ANTIMICROBIAL ACTIVITIES OF VEGETABLE OIL-EXTRACTED ASTAXANTHIN FROM MICROALGAE *Haematococcus pluvialis*

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Abstract

This study aimed to extract astaxanthin from algae using bio-solvent vegetables oils and investigate for antimicrobial potential of the crude extract. Microalgae *Haematococcus pluvialis* was cultivated in appropriated conditions and extracted for astaxanthin. The crude extracts were tested for antimicrobial activities with the panel of test microorganisms; four strain of bacteria and one strain of yeast. The highest astaxanthin content was expressed in crude extract of coconut oil after 48 hrs of extraction. The coconut oil extract susceptible to all 5 tested microorganisms, while tea seed oil, olive oil, rice bran oil extracts susceptible to 2 test microorganisms, sesame and grapes seed oil extracts susceptible to 1 test microorganism, where as astaxanthin extracted with palm and sweet almond oils did not show any antimicrobial activity. Bacterial tested microorganisms showed more susceptibility to oil extracted than yeast since *Escherichia coli* susceptible to 4 extracts (coconut, tea seed, sesame, and olive oils), *Salmonella typhimurium* susceptible to 3 extracts (coconut, rice bran, and grapes seed oils), *Pseudomonas aeruginosa* susceptible to 2 extracts (coconut and olive oils), *Staphylococcus aureus* susceptible to 3 extracts (coconut, tea seed, and rice bran oils), while yeast *Candida albicans* susceptible to only coconut oil extract. A 100% of microorganism was susceptible to coconut oil extracted astaxanthin, while 100% of microorganism resistant to palm and sweet almond oil extracts.

Keywords: algae, antimicrobial activity, astaxanthin, Haematococcus pluvialis, vegetable oil

Introduction

The growing interest in the cosmetic formulation with natural ingredients continues to raise the demand. Along with this trend, the possibility of replacing synthetic preservatives with natural ones receiving much attention. These natural preservatives are preferred to come from plants, bacteria, fungi, and algae. Several reports have been explored the antimicrobial activities of different liquid extracts obtained from microalgae *H. pluvialis* and found that the highest antimicrobial activities of the ethanolic extract corresponding to red cyst of *H. pluvialis* (Santoyo *et al*. 2009).

The red cyst of *H. pluvialis* enclosing the red pigment which was identified the principal carotenoid as astaxanthin. *Haematococcus pluvialis* is potential to synthesis and accumulate a large amount of astaxanthin up to 80% of dry cell weight under induced condition of various stresses (Park *et al.* 2009, Kim *et al.* 2011). Astaxanthin is an attractive compound in food, cosmetics, and various pharmaceutical applications (Kim *et al.* 2011) since it has several essential biological functions including strong potent antioxidant, protection against UV light, enhance immune response, and strong coloring agent (Guerin *et al.* 2003).

Nevertheless, the thicken wall of red cyst *H. pluvialis* is a major difficulty to extract astaxanthin. This problem resulted to extraction method using organic solvent (e.g. ethanol and hexane) comprise high energy consumption, multiple separation steps, and remain undesirable chemicals which not suitable for cosmetic formulation (Kang and Sim 2008), and those extracts were signified on the benefits and activities of astaxanthin in antioxidant activity, but less emphasized for its antimicrobial activity.
This study aimed to utilize the antimicrobial property of astaxanthin extracted from *H. pluvialis* using bio-solvent vegetable oils for further application as natural preservative in cosmetic formulation.

Materials and Methods

Cultivation of *H. pluvialis* for astaxanthin accumulation
Haematococcus pluvialis was obtained from Thailand Institute of Scientific and Technological Research, Thailand, and cultured in Modified COMBO Medium (MCM) which composed of the following (mg/L): KNO_3 200, KH_2PO_4 20, $NaHCO_3$ 450, $MgSO_4 \cdot 7H_2O$ 100, V_{B12} 4×10^{-3}, $Na_2EDTA \cdot 2H_2O$ 3.36, $FeCl_3 \cdot 6H_2O$ 2.44, $ZnCl_2$ 4.1×10^{-3}, H_3BO_3 6.1×10^{-3}, $CoCl_2 \cdot 6H_2O$ 5.1×10^{-3}, $MnCl_2 \cdot 4H_2O$ 4.1×10^{-3}, $(NH_4)_6Mo_7O_{24} \cdot 7H_2O$ 38×10^{-3} (Liu *et al.* 2002). The culture flasks were incubated on 60 rpm shaker. To induce high astaxanthin biosynthesis (red phase), exponentially grown cultures were continuously illuminate with 4000 Lux at 28±1 °C. Cell morphology and cell number were observed daily. After day 3, 6, 9, and 12 of cultivation, cells were harvested by centrifugation at 8000 rpm for 10 min. The culture broth was discarded. The packed cell were re-suspended in new culture broth medium and counted the cell using hemacytometer under light compound microscope, and further extraction for astaxanthin.

Extraction of astaxanthin from *H. pluvialis* with vegetable oils
The extraction method was performed following Kang and Sim (2008) with some modification. A 5 ml of 3.75×10^6 cells/ml of induced cyst culture was mixed with 5 ml of each commercial vegetable oil (coconut oil, grapes seed oil, olive oil, palm oil, rice bran oil, tea seed oil, sesame oil, and sweet almond oil). The mixture was allowed to vigorous stirring on magnetic stirrer at room temperature for 48 hrs. The mixture was allowed to settle under gravity for 10 min after stopped the stirring before collected the oil extracted supernatant (top layer) (Figure 1). The absorbance of the oil extract was scanned for astaxantain content with UV spectrophotometer at 350 to 650 nm using original oil as blank and astaxanthin was quantified at 480 nm compared with standard astaxanthin (0.5 µg/ml). The vegetable oil which provided the greatest astaxanthin content was selected for further preparation of astaxanthin.

Figure 1 The separation layer of the oil phase and water phase of astaxanthin extraction

Antimicrobial activity assay

The astaxanthin extracts were individually tested against a panel of microorganisms including one strain of yeast *C. albicans*, and four strains of bacteria *E. coli*, *P. aeruginosa*, *S. typhimurium*, and *S. aureus*. The antimicrobial activities were tested using disc diffusion method. A 1 ml of test organism at concentration of 10^8 cells/ml was spread on Mueller-Hinton agar plate, and yeast was spread on Sabouraud dextrose agar (SDA) plate. The sterile paper discs (6 mm diameter) were aseptically soaked with 15 µl astaxanthin extract, dried out in laminar air flow, and placed on the spread plate. Negative control was a paper disc containing the counterpart vegetable oils. Positive controls for *C. albicans* was Ampicillin (AM-10µg, Difco), for *P. aeruginosa* was Chloramphenicol (C-30 µg, Difco), for *E. coli* was Streptomycin (S-10 µg, Difco), for *S. aureus* and *S. typhimurium* was Tetracycline (TE-30 µg, Difco). The assayed plates of bacterial strain were incubated at 37 °C for 24 hrs and *C. albicans* culture plates were incubated at 30 °C for 72 hrs. The inhibition zone around discs was observed and the diameter of clear zone was measured.

Results

Cell growth and astaxanthin accumulation.

Throughout twelve days of cultivation, cellular accumulation of astaxanthin was progressively increased during the transformation of green to red cells. The accumulation of astaxanthin was started on day one of cultivation while an increasing in cell size started on day six. At the end of the induction period, most cells had formed enlarge red cysts with a thick cell wall (Figure 2). The amount of astaxanthin was gradually increased to the highest since the cultivation was nine days, which was equal to twelve days cultivation (Figure 3). Therefore, to save the time, energy and obtained the freshest cells, a 9-day cultivation of *H. pluvialis* were prepared for astaxanthin extraction. A distinctive developmental feature of the green algae *H. pluvialis* is the transition from a green to a red stage with an auxiliary accumulation of astaxanthin in the cytoplasm under the inductive conditions and dramatic change in cell morphology including of thickening of the cell wall and significant increase in cell volume (He *et al.* 2007).

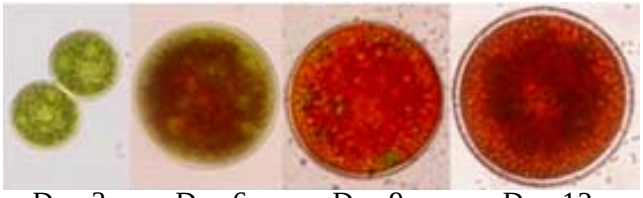

Figure 2 Cell morphology differentiated throughout twelve days of cultivation examined under light microscope

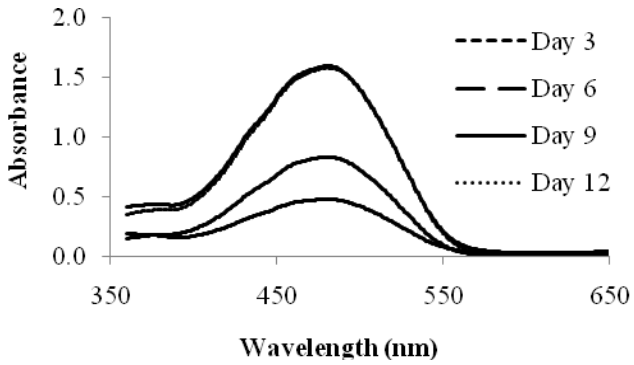

Figure 3 Absorbance spectra of astaxanthin extract throughout twelve days of cultivation. All samples were diluted 10 fold before measuring the absorbance

Extraction of astaxanthin from *H. pluvialis* with appropriate vegetable oils.

The red pigment astaxanthin was extracted into vegetable oil phase (top layer) due to their hydrophobic property. The different of polarity between cell culture (water phase) and vegetable oils can creates a phase separation, and the oil can separate from cell culture by gravity. Almost of all astaxanthin was extracted from the red cyst cell to each vegetable oil phase after extraction for 48 hrs. The redness color of the oil phase deepened in which astaxanthin has specific absorbance peak at 480 nm (Kang and Sim 2008, Sindhu and Sherief 2011). The absorbance peak at 480 nm of individual 8 oil extracts was compared with the counterpart base oil and standard astaxanthin (Figure 4). Among 8 oil extracts, the highest astaxanthin content was in coconut oil extract (Figure 4).

Antimicrobial activities of oil extracted astaxanthin.

The data pertaining to the antimicrobial potential of the oil-extracted astaxanthin extracts are presented in Table 1. The astaxanthin extracted with coconut oil, tea seed oil, sesame oil, olive oil, rice bran oil, and grapes seed oil presented antimicrobial activity to at least one of tested microorganisms.
The tested bacteria showed more susceptibility to oil extracted astaxanthin than yeast since *E. coli* showed susceptible to 4 extracts (coconut, tea seed, sesame, and olive oils), *S. typhimurium* showed susceptible to 3 extracts (coconut, rice bran, and grapes seed oils), *P. aeruginosa* showed susceptible to 2 extracts (coconut and olive oils), *S. aureus* showed susceptible to 3 extracts (coconut, tea seed, and rice bran oils), while yeast *C. albicans* showed susceptible to only one extract of coconut oil (Table 1). A 100% of microorganism was susceptible to coconut oil extracted astaxanthin, while 100% of microorganism resistant to palm and sweet almond oil extracts.
Since coconut oil extract is interested as its susceptibility against all tested microorganisms. The point of concentration of astaxanthin in the coconut oil extract was determined for the correlation of extract dilution to the absorbance at 480 nm (Figure 5), which known to be the peak absorption of astaxanthin. As Figure 5, the linearity value of R^2 of 0.9973 was highly related between extract concentrations and absorption of astaxanthin implied that astaxanthin in the extract would be the active compound against tested microorganisms.

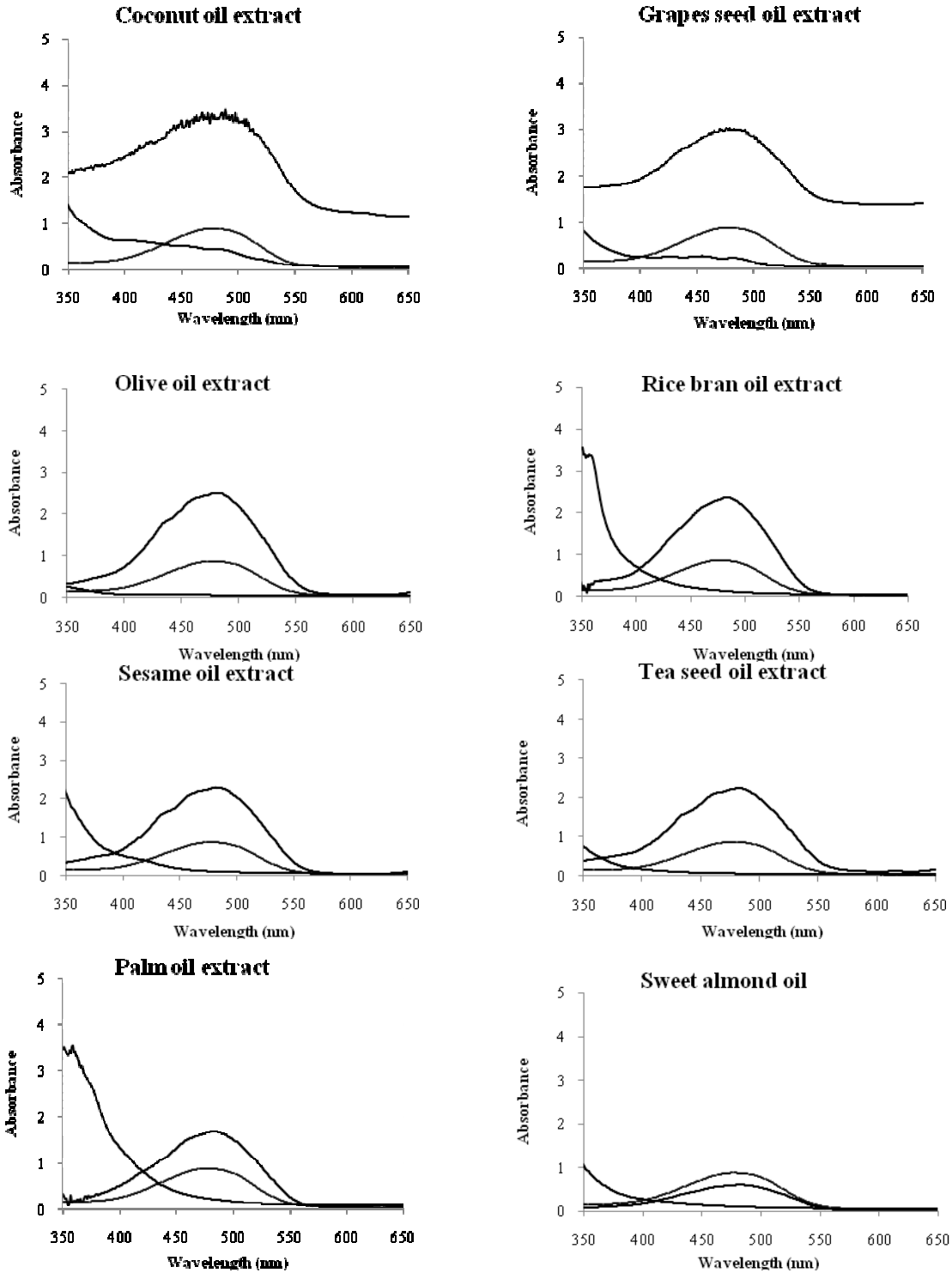

Figure 4 Absorbance spectra of each oil extract compared with reference base oil and astaxanthin standard; ——= Extract, – –= Reference oil, ····· = Astaxanthin standard

Table 1 Antimicrobial activities of undiluted oil extracted astaxanthin throughout agar diffusion method

Tested disc	Inhibition zone of the oil extracted astaxanthin (mm)							
	Coconut	Tea seed	Sesame	Olive	Rice bran	Grapes seed	Palm	Sweet almond
E. coli								
Reference oil	8.5±1.0	7.5±1.5	7.0±1.5	7.5±1.25	-	-	-	-
Test sample	11.5±1.5 (+)	10.0±1.5 (+)	9.5±1.0 (+)	9.5±1.5 (+)	-	-	-	-
*Streptomycin	12.0±1.5	12.0±1.5	12.0±1.5	12.0±1.5	12.0±1.5	12.0±1.5	12.0±1.5	12.0±1.5
S. typhimurium								
Reference oil	7.5±0.5	-	-	-	7.0±0.5	7.5±0.5	-	-
Test sample	9.5±1.0 (+)	-	-	-	9.5±0.5 (+)	10.0±1.5 (+)	-	-
*Tetracycline	26.0±1.5	26.0±1.5	26.0±1.5	26.0±1.5	26.0±1.5	26.0±1.5	26.0±1.5	26.0±1.5
P. aeruginosa								
Reference oil	7.5±0.5	-	-	6.5±0.5	-	-	-	-
Test sample	10.5±1.5 (+)	-	-	9.5±1.0 (+)	-	-	-	-
*Chloramphenicol	14.0±1.5	14.0±1.5	14.0±1.5	14.0±1.5	14.0±1.5	14.0±1.5	14.0±1.5	14.0±1.5
S. aureus								
Reference oil	7.5±0.5	8.5±1.0	-	-	6.5±0.5	-	-	-
Test sample	11.0±1.5 (+)	10.5±1.5 (+)	-	-	8.5± 0.5 (+)	-	-	-
*Tetracycline	36.0±2.0	36.0±2.0	36.0±2.0	36.0±2.0	36.0±2.0	36.0±2.0	36.0±2.0	36.0±2.0
C. albicans								
Reference oil	7.5±0.5	-	-	-	-	-	-	-
Test sample	10.0±1.0 (+)	-	-	-	-	-	-	-
*Ampicillin	17.5±1.5	17.5±1.5	17.5±1.5	17.5±1.5	17.5±1.5	17.5±1.5	17.5±1.5	17.±1.5

* = Positive control

\- = Absence of inhibition zone

(+) = Susceptible (inhibition zone wider than the reference oil)

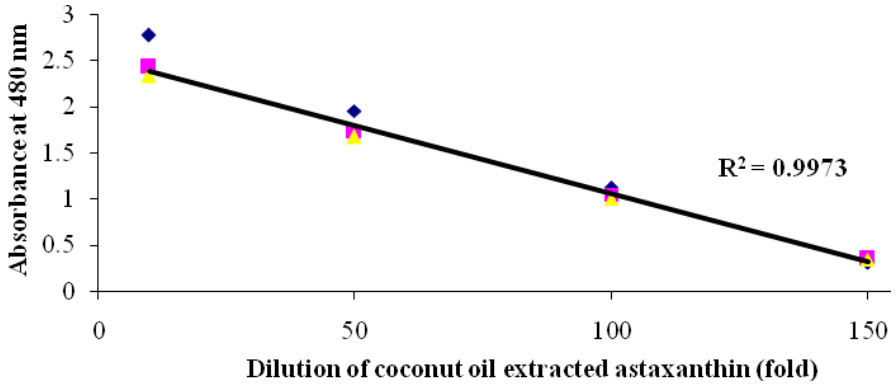

Figure 5 The correlations of coconut oil extract dilutions and astaxanthin concentration

Discussion

The direct extraction of *Haematococcus* astaxanthin have been performed by using common vegetable oils such as soybean oil, corn oil, grapes seed oil, and olive oil, with recovery yield of over 87.5%. The highest recovery yield of astaxanthin has been indicated in olive oil extract (Kang and Sim 2008). Current study, the olive oil was also included and compared with other 7 vegetable oils which commonly use as cosmetic ingredients. Coconut oil extract and grapes seed oil extract were efficiently provided higher astaxanthin content than olive oil extract. Therefore, the high performance extraction of astaxanthin from *H. pluvialis* was now recommended using of coconut oil.

Coconut oil extracted astaxanthin was susceptible to inhibited growth of both Gram-positive and Gram-negative bacteria, and also inhibit fungal strain. Coconut oil, itself has been confirmed to possess antimicrobial, antiviral and antiprotozoal. The antimicrobial activity of coconut oil had been attributed to the carboxylic acid–monolaurin metabolized to lauric acid in the body (Obi *et al.* 2005, Oyi *et al.* 2010).

Conclusion

Astaxanthin received from direct extraction of encysted *H. pluvialis* culture using bio-solvent vegetable oils without using of organic solvent was an environmental friendly and a green process with reduction of separation steps and energy consumption, and may able to apply in cosmetic formulation as natural preservative ingredient.

Acknowledgments

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