสารแอสตาแซนธินในรูปของ oil และ oleoresin มีความสามารถในการออกฤทธิ์ที่แตกต่างกันอย่างมีนัยสำคัญ โดยพบว่าสารแอสตาแซนธินในรูปของ oil มีความเหมาะสมที่จะใช้เป็นสารออกฤทธิ์ทางยาและเครื่องสำอางเพื่อหวังผลในด้านฤทธิ์ต้านอนุมูลอิสระ

คณะเภสัชศาสตร์ ลายมือชื่อนิสิต ภัทรสุดา พงศ์ภัทรานนท์

จุฬาลงกรณ์มหาวิทยาลัย ลายมือชื่ออาจารย์ที่ปรึกษา Ramhat Chutoprapat

Abstract

Senior project title : Evaluation of the anti-tyrosinase, antioxidant and metal chelating activities of astaxanthin in *Haematococcus pluvialis*

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Astaxanthin, a xanthophyll carotenoid, a red fat-soluble pigment, is naturally found in marine organisms. The richest source of natural astaxanthin is *H. pluvialis. Astaxanthin from H. pluvialis* has attracted researchers' attention in the pharmaceutical and cosmetic industries as its potential benefits, especially its potent antioxidant activity. However, there was no research had been conducted on the effect of the different forms of *Astaxanthin extract such as oil and resin on its biological activity*. This study investigated three activities, including antioxidant, metal chelating activity, and the anti-tyrosinase (whitening potential), of commercially available astaxanthin *extracts from H. pluvialis* in oil and oleoresin forms. Characterization of astaxanthin oil and oleoresin was examined by UV spectrophotometry at the 300–700 nm wavelength. The antioxidant activity was measured using the DPPH assay with α-Tocopherol as a positive control. The metal chelating activity was determined by the ferrous ion chelating activity method with EDTA as a positive control. The anti-tyrosinase activity was determined by using L-Tyrosine as a substrate together with mushroom tyrosinase to form melanin pigment. Kojic acid was used as a positive control. Astaxanthin oil and oleoresin had similar light absorbance characteristics with λ_{max} at 476 nm. Astaxanthin oil showed an excellent antioxidant with the half maximal inhibitory concentration (IC_{50}) of 0.4981 ± 0.17 mg/mL. The radical scavenging activity of astaxanthin oil, at the concentration of 500 µg/mL, was 3 times higher than that of oleoresin. The α-Tocopherol gave a radical scavenging activity with IC_{50} of 19.25 ± 0.08 µg/mL. Both astaxanthin oil and oleoresin demonstrated no metal chelating activity, but oleoresin at the low concentrations increased a metal chelation. The EDTA showed a metal chelating activity with IC_{50} of 20.65 ± 0.98 µg/mL. Unfortunately, anti-tyrosinase activity was not conducted in this study due to a problem with mushroom tyrosinase (degradation of enzyme). These findings indicated that the different forms of the extract, astaxanthin oil and oleoresin, could give significant different effect on their biological activities. Astaxanthin oil from *H. pluvialis* can be preferably used as an active ingredient in pharmaceutical products and skincare or cosmetic formulation because of its antioxidant property.

Faculty of Pharmaceutical Sciences Student's signature Patsuda Pongpattranont

Chulalongkorn University Advisor's signature Romchat Chutoprapat

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Chapter 1 Introduction

1.1 Background and Rationale

Haematococcus pluvialis is a green alga (Chlorophyta). This alga has two stages during its life cycle. The first stage is called a green stage which grows under favorable conditions. *H. pluvialis* cells display a green color in this stage. The later stage is called a red stage which grows under the condition of limiting cell division. The cells in the red stage are called aplanospores which show a deep red pigmentation. The color change is the outcome of chlorophyll degradation and astaxanthin synthesis. The conditions that enhance numerous astaxanthin are (1) nutrient starvation and nitrate limitation; (2) carbon bioavailability as carbon dioxide or organic carbon; and (3) well distributed high irradiance.[1]

Astaxanthin is a xanthophyll carotenoid which is a red fat-soluble pigment.[2] Astaxanthin is naturally found in marine organisms, for example, salmonids, shrimps, and crabs.[3, 4] It is also biosynthesized by plants, bacteria, and microalgae.[4] *H. pluvialis* is the richest source of natural astaxanthin.[2] It can accumulate more than 3 grams of astaxanthin kg^{-1} dry biomass.[5] According to the study of Ranga Rao Ambati et al., *H. pluvialis* has the most content of astaxanthin (3.8% on the dry weight basis) compared with other types of algae.[2] Astaxanthin from *H. pluvialis* is the primary source for various human applications such as food, dietary supplements, and cosmetics.[4]

Nowadays, astaxanthin from microalgae has a very high market value due to its considerable potentials. Following a recently published market research report, the total market value of astaxanthin is reported to be over 550 million U.S. dollars in 2017 and is expected to reach 800 million U.S. dollars by 2022 with a CAGR (Compound annual growth rate) of 8.0%.[6] As a result of its potential, astaxanthin has become widely used in the cosmetic industry. According to the study of S. Villaró et al., astaxanthin was recently recommended by one of the world's leading market intelligence agencies to characterize one of the three main ingredients to observe and a niche ingredient with powerful potential.[7]

Astaxanthin has a potent antioxidant activity and unique molecular and biochemical messenger properties in treating and preventing skin disease. The first potential of astaxanthin is the antioxidant property that is better than α-tocopherol.[8] Specifically, astaxanthin inhibits reactive oxygen species (ROS) formation.[4] Previous studies addressed that natural astaxanthin from *H. pluvialis* has a notably greater

antioxidant capacity than synthetic.[9] Kumi Tominaga et al. study suggested that astaxanthin from *H. pluvialis* can improve skin condition in all layers, such as the epidermis and dermis, by combining oral supplementation and topical treatment astaxanthin.[3] However, most studies about astaxanthin's antioxidant activity use astaxanthin in combination with other active ingredients that can also provide antioxidant activity;[3] therefore, few studies research only the antioxidant activity of astaxanthin. In addition, there is no research regarding the effect of the different forms of astaxanthin extract.

The second potential is anti-tyrosinase activity. Tyrosinase is the critical enzyme of melanin synthesis.[5] According to J.-B. Guillerme et al. studies recently focused on finding skin-whitening compounds from new marine microorganisms. Astaxanthin shows attractive depigmentation properties that protect skin from age spots by reducing melatonin synthesis by 40%.[5] Nevertheless, there are few studies about anti-tyrosinase activity in marine microalgae, and more studies of whitening activity are required before their use in cosmetic formulations. The last potential of astaxanthin is metal chelation activity. Metal chelating activity is one of the antioxidant mechanisms that is also interesting and has limited investigations in microalgae.

Consequently, this study evaluated three activities, including antioxidant, metal chelating activity, and anti-tyrosinase (whitening potential), of commercially available astaxanthin extracts from *H. pluvialis* in oil and oleoresin forms for use as an active ingredient in pharmaceutical products and skincare or cosmetic formulation.

Chapter 2 Literature Review

2.1 Astaxanthin from *Haematococcus pluvialis*

Astaxanthin (3,3' -dihydroxy-b,b-carotene-4,4'-dione)[10] is a xanthophyll carotenoid, a red fat-soluble pigment with the molecular formula $C_{40}H_{52}O_4$ [Figure 1], and its molecular weight is 596.84 g/mol.[2] The sources of astaxanthin are various microorganisms and marine animals, for instance, algae, yeast, salmon, shrimp, and crayfish. [3, 4] *Haematococcus pluvialis*, a unicellular microalga, is one of the rich sources of natural astaxanthin; according to the study of Ranga Rao Ambati et al., H. pluvialis has the most content of astaxanthin (3.8% on the dry weight basis) compared to other microorganism sources as seen in [Table 1].[2]

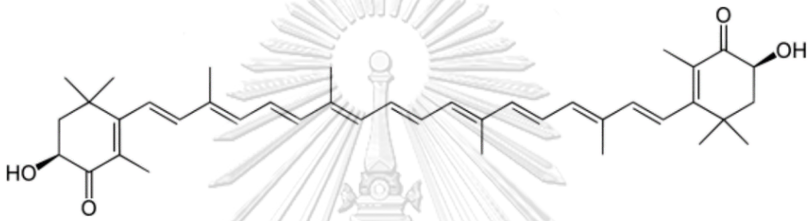

Figure 1 Chemical structure of astaxanthin[11]

Table 1 Microorganism sources of astaxanthin[2]

Sources	Astaxanthin (%) on the Dry Weight Basis	References
Chlorophyceae		
Haematococcus pluvialis	3.8	[17,18]
Haematococcus pluvialis (K-0084)	3.8	[22]
Haematococcus pluvialis (Local isolation)	3.6	[23]
Haematococcus pluvialis (AQSE002)	3.4	[24]
Haematococcus pluvialis (K-0084)	2.7	[25]
Chlorococcum	0.2	[26,27]
Chlorella zofingiensis	0.001	[28]
Neochloris wimmeri	0.6	[29]
Ulvophyceae		
Enteromorpha intestinalis	0.02	[30]
Ulva lactuca	0.01	[30]
Florideophyceae		
Catenella repens	0.02	[30]
Alphaproteobacteria		
Agrobacterium aurantiacum	0.01	[31]
Paracoccus carotinifaciens (NITE SD 00017)	2.2	[32]
Tremellomycetes		
Xanthophyllomyces dendrorhous (JH)	0.5	[33]
Xanthophyllomyces dendrorhous (VKPM Y2476)	0.5	[34]
Labyrinthulomycetes		
Thraustochytrium sp. CHN-3 (FERM P-18556)	0.2	[35]
Malacostraca		
Pandalus borealis	0.12	[20]
Pandalus clarkia	0.015	[36]

2.2 Biological activities of astaxanthin

2.2.1 Antioxidant effect

Astaxanthin has an efficient antioxidant activity 10-fold greater than other carotenoids, including β-carotene, lutein, zeaxanthin, and canthaxanthin,[2, 8, 11] and 500-fold better than an α-tocopherol.[2, 8] Astaxanthin from *H. pluvialis* demonstrated the best protection from free radicals in rats compared with different carotenoids.[2] In addition, the natural extracts of astaxanthin from *H. pluvialis* showed notably more excellent antioxidant activity than synthetic astaxanthin.[9]

2.2.2 Whitening effect

Astaxanthin showed fascinating depigmentation properties. It would protect skin from age spots by reducing melanin production by 40%.[5]

2.2.3 Metal chelating effect

Astaxanthin acts as a metal chelator by converting metal prooxidants into innocuous molecules.[12]

2.3 Characterization of astaxanthin

The UV spectrum of astaxanthin in ethanol solution (3 μg/mL) scanned with ultraviolet and visible light at the 200–800 nm wavelength using a UV spectrophotometer is shown in [Figure 2]. The characteristic absorption peak of astaxanthin is at 476 nm.[13]

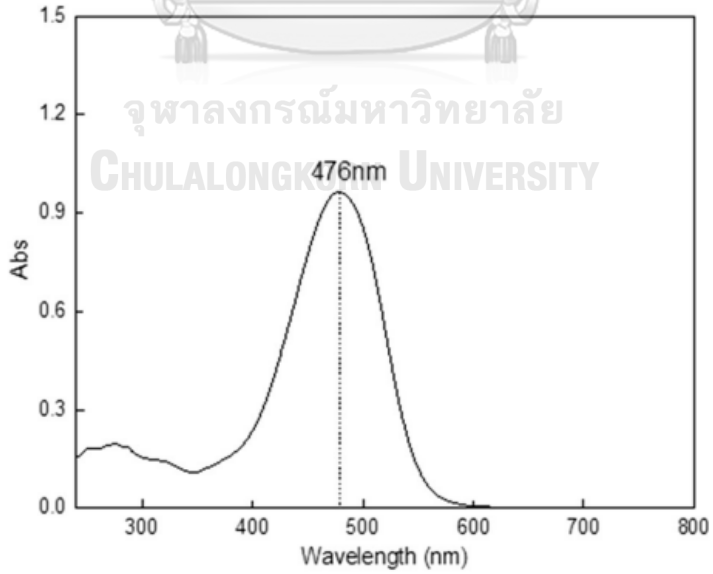

Figure 2 UV-VIS spectrum of astaxanthin dissolved in ethanol[13]

2.4 Determination of antioxidant activity

DPPH method is a rapid, simple, and inexpensive method to evaluate a sample's antioxidant capacity compared to other assays.[14, 15] 1, 1-diphenyl-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl; DPPH) is a stable free radical that acts as a scavenger for hydrogen radical by the delocalization of the spare electron over the molecule so that its molecule does not dimerize. The delocalization of electrons results in deep violet color characterized by an absorption band in ethanol solution at 517 nm. When a solution of DPPH is mixed with a substrate (AH) that can donate a hydrogen atom, this causes the reduced form of DPPH with the loss of violet color, which decreases in absorbance.[14] [Figure 3]

Diphenylpicrylhydrazyl (free radical) Diphenylpicrylhydrazine (nonradical)

Figure 3 Proposed chemical reaction of DPPH assay[14]

2.5 Determination of metal chelating activity

Metal chelating activity is one of the antioxidant mechanisms.[16] Excess transition metal ions can generate hydroxyl radicals [•OH] in biological systems, such as ferrous ions (Fe(II)), the most potent pro-oxidant, and induce hydroxyl radicals via Fenton-like reactions.[17] Ferrozine assay is the one of metal chelating activity evaluation and measures the ability of a sample to compete with ferrozine for the ferrous ions by spectrophotometer. Ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazinep,p′-disulfonic acid) can form a complex with free Fe(II), resulting in a chromophore with absorbance at about 560 nm. Compounds that are able to chelate Fe(II) give rise to decreasing the amount of free Fe(II) in the solution, then reduce the Ferrozine-Fe(II) complex concentration, which results in a loss of absorbance.[17] [Figure 4]

Fe(II) + Ferrozine ⟶ Ferrozine * Fe(II) (highly colored)

Fe(II) + Chelator + Ferrozine ⟶ Chelator*Fe(II) + Ferrozine (less color)
[Fe(II) chelators reduce Ferrozine-Fe(II) formation which generates less color]

Figure 4 Proposed chemical reaction of ferrozine assay[17]

2.6 Determination of anti-tyrosinase activity (Whitening effect)

Naturally, skin stimulates tyrosinase for melanin production to prevent skin damage from ultraviolet A (UVA) and ultraviolet B (UVB) in sunlight. Melanin accumulation results in hyperpigmentation of skin darkening, leading to dermatological disorders, e.g., freckling and age spots. Anti-tyrosinase activity inhibits the tyrosinase enzyme, which converts L-tyrosine to melanin.[18] The melanin synthesis pathway is shown in [Figure 5]. Anti-tyrosinase molecule inhibits tyrosinase activity by chelating copper (Cu) atoms of the tyrosinase molecule. Tyrosinase enzyme without Cu atoms slows down L-tyrosine to melanin transformation and decreases melatonin accumulation, reducing skin hyperpigmentation.[18]

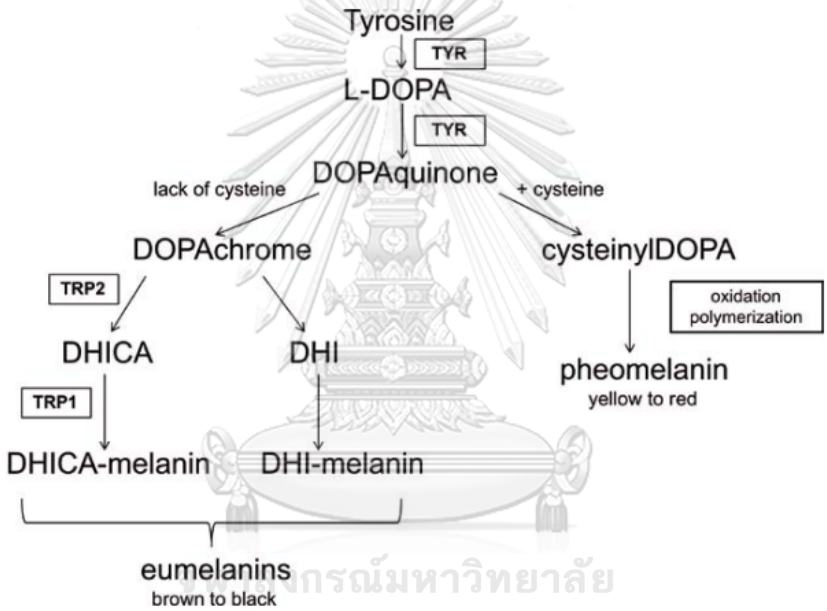

Figure 5 The simplified scheme of the melanin synthesis – tyrosinase (TYR), tyrosine-related protein 1 (TRP1) and 2 (TRP2).[19]

Chapter 3 Materials and Methods

3.1 Materials

Astaxanthin oil (AstaTROL® Hp), which contains NLT 5.0% as a free form of astaxanthin extracted from *Haematococcus pluvialis* was purchased from AstaReal Co., Ltd. Astaxanthin oleoresin (AstaKey K100), which contains NLT 10% astaxanthin extracted from dried *Haematococcus pluvialis* was purchased from SagaNatura ehf. Ethanol was purchased from RCI Labscan Co Ltd. Kojic acid, L-Tyrosine, mushroom tyrosinase, 2,2-Diphenyl-1-picrylhydrazyl, (±)-α-Tocopherol, iron (II) chloride tetrahydrate and 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p, p′-disulfonic acid monosodium salt hydrate were purchased from Sigma-Aldrich. Ethylenediaminetetraacetic acid was purchased from Carlo Erba Reagents S.A.S. Ultra-water from the faculty of pharmaceutical sciences at Chulalongkorn University.

3.2 Methods

3.2.1 Preparation of astaxanthin concentrations

A stock solution of Astaxanthin oil (5,000 µg/mL) was prepared in ethanol. A stock solution of astaxanthin oleoresin (720 µg/mL and 5,000 µg/mL) was prepared in ethanol using a gentle magnetic stirrer to make it clear and all dissolved. Different diluted concentrations of astaxanthin oil and oleoresin in ethanol were prepared using a micropipette. The samples were wrapped with parafilm and aluminum foil and stored in the refrigerator.

3.2.2 Characterization of astaxanthin oils and oleoresins

Characterization of astaxanthin oils and oleoresins was analyzed by UV spectrophotometry. Diluted concentrations (400-800 µg/mL) of astaxanthin oil (200 µL) and oleoresin (100 µL) were pipetted into a cuvette. Ethanol was added to make the final volume which is 2,000 µL. Sample and blank (ethanol) were scanned with ultraviolet and visible light at the 300–700 nm wavelength using a UV-VIS spectrophotometer (Thermo Scientific Evolution 300).

3.2.3 Determination of antioxidant activity

Antioxidant activity was determined by DPPH radical-scavenging capacity assay according to the modified method of Takei et al. (2017)[19]. 100 µL of diluted astaxanthin oil (600, 800, 1,000, 1,200, and 1,400 µg/mL) or oleoresin (100, 200, 300, 400, and 500 µg/mL) and 100 µL of ethanol were added into a 96-well microplate. Absorbance at 517 nm **(A)** was measured using a microplate reader (CALIOstar). Subsequently, 25 µL of 1 mmol/L DPPH was added to the earlier solution. Then, the 96-well microplate was wrapped with aluminum foil and incubated at room temperature for 30 minutes. Absorbance at 517 nm **(B)** will be measured using a microplate reader (CALIOstar). **α**-Tocopherol (4.5, 9, 18, 22.5, and 27 µg/mL) and ethanol were used as positive control and blank, respectively. DPPH radical scavenging capacity (%) was calculated as:

DPPH radical scavenging capacity (%)

$$= [(\text{Blank} - \text{Sample})/\text{Blank}] \times 100$$

$$= [(B_{blank} - A_{blank}) - (B_{sample} - A_{sample})/(B_{blank} - A_{blank})] \times 100$$

Where, A: Absorbance at 517 nm of samples and ethanol

B: Absorbance at 517 nm of samples after adding all reagents and incubating for 30 min

All assays were carried out in triplicate, and results were expressed as IC_{50}, as the concentration yielding 50% DPPH radical scavenging capacity, calculated by interpolation from the Radical scavenging capacity (%) vs. concentration curve.

3.2.4 Determination of metal chelating activity

Metal chelating activity was determined according to the ferrous ion chelating activity method of Murugan et al. (2013)[20] with minor modifications. 100 µL of diluted astaxanthin oil (400, 500, 600, 700, and 800 µg/mL) or oleoresin (18, 36, 72, 144, and 288 µg/mL), 100 µL of ultra-water, and 25 µL of 0.5 mM ferrous chloride solution were added in a 96-well microplate. Absorbance **(A)** at 544 nm will be measured instantly using a microplate reader (CALIOstar). Then, 25 µL of 2.5 mM ferrozine solution was added. The 96-well microplate was wrapped with aluminum foil and incubated in the dark for 20 minutes at room temperature. Absorbance **(B)** at 544 nm will be detected. EDTA (1.25, 2.5, 12.5, 25, and 37.5 µg/mL) and ethanol were used as positive control and blank, respectively. Percentage of ferrous ion chelating activity was calculated as:

Ferrous ion chelating activity (%)

$$= [(\text{Blank} - \text{Sample})/\text{Blank}] \times 100$$

$$= [(B_{blank} - A_{blank}) - (B_{sample} - A_{sample})/(B_{blank} - A_{blank})] \times 100$$

Where, A: Absorbance at 544 nm of samples, ultra-water, and ferrous chloride

B: Absorbance at 544 nm of samples after adding all reagents and incubating for 20 min

All assays were carried out in triplicate, and results were expressed as IC50, the concentration of the sample that chelated 50% of the ferrous ion, calculated by interpolation from the ferrous ion chelating activity (%) vs. concentration curve.

3.2.5 Determination of anti-tyrosinase activity

Anti-tyrosinase activity was determined according to Namjoyan et al. (2019)[21] with slight modifications. 100 µL of 200 units/mL mushroom tyrosinase in phosphate buffer was added to 50 µL of diluted astaxanthin oil or oleoresin (125, 250, 500, 1,000, and 2,000 µg/mL) in a 96-well microplate. The absorbance **(A)** of wells will be recorded at 475 nm with a microplate reader (CALIOstar). Then, 100 µL of 1.5 mM L-tyrosine was added to the mixture reaction. The mixture will be incubated at room temperature for 10 min, and absorbance **(B)** was then measured at 475 nm. Kojic acid (250, 375, 500, 750, and 1,000 µg/mL) and ethanol were used as positive control and blank, respectively. Percentage of inhibition of tyrosinase activity was calculated as:

Inhibition (%) $= [(\text{Blank} - \text{Sample})/\text{Blank}] \times 100$

$$= [(B_{blank} - A_{blank}) - (B_{sample} - A_{sample})/(B_{blank} - A_{blank})] \times 100$$

Where, A: Absorbance of the enzyme and sample solution/blank

B: Absorbance of the enzyme, L-tyrosine, and sample solution/blank

All assays were carried out in triplicate, and results were expressed as IC50, which is the concentration of the sample that inhibits 50% of the enzyme activity, calculated by interpolation from the % tyrosinase inhibition vs. concentration curve.

3.2.6 Statistic and analysis

The results will be analyzed by using Student's t-test as applicable.

Chapter 4 Results

4.1 Characterization of astaxanthin oils and oleoresins

UV-VIS spectrum of astaxanthin oils and oleoresins scanned at 300–700 nm wavelength as shown in [Figure 6]. The spectrum of astaxanthin oils, whose concentrations were higher than oleoresins two times, was similar to that of astaxanthin oleoresins. The characteristic absorbance peak of both is at 476 nm.

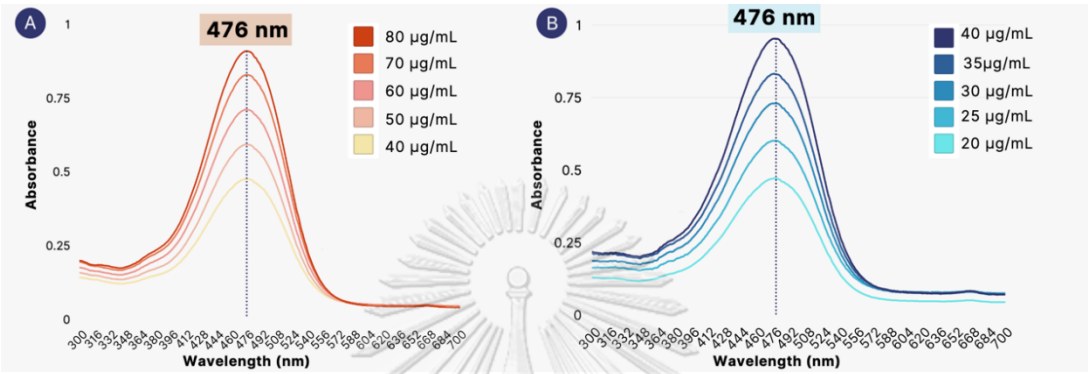

Figure 6 UV-VIS spectrum of astaxanthin oil (A) and oleoresin (B) dissolved in ethanol

4.2 Determination of antioxidant activity

DPPH radical-scavenging capacity assay, five different concentrations of astaxanthin oil (600, 800, 1,000, 1,200, and 1,400 µg/mL) and oleoresin (100, 200, 300, 400, and 500 µg/mL) were tested with alpha tocopherol (4.5, 9, 18, 22.5, and 27 µg/mL) as positive control. Graphs were plotted [Figure 7-9] and IC50 value of astaxanthin oil on DPPH was 0.4981 ± 0.17 mg/mL [Table 2].

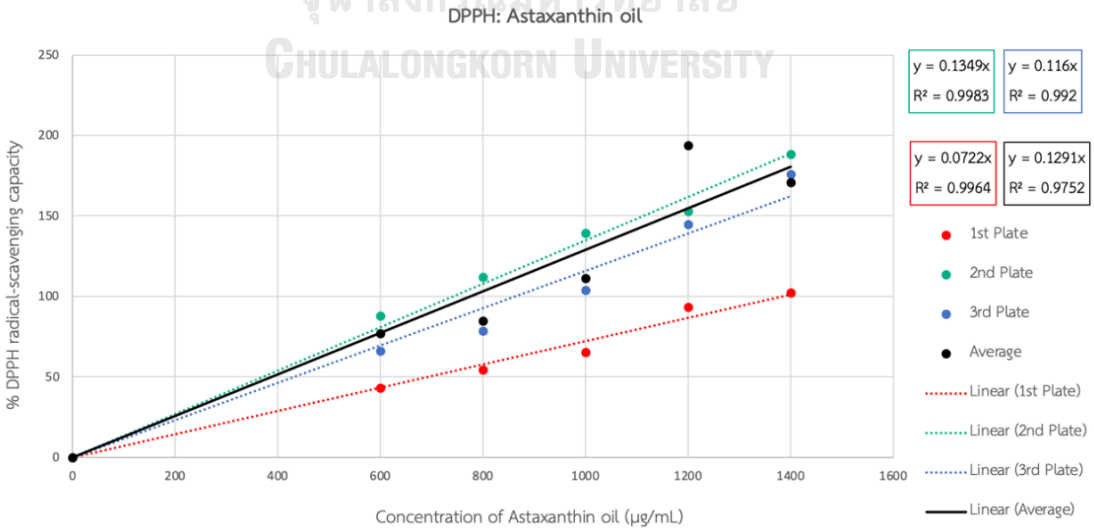

Figure 7 Scavenging activity of astaxanthin oil (600, 800, 1,000, 1,200, and 1,400 µg/mL)

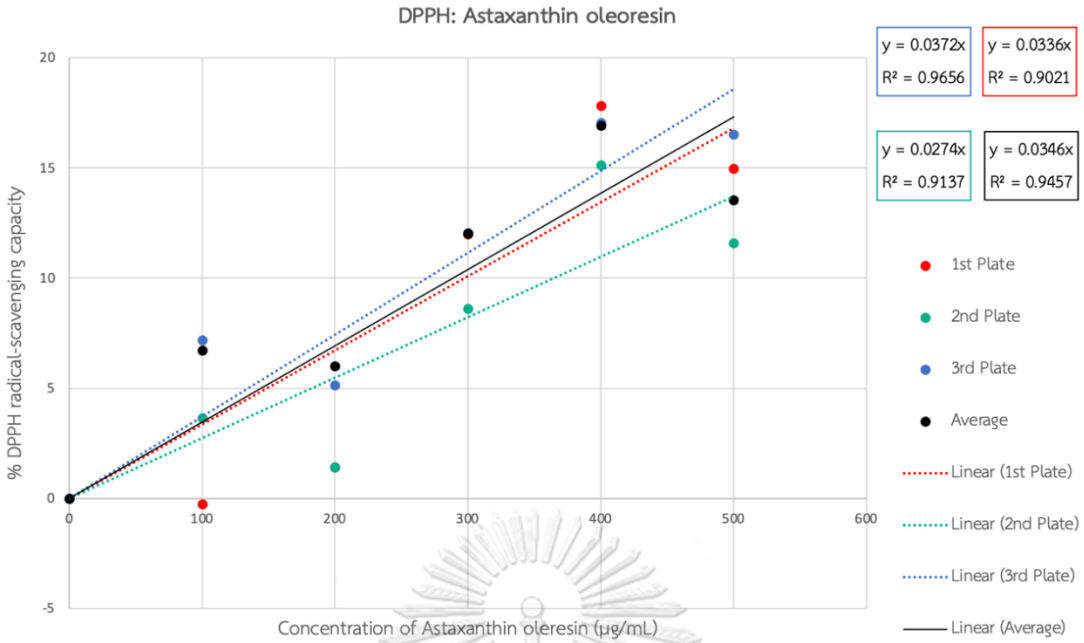

Figure 8 Scavenging activity of astaxanthin oleoresin (100, 200, 300, 400, and 500 µg/mL)

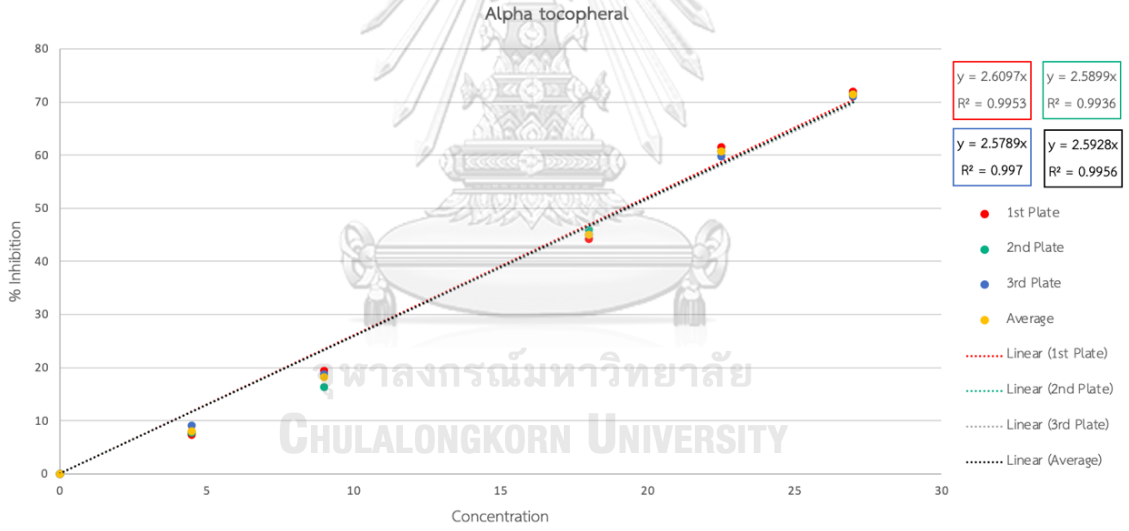

Figure 9 Scavenging activity of alpha tocopherol (4.5, 9, 18, 22.5, and 27 µg/mL)

4.3 Determination of metal chelating activity

Ferrous ion chelating activity assay, five different concentrations of astaxanthin oil (400, 500, 600, 700, and 800 µg/mL) and oleoresin (18, 36, 72, 144, and 288 µg/mL) were tested with EDTA (1.25, 2.5, 12.5, 25, and 37.5 µg/mL) as positive control. Graphs were plotted [Figure 10-12], indicating that astaxanthin oil and oleoresin were no ferrous chelating activity.

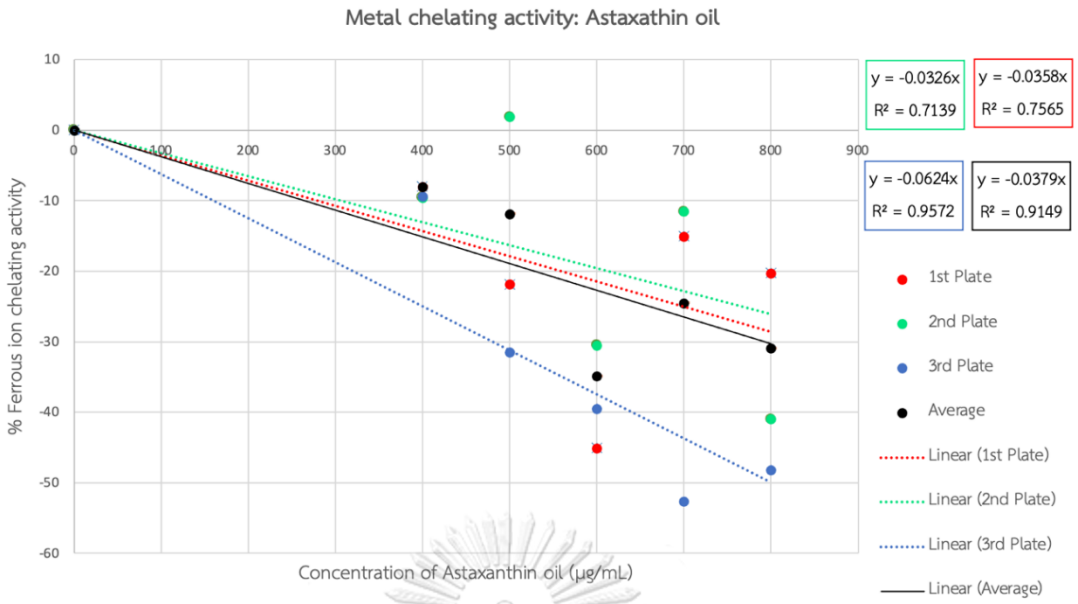

Figure 10 Metal chelating activity of astaxanthin oil (400, 500, 600, 700, and 800 µg/mL)

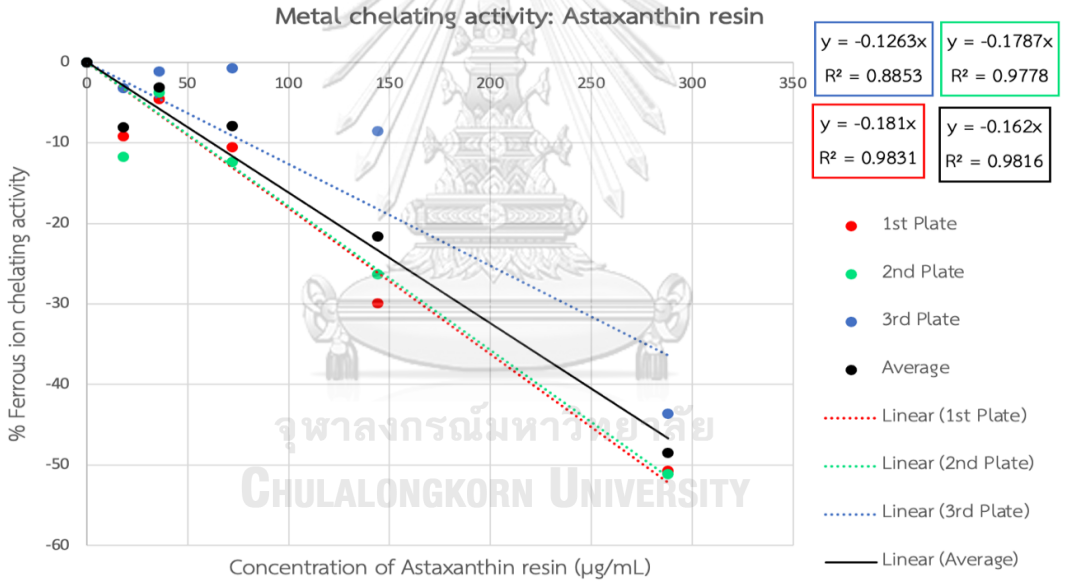

Figure 11 Metal chelating activity of astaxanthin oleoresin (18, 36, 72, 144, and 288 µg/mL)

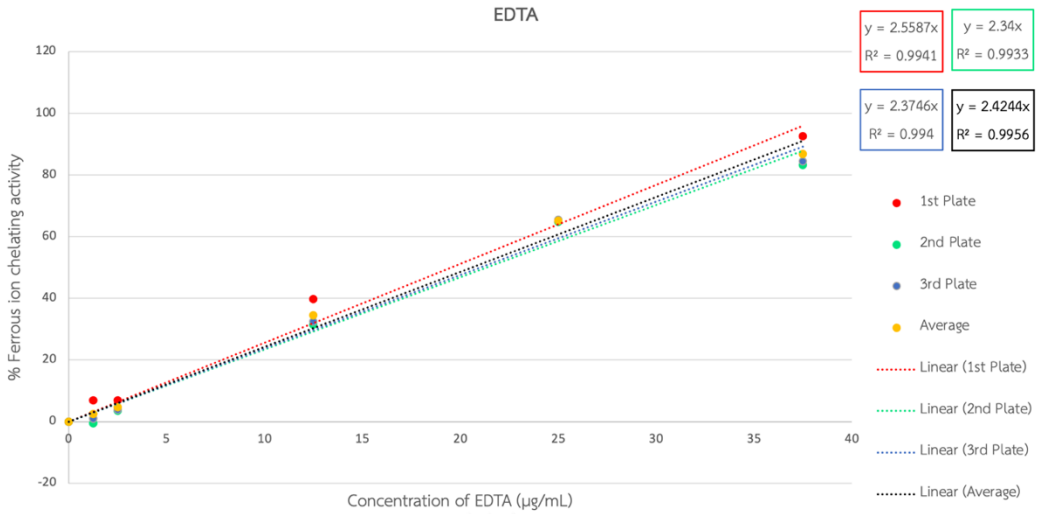

Figure 12 Metal chelating activity of EDTA (1.25, 2.5, 12.5, 25, and 37.5 µg/mL)

Table 2 Activity of astaxanthin oil and oleoresin in assays

Assay	Sample	Result	N
DPPH radical-scavenging capacity	AST* oil	IC_{50}** = 0.4981 ± 0.17 mg/mL	3
		At 500 µg/mL: %Radical scavenging capacity = 53.85%	
	AST oleoresin	At 500 µg/mL: %Radical scavenging capacity = 16.22%	3
	Alpha tocopherol	IC_{50}** = 19.25 ± 0.08 µg/mL	3
Ferrous ion chelating activity	AST oil	No metal chelating activity	6
	AST oleoresin	No metal chelating activity with concentration-dependent at low concentrations	6
	EDTA	IC_{50}** = 20.65 ± 0.98 µg/mL	3
Anti-tyrosinase activity	-	No experiment was conducted	-
**AST = astaxanthin, *IC_{50} values are mean ± SD			

Chapter 5 Discussion and Conclusion

5.1 Discussion

Astaxanthin from *H. pluvialis* in different forms and extraction methods could cause different potential activities. Although the characteristics profiles of astaxanthin oil and oleoresin were identical, their activities differed. Astaxanthin oil and oleoresin had a similar UV-VIS spectrum at the wavelength of 300 – 700 nm, and the absorbance peak (λ_{max}) of both extracts was at 476 nm, which was in accordance with previous studies conducted on the characterization of astaxanthin dissolved in ethanol.[13] The UV-VIS spectrum of astaxanthin oils and oleoresins demonstrated that oleoresins at lower concentrations than oil gave similar absorbance to higher oil concentrations. Of note, the oleoresin form has an astaxanthin concentration than the oil form. However, the oil form was more potent in antioxidant capacity than the oleoresin form when conducting the DPPH assay. The IC_{50} value of DPPH radical-scavenging capacity for astaxanthin oil is 0.4981 ± 0.17 mg/mL, and its scavenging capacity is robustly correlated with concentration. In addition, the radical-scavenging capacity of astaxanthin oil was three times higher than that of oleoresin by comparing at the concentration of 500 µg/mL. A possible explanation for this might be that the composition of oil form comprised tocopherol with astaxanthin, whereas oleoresin did not compose tocopherol, enhancing antioxidant capacity. Regardless, the antioxidant activity of astaxanthin oil was less potent than alpha-tocopherol (positive control). Our results differ from studies that reported that the antioxidant activity of astaxanthin was better than alpha-tocopherol.[2, 8]

Furthermore, the metal-chelating activity of astaxanthin oil and oleoresin negatively related to concentration exhibited that both oil and oleoresin had no ferrous chelating activity. It seems that oil and oleoresin could increase free Fe(II) in the solution, then increase the complex concentration between Fe(II) and ferrozine, increasing absorbance. This result also indicated that the antioxidant activity of astaxanthin oil and oleoresin is not due to metal chelating activity, even though metal chelating activity is one of the antioxidant mechanisms.[16] Additionally, the oleoresin form showed an interesting result that was at the low concentrations (18, 36, 72, 144, and 288), the metal-chelating effects on Fe(II) decreased when extract concentrations increased. Unfortunately, anti-tyrosinase activity was not conducted in this study due to enzyme degradation. Finally, this study speculated that the extraction form and techniques of astaxanthin could affect the biological activities of astaxanthin.

5.2 Conclusion

Astaxanthin oil from *H. pluvialis* can be preferably used as an active ingredient in pharmaceutical products and cosmetic formulations because of its antioxidant property. Astaxanthin oil and oleoresin from *H. pluvialis* have significantly different biological activities. The form, composition, and extraction technique of astaxanthin extract from *H. pluvialis* could affect the activities of astaxanthin. This study can be applied to select suitable extract forms of astaxanthin that could be used to research and develop astaxanthin products.

5.3 Recommendations for future research

Further studies are required to confirm the metal chelating activity of astaxanthin extract from *H. pluvialis* with other optimal assays and investigate the anti-tyrosinase activity of astaxanthin, which could be attractively used in cosmetic products for whitening effects.

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