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Antioxidant and antimicrobial activities of astaxanthin from *Penaeus monodon* in comparison between chemical extraction and High Pressure Processing (HPP)

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Abstract

The use of High Pressure Processing as an extraction method was studied by evaluating the yield of astaxanthin from shrimp carapace as a model. Previous studies have demonstrated the antioxidant and antimicrobial properties of astaxanthin. The aim of this research was to compare these properties of astaxanthin as a surrogate for its yield from High Pressure Processing (HPP) extraction with the effect of hydrostatic pressure, holding time and amount of solvents versus chemical extraction method. A solvent mixture of acetone and methanol 7:3 (v/v) was used in both methods. The pressure treated was at 238 MPa with 16.29 min of holding time and 6.59 ml of solvents for HPP method. Antioxidant activity was evaluated using scavenging activity of DPPH radical, the reducing activity of Ferrum redox reaction and oxygen radical absorption capacity. Antimicrobial activity was evaluated using a zone of inhibition test against four strain of bacteria: *E. coli, E. aerogenes, S. aureus* and *B. subtilis*. The sample of astaxanthin demonstrated a significant increase in DPPH radical scavenging activity (25.47% to 87.90%), reducing activity of Ferrum redox reaction (2.86 µmol TE/g to 8.13 µmol TE/g) and oxygen radical absorption capacity (2,000 µmol TE/100 g to 4,000 µmol TE/100 g) compared to the chemical extraction sample. The antimicrobial activity of the astaxanthin from the HPP sample produced a greater zone of inhibition against all four strains of bacteria when compared to the chemically extracted sample. A higher quality of astaxanthin was achieved with the HPP extraction method compared to chemical extraction.

Introduction

Astaxanthin extraction from shrimp carapace is a cost effective way to produce astaxanthin. This carapace offers a low-cost raw material source (Jeddi *et al*., 2013). Previous studies have demonstrated antioxidant and antimicrobial properties of this bioactive compound (Kumaresan *et al*., 2008; Ushakumari and Ramanujan, 2013). The potential immune enhancing and health benefits of astaxanthin would be of interest given its use in the aforesaid industries (Guerin *et al*., 2003; Hussein *et al*., 2006; Chang-hwan *et al.,* 2012; Zhang *et al*., 2014). The study of the power of astaxanthin as antioxidant has proven (Yamashita, 2013). Astaxanthin is the best antioxidant than another antioxidant even though in general vitamin E has been touted for cosmetic properties. It is also interesting to note that although β-carotene has a similar structure with astaxanthin, the antioxidant power is not the same (Yamashita, 2013).

Currently, HPP technology has been applied to the extraction of bioactive compounds from various sources such as tomato, papaya and potato (Jun, 2006; Cardoso *et al*., 2013; Uribe, 2015). This advanced technology is a powerful way to extract bioactive compounds, leaving less structural damage and colour changes, as well as the higher stability of substrate (Gamlath and Wakeling, 2011). It is present to be more effective at increasing the yield of astaxanthin in order to increase the antioxidant activity. Studies have shown that quality of products processed by HPP is superior to those processed by chemical extraction (Yordanov and Angelova, 2010; Penchalaraju and Shireesha, 2013).

The quality of food treated using HPP is higher

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than other methods because HPP is more effective at reducing spoilage and inactivating microorganisms (Penchalaraju and Shireesha, 2013). Therefore, it is possible to drive consumers demand for HPP products as a way to maintain the natural appearance and the shelf life. These benefits of this innovation – namely the convenience of longer shelf-life and optimal food quality – in food preservation may lead consumers to opt for HPP technology (Hjelmqwist, 2007).

There are two methods that used in this study, chemical extraction and High Pressure Processing (HPP). Efficiency between these methods were investigated according to additional pressure that affects of HPP. In this study, a comparison of antioxidant (DPPH, FRAP and ORAC) and antimirobial activities of astaxanthin extracted from shrimp carapace (Penaeus monodon) was reported. Four strains of bacteria tested were *Escherichia coli, Enterobacter aerogenes, Staphylococcus aureus* and *Bacillus subtilis.*

Material and methods

Chemical Extraction

Extraction of astaxanthin was carried out by chemically (Othman, 2009; Radzali *et al*., 2014). One gram of powdered sample of each species of shrimp carapace was extracted at room temperature by adding 5 ml of a mixture of acetone and methanol 7:3 (v/v) until the discolouration was noted. Then, samples were centrifuged for 10 min at 10,000 x g in a centrifuge. Subsequently, an equal volume of distilled water and hexane 1:1 (v/v) were added to the supernatant. The upper layer was collected and dried under a gentle stream of oxygen-free nitrogen. The extracted sample was immediately stored at -80°C until further analysis.

High Pressure Processing (HPP)

Extraction of astaxanthin was carried out using the HPP method as described by (Du *et al*., 2013). One gram of powdered sample of each species of shrimp carapace was extracted at room temperature by adding 5 ml of a mixture of acetone and methanol 7:3 (v/v) into a plastic bag which was then sealed. The sealed bag was then placed into the high pressure vessel. The condition of HPP was set according to the previous study (Du, 2013), namely at 210 MPa pressure for 10 minutes at room temperature. Subsequently, the samples were centrifuged for 10 minutes at 10,000 x g in a centrifuge. An equal volume of distilled water and hexane 1:1 (v/v) was added to the resultant supernatant. The upper layer was collected and dried under a gentle stream of oxygen-free nitrogen. The extracted sample was immediately stored at -80°C until further analysis.

2,2-diphenyl-1-1picrylhydrazyl (DPPH) Assay

DPPH radical scavenging activity was determined as described in previous studies (Brand-Williams *et al*., 1995; Braca *et al*., 2001). 3.9 ml methanolic DPPH solution was added with 100 µl of the sample solution. DPPH radical scavenging activity was expressed as a percentage of inhibition.

Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay was done according to previous study and reagents was prepared and used freshly (Pulido *et al*., 2000). Several solutions prepared were acetate buffer pH 3.6, 2,4,6-tripydyl-s-triazine 10 mM and $FeCl_3$ 20 mM to make FRAP reagent. FRAP assay was determined by adding 3 ml of FRAP reagent with 100 µl of the sample solution and was heated in a 37°C water bath for 30 min. The FRAP test result was calculated according to Fe (II) standard curve.

Oxygen Radical Absorption Capacity (ORAC) Assay

The ORAC assay was performed essentially as described (Huang *et al*., 2002). The sample was prepared by dissolving 20 mg of the sample in 10 ml of water solution. The standard solution was 800 µM Trolox solution. Each well of microplate reader was filled with 150 µl of working solution and 25 µl of the sample, 25 µl of phosphate and 25 µl of Trolox then incubated for 30 seconds before recording the initial fluorescence (f_0). The fluorescence was monitored kinetically (f_1, f_2, f_3,..). BMG Omega Fluostar Fluorescent Spectrophotometer was used to carry out fluorescence The ORAC value was calculated using the equation below and expressed as µM Trolox equivalent (µmol TE/100g).

$$\text{Orac (µM TE)} = \frac{C_{\text{Trolox}} \cdot (AUC_{\text{Sample}} - AUC_{\text{Blank}}) \cdot k}{AUC_{\text{Trolox}} - AUC_{\text{Blank}}}$$

Where

AUC : $(0.5 + f_5/f_0 + f_{10}/f_0 + ... + f_{n+5}/f_0)$

Antimicrobial Test

The microbial activity was tested using disc diffusion method with four strains of bacteria, *E. coli, E. aerogenes, S. aureus* and *B. subtilis* (Valgas *et al*., 2007). The bacteria were cultured in LB Broth for 8-12 hours then spread on the Nutrient Agar plate. The sterile paper discs (6 mm diameter) were aseptically soaked at different concentration of extracted astaxanthin (25 µg/ml, 50 µg/ml and 75 µg/ml) and placed on the plate. The plates were

incubated at 37o for 12 hours and measured for zones of growth inhibition.

Results and discussion

2,2-diphenyl-1-1picrylhydrazyl (DPPH) radical scavenging capacity

The DPPH scavenging assay has an absorption characteristic that can neutralize free radicals. Investigation of antioxidant activity using DPPH was carried out for astaxanthin extracted chemically and by the HPP method. Figure 1. shows the variation in colour between chemical extraction and HPP method. Percentage of scavenging activity of astaxanthin extracted was 25.47% for chemical extraction and 87.90% for HPP method. It showed a significant increase in DPPH radical scavenging capacity using the HPP method, suggesting that treatment with HPP produced a sample with a higher level of antioxidant activity relative to chemical extraction.

Moreover, Figure 1 demonstrates that in the case of HPP, the DPPH assay shifted towards a lighter colour. This is explained by the absorption of hydrogen of an antioxidant (Moon and Shibamoto, 2009; Badarinath *et al.*, 2010). The percentage of scavenging capacity of HPP-extracted astaxanthin from *Penaeus monodon* carapace was 9.9% higher compared to a previous study that used *Penaeus indicus* carapace and 0.5% enzyme Alcalase (Sowmya *et al.* 2014)

Ferric Reducing Antioxidant Power (FRAP) value

The FRAP assay is an antioxidant analysis which is based on the redox reaction. The total antioxidant capacity is represented as the total reducing power of a sample which is expressed as the reducing capacity (Berker *et al*., 2010). shows the colour between chemical extraction and HPP method. The FRAP value was 2.86 µmol TE/g for chemical extraction and 8.13 µmol TE/g for HPP method. Although there was no significantly different Prussian blue colour between HPP and chemical extraction in., results indicate that methods resulted in a significant increase in FRAP reducing capacity.

The FRAP value of astaxanthin from P*enaeus monodon* using HPP was higher than a previous study which was 7.13 µmol TE/g, involving extracts from the cephalothorax of *Litopenaeus vannamei* using ethanol (Binsan *et al*., 2008). The FRAP value is different due to the difference in species and methodology (Ou *et al.* 2001; Berker *et al*., 2010; Binsan *et al.* 2008). Chemical extraction (using a single solvent) resulted in a lower yield of astaxanthin as indicated by the lower value of FRAP.

Figure 1. DPPH test for chemical extraction (right) and HPP (left) method

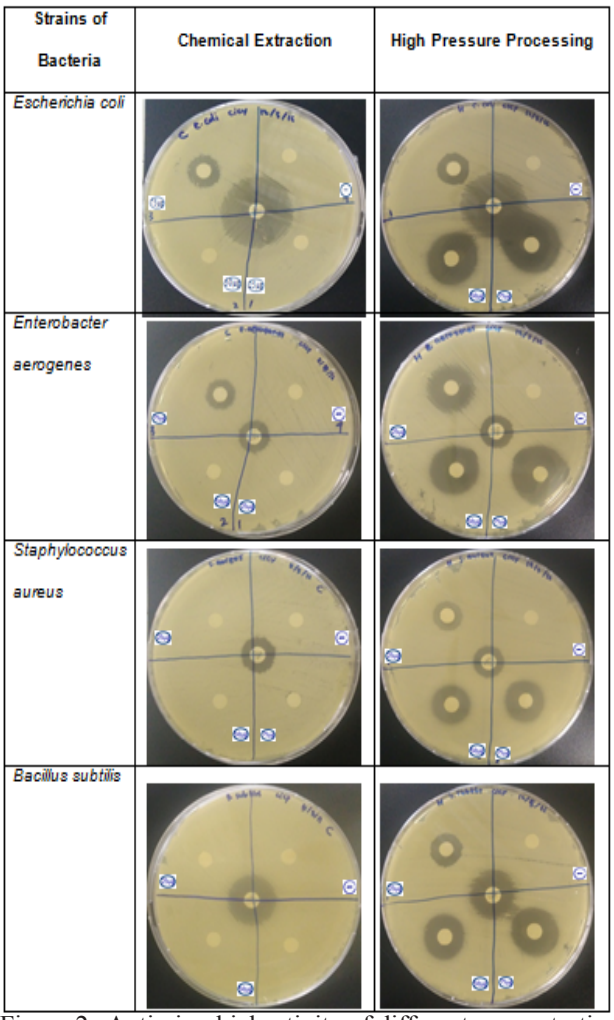

Figure 2 . Antimicrobial activity of different concentration of astaxanthin (25µg/ml, 50 µg/ml and 75 µg/ml) against four types of bacteria (*E. coli, E. aerogenes, S.aureus,* and *B. subtilis*)

Oxygen Radical Absorption Capacity ORAC value

The ORAC assay is an antioxidant assay based on a fluorescent indicator that depends on both the inhibition degree and inhibition time. The ORAC values were 2,000 µmol TE/100g and 4,000 µmol TE/100g for HPP method. The value was calculated using MARS data analysis software that comes

Table 1. Zone inhibition of different concentration of astaxanthin against four strains of bacteria

Strains of Bacteria	Zone of Inhibition							
	Chemical Extraction (mm)				High Pressure Processing (mm)			
	(+)	25 µg/ml	50 µg/ml	75 µg/ml	(+)	25 µg/ml	50 µg/ml	75 µg/ml
Escherichia coli	27.67±1.52	-	-	12.33±0.58	27.67±1.53	13.83±0.29	20.83±0.76	22.67±0.58
Enterobacter aerogenes	13.00±1.00	-	-	12.68±0.58	13.00±1.00	18.33±0.58	22.50±0.50	26.00±1.00
Staphylococcus aureus	13.00±1.00	-	-	-	13.00±1.00	11.83±0.29	14.50±0.50	17.67±0.58
Bacillus subtilis	19.33±0.58	-	-	-	19.33±0.58	13.68±0.58	18.33±0.58	20.00±1.00

The results are mean ± SD (n=3)

together with the instrument at Forest Reseacrh Institute Malaysia.

The antioxidant activity of the HPP treated sample was twice greater than that of chemical extraction based on the elevated inhibition to fluorescence intensity. Both, the ORAC value for HPP and chemical extraction was higher than a previous study using Dimethyl Sulfoxide (ORAC value: 8.1 µmol TE/g of astaxanthin) on extractions from *Haematococcus pluvialis* (Regnier *et al*., 2015). The ORAC value is different due the different species sourced and methodology used in extraction (Ou *et al*., 2001; Binsan *et al*., 2008; Zulueta *et al.*, 2009). Chemical extraction (single solvent) resulted in a lower yield of astaxanthin which affected the value of ORAC.

Antimicrobial activity

Antimicrobial activity of astaxanthin was tested against four strains of bacteria, two gram (+) and two gram (-), in three different concentrations (25 µg/ml, 50 µg/ml and 75 µg/ml). Comparison of antimicrobial activity from three different concentration of astaxanthin against four different strains of bacteria between chemical extraction and high pressure processing is shown in Figure 2.

Results indicate that astaxanthin extracted using HPP produced a zone of inhibition against all four strains of bacteria, while the chemical extraction sample produced a zone of inhibition against *Escherichia coli* and *Enterobacter aerogenes*. In addition, the zone of inhibition produced by HPP derived astaxanthin was larger than that seen with chemical extraction. On the other hand, there was no zone of inhibition observed at any concentration for *Staphylococcus aureus* and *Bacillus subtilis* within the chemical extraction group.

The highest zone of inhibition was noted at an HPP-astaxanthin concentration of 75µg/ml, against *Enterobacter aerogenes* (26.00±1.00 mm), followed by astaxanthin extracted at the same concentration against *Escherichia coli* (22.67±0.58 mm). In comparison, the zone of inhibition from chemically extracted astaxanthin of equivalent concentration, against Enterobacter aerogenes, was less than half (12.68±0.58 mm) that of the HPP sample (Table 2).

A previous study investigating the zone of inhibition of astaxanthin against *E. coli* noted 12.06 mm as the finding, using acetone at 50 µg with a yield of astaxanthin 29.01 µg/g. This was lower than the zone of inhibition of astaxanthin from crab shells extracted using HPP at 50 µg/ml (Suganya and Asheeba, 2015).

In addition, others have reported the zone of inhibition of astaxanthin against *P. Aeruginosa, B. subtilis,* and *S. aureus,* as being 22 mm, 18 mm, and 16 mm, respectively. These extracts were from shrimp in Cochin, Kerala (India) using hexane, at 75 µg lower than the zone of inhibition of astaxanthin extracted using HPP at 75 µg/ml (Ushakumari and Ramanujan, 2013). All four strains of bacteria produced zone of inhibition from the HPP method which was greater compare to chemical extraction. High Pressure Processing was susceptible to inhibit growth of both Gram-positive and Gram-negative bacteria (Ushakumari and Ramanujan 2013). The antimicrobial activity of astaxanthin from High Pressure Processing had been attributed to its phytochemicals.

Conclusion

HPP facilitates higher antioxidant and antimicrobial activities as demonstrated with a higher percentage of scavenging capacity, reducing the capability of Ferrum redox oxygen, radical absorption capacity value and zone of inhibition compared to chemical extraction. It is recommended that for

further studies be performed to design and perform antifungal testing of agents in association with HPP in order to further delineate the antimicrobial benefits of this techonology.

Conflict of Interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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