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RESEARCH ARTICLE

ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF ASTAXANTHIN ISOLATED FROM THREE VARIETIES OF CRABS

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ABSTRACT

The aim of the present study was to isolate and evaluate the antioxidant and antimicrobial activity of astaxanthin pigment. Astaxanthin was isolated from *Portunus sanguinolentus* (Three Spotted Crab), *Callinectes sapidus* (Blue Crab) and *Paralithodes brevipes* (Spiny King Crab) using DMSO: Acetone solvent mixture. The yield of astaxanthin from *Portunus sanguinolentus* (Three Spotted Crab), *Callinectes sapidus* (Blue Crab) and *Paralithodes brevipes* (Spiny King Crab) was found to be 29.01 µg/g, 38.98 µg/g and 18.06 µg/g. The isolated astaxanthin along with the astaxanthin standard was tested for antioxidant activity viz., Total Antioxidant, Reducing Power, Hydrogen Per Oxide Radicals Scavenging Assay, DPPH Free Radicals Scavenging Activity, Ferrous Metal Ion Chelating Activity. Total antioxidant activity of astaxanthin (three crabs) ranged from 50 % to 83 % and the maximum of 58 % was found in astaxanthin standard. The increase in O.D was seen, which reflected a good reducing activity in both the astaxanthin standard and test samples. A good hydrogen peroxide radicals scavenging property was gained which ranged from 33 % to 91 %. Also, it is found that all the samples of astaxanthin possessed a good DPPH activity. A good chelating activity of 40 % to 64 % was gained by both isolated and standard astaxanthin. The antimicrobial activity of isolated and standard astaxanthin was also studied against the isolated organism (*E.coli*) from spoiled milk and rotten meat. The astaxanthin pigment was found to be more effective against isolated strains at concentration of 50 µg where the zone of inhibition was found to be in the range of 10.05 ± 0.53 mm to 12.11 ± 0.95 mm. The study indicate that the astaxanthin isolated from three crab varieties have promising antioxidant and antimicrobial activities which can be used in food and pharmaceutical industries.

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INTRODUCTION

Crabs among various other invertebrates are considered as an essential shell fishery product (Nalan G *et al.,* 2003).There are many aquatic crabs, semi-terrestrial crabs and terrestrial crabs, particularly in tropical regions (FAO 2006).

About 80 % of the weight of crab is lost as waste material which consists of raw carapace, shells of cooked appendages, viscera and meat residues, etc. only live crab are used and thus the dead crabs also contribute to the waste problems. One possible use of this waste is as a source of carotenoids, protein and lipid for the diet of salmonids raised in aquaculture. While the protein and lipids can be supplied from other sources, there is no source of the carotenoids astaxanthin other than from dietary crustacean. The recovery of these valuable components from the waste would not only improve the economy for crustacean processors, but also would minimize the pollution potential of the crustacean waste (Kuo H C *et al.,* 1976).

The most abundant carotenoids present in the human organism are -carotene, lycopene, astaxanthin and lutein. These substancesare not produced by the human body, and therefore must be supplied by the human diet. Besides their use as pigments, carotenoids have several applications due to their positive effect on health such as the antioxidant potential (Skonberg D I and Perkins B L, 2002).

Astaxanthin, a naturally occurring carotenoid, which is mainly present in seafood products, especially in shells of crab, lobster and shrimp, represents animportant biological compound. It has unique chemical properties based on its molecular structure. The presence of the hydroxyl and keto moieties on each ionone ring is responsible for its higher antioxidant activity (Kishimoto Y*et al.,* 2010). The oxo function is capable to resonance-stabilize carbon-centered radicals, which may explain the powerful antioxidative properties of astaxanthin without pro-oxidative contributions (Martin H D *et al.,* 1999) and exert a protective effect against chronic diseases such as cancer (Jyonouchi H *et al.,* 2000, Kurihara H *et al.,* 2002).

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It has been generalized that astaxanthin has an antioxidant activity, as high as ten times more than other carotenoids such as zeaxanthin, lutein, canthaxanthin and -carotene and 100 times more than -tocopherol and thus has been dubbed a super vitamin-E (Higuera-Ciapara I *et al.,* 2006).

The antioxidant enzyme Catalase, Superoxide dismutase, Peroxidase and ThioBarbituric Acid Reactive Substances (TBARS) were high in rat plasma and liver after feeding *Haematococcus* biomass as source of astaxanthin. The polyene chain of astaxanthin trapes radicals in the cells membrane, whereas, the terminal ring of astaxanthin could scavenge radicals at the outer and inner parts of cell membrane. Antioxidant enzyme activity was evaluated in the serum after astaxanthin was supplemented in the diet of rabbits, showing enhanced activity of superoxide dismutase and thioredoxine reductase whereas, paraxonase was inhibited in the oxidative induced rabbits (Augusti P R *et al.,* 2012). Antioxidant enzyme levels were increased when astaxanthin fed to ethanol induced gastric ulcer rats (Kamath B S *et al.,* 2008).

Several reports have been explored on the antimicrobial activities of astaxanthin extracts obtained from microalgae *Haematococcus pluvialis* and found highest antimicrobial activities of ethanolic extract corresponding to red cyst of *Haematococcus pluvialis* (Santoyo S *et al.,* 2009). Astaxanthin is an attractive compound in food, cosmetics and various pharmaceutical applications (Kim D K *et al.,* 2011).

The aim of the present research work is to study the antioxidant and antimicrobial activity of astaxanthin extracted from three different types of sea crabs i.e. Three spotted crab (*Portunus sanguinolentus*),Blue crab (*Callinectes sapidus*), and Spiny king crab (*Paralithodes brevipes*) along with the standard astaxanthin.

MATERIALS AND METHODS

Extraction of astaxanthin using DMSO: Acetone (Persike D S et al., 2002)

To 0.5 g of powdered crab shells,, 2 ml of DMSO was added in centrifuge tube, pre-warmed at 55°C for 30 minutes, shaken well and vortexed for 1 min. It was then, kept undisturbed for 30 minutes. To this, 6 ml of acetone was added. The solution was centrifuged at 1745 x g for 10 min and the supernatant was collected. The same procedure was repeated for the precipitate. Supernatants were gathered for determining the astaxanthin specific concentration.

Quantification of astaxanthin(Uma Nath Ushakumari and Ravi Ramanujan 2012)

The extracted astaxanthin is redissolved in 3 ml of acetone and read @ 470 nm.

$$AST\ (\mu g/g) = \frac{A \times D \times 10^6}{100 \times G \times d \times E^{1\%}{}_{1}cm}$$

Where,
AST concentration in µg/g
A is absorbance
D is volume of extract in acetone
10^6 is dilution multiple
G is weight of sample in g
D is the cuvette width (1 cm)
E is extinction coefficient 2100.
OD @470 nm.

Antioxidant activity of astaxanthin standard and isolated astaxanthin

Total Antioxidant Activity (Khan M A et al., 2009)

Total Antioxidant Capacity (TAC) reagent were prepared by mixing 7.45 ml of sulphuric acid (0.6 mM solution), 0.9942 g of sodium sulphate (28 mM solution) and 1.2359 g of ammonium molybdate (4 mM solution) in 250 ml of distilled water. 300 µl of isolated astaxanthin from three carb varieties was dissolved in 3 ml of TAC reagent. Distilled water was used as blank. Absorbance of all sample mixtures was measured at 695 nm. Gallic acid was used as standard to determine total antioxidant activity.

Reducing Power (Govindarajan R et al., 2003)

1.0 ml of isolated astaxanthin from three carb varieties were mixed with 2.5 ml of phosphate buffer (0.2 M, pH-6.6) and 2.5 ml of potassium ferric cyanide (1%). Reaction mixture was kept in a water bath at 50°C for 20 minutes. After incubation, 2.5 ml of Trichloroacetic acid (10%) were added and centrifuged at 650 rpm for 10 minutes. From the supernatant, 2.5 ml solution were mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (0.1%). Absorbance of all the solution was measured at 700 nm after every 10 minutes intervals up to 30 minutes. Increased absorbance indicated increased reducing power.

Hydrogen Peroxide Radical Scavenging Assay (Govindarajan R et al., 2003)

Hydrogen peroxide (10 mM) solution were prepared in the phosphate buffer saline (0.1 M, PH-7.4). 1 ml (0.25 mg) of the isolated astaxanthin from three carb varieties were rapidly mixed with 2 ml of hydrogen peroxide solution. The absorbance were measured at 230 nm against a blank (without hydrogen peroxide) after 10 minutes of incubation at 37°C. The percentage of scavenging of hydrogen peroxide was calculated using the following formula:

% Scavenging = [Absorbance of control - Absorbance of test sample/Absorbance of control] X 100

Where blank – phosphate buffer; control – hydrogen per oxide without astaxanthin; test – astaxanthin with hydrogen per oxide.

DPPH Free Radicals Scavenging Activity (Shimada K et al., 1992)

1 ml of test solution were mixed with 1 ml of 0.1 mM DPPH in methanol. The mixture was incubated in dark place for 30 min at 25°C. After standing for 30 minutes, absorbance was recorded at 517 nm. The percentage of DPPH free radicals scavenging activity was calculated by following equation:

% Scavenging = [Absorbance of control - Absorbance of test sample/Absorbance of control] X 100

Where blank – methanol; control - DPPH in methanol without astaxanthin; test – astaxanthin with DPPH in methanol.

Ferrous Metal Ion Chelating Activity (Khan M A et al., 2009)

1 mL of astaxanthin was mixed with 3.7 mL methanol and 0.1 mL ferrous chloride (2 mmol/L). To the mixture, 0.2 mL of ferrozine (5 mmol/L) was added to initiate the reaction. The mixture was shaken vigorously and left to stand in the dark at room temperature for 10 min. The absorbance of the sample solution was measured at 562 nm. Calculation for percentage of inhibition of the ferrozine-Fe2 complex formation was done according to the following Equation

% Chelating = [Absorbance of control - Absorbance of test sample/Absorbance of control] X 100

Where blank – methanol; control – methanol, ferrous chloride and ferrozine without astaxanthin; test – astaxanthin with methanol, ferrous chloride and ferrozine.

Isolation and characterization of organism

A loop full of samples were taken from spoiled milk and meat. It was inoculated in petri plates containing nutrient agar. The plates were incubated at 37°C for 24 hours. After 24 hours of incubation the isolated organisms were subjected to biochemical tests to identify the unknown organism.

Antimicrobial activity of astaxanthin using agar diffusion method (Uma Nath Ushakumari and Ravi Ramanujan, 2013)

Petri plates containing 20ml Muller Hinton medium were seeded with 24hr culture of bacterial strains. Wells were cut and different concentration of astaxanthin extracts of 10 µg, 30 µg and 50µg (acetone extracts) were added. Chloramphenicol (30 µg/ml) was used as a positive control. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well.

Statistical analysis

Antimicrobial activity of astaxanthin standard and astaxanthin isolated from various samples were analyzed in triplicate and the results were given as mean ± S.D.

RESULTS AND DISCUSSION

Quantification of astaxanthin

Table 1 Yield of astaxanthin obtained using DMSO: Acetone

Sources	Astaxanthin (µg/g)
Portunus sanguinolentus(Three Spotted Crab)	29.01 ± 0.10
Callinectes sapidus(Blue Crab)	38.98± 0.43
Paralithodes brevipes (Spiny King Crab)	18.06 ± 0.23

The concentration of astaxanthin obtained from *Portunus sanguinolentus* (Three Spotted Crab), *Callinectes sapidus*(Blue Crab), *Paralithodes brevipes* (Spiny King Crab) and *Tagetes patula* (Red Marigold) using DMSO: Acetone was found to be 29.01 ± 0.10 (µg/g), 38.98 ± 0.43 (µg/g), and 18.06 ± 0.23 (µg/g) which is depicted in Table 1.The present investigation was correlated with the findings of Renata Aline dos Santos da Fonseca *et al.,* 2011 and Shahidi F and Synowiecki J, 1990.

Antioxidant activity of astaxanthin standard and isolated astaxanthin

Table 2 Total Antioxidant Activity

S.No	Content	% of antioxidant activity
1.	Standard (Gallic Acid)	67
2.	Astaxanthin standard	58
3.	*Portunus sanguinolentus* (Three Spotted Crab)	50
4.	*Callinectes sapidus* (Blue Crab)	83
5.	*Paralithodes brevipes* (Spiny King Crab)	75

Antioxidant properties of astaxanthin was evaluated since, astaxanthin possess the properties of free radical scavenging, antiaging, anti-cancer, anti-inflammatory etc. In the present investigation, total antioxidant property of astaxanthin was evaluated in Table 2. The total antioxidant potential of three crabs ranges from 50 % to 83 % and the maximum antioxidant potential of 58 % was found in astaxanthin standard, but it is lower than that of gallic acid the standard drug i.e. 67 %. Sudhakar M *et al.,* 2011 recorded the total antioxidant activity ranged from 28.52 % to 80.26 % in *P.Sanguinolentus* crab shell chitosan sample. The present test was also correlated with the study of Soundarapandian P *et al.,* 2014 in which the total antioxidant activity was found to be 32 to 49% in soft and hard shelled crabs of *C. Lucifera* and 86 % for gallic acid.

Table 3 Reducing Power Assay

S.No	Content	OD at 700 nm after 10 minutes	OD at 700 nm after 20 minutes	OD at 700 nm after 30 minutes
1.	Standard (Ascorbic Acid)	0.05	0.07	0.08
2.	Astaxanthin standard	0.04	0.05	0.07
3.	*Portunus sanguinolentus* (Three Spotted Crab)	0.03	0.04	0.06
4.	*Callinectes sapidus* (Blue Crab)	0.03	0.05	0.06
5.	*Paralithodes brevipes* (Spiny King Crab)	0.02	0.03	0.05

The present results revealed the reducing effect of astaxanthin. Astaxanthin possess the ability to donate an electron to free radicals which leads to neutralization of the radicals. Reducing power was evalutaed using ferric ions. The results are formulated in Table 3. There was increase in O.D for every 10

mins in both the standard and the test samples. Due to increase in O.D, the result reveals the good reduicng property.In reducing power assay, the drug ascorbic acid was dominating agent that shows the maximum reducing ability i.e 0.05 for 10mins and 0.08 for 30 mins, which is followed by astaxanthin standard that shows 0.04 for 10 mins and 0.07 for 30 mins. The reducing ability of Three spotted crab (*Portunus sanguinolentus*) and Blue crab (*Callinectus sapidus*) were found to be equal which shows 0.03 for 10 mins and 0.06 for 30 mins. The low reducing ability was recorded in Spiny king Crab (*Paralithodes brevipes*) that produced 0.02 for 10 mins and 0.05 for 30 mins..Thus, the results correlates astaxanthin as a good reducing agents. The present investigation was interrelated with the study of Soundarapandian P *et al.,* 2014.

Table 4 Hydrogen Peroxide Radical Scavenging Assay

S.No	Content	% Scavenging
1.	Standard (Ascorbic acid)	91
2.	Astaxanthin standard	71
3.	*Portunus sanguinolentus* (Three Spotted Crab)	62
4.	*Callinectes sapidus* (Blue Crab)	52
5.	*Paralithodes brevipes* (Spiny King Crab)	33

Hydrogen per oxide is a standard free radical compound which enchances the lipid peroxidase acitivity. In the present investigation, the ability of astaxanthin to scavenge hydrogen per oxide was assayed and tabulated in Table 4. The maximum acitivity of 91 % inhibition was seen in ascorbic acid followed by standard astaxanthin of 71 % inhibition. The scavenging activity of 62 % was seen in *Portunus sanguinolentus* (Three spotted crab),52 % in *Callinectus sapidus* (Blue crab) and 33 % in *Paralithodes brevipes*(Spiny king Crab). By viewing the results, a good scavenging property was gained. This results further made us to prove that astaxanthin has a good antioxidant property. The present investigation was interrelated with the study of Soundarapandian P *et al*., 2014.

Table 5 DPPH Free Radicals Scavenging Activity

S.No	Content	% Scavenging
1.	Standard (Gallic Acid)	88
2.	Astaxanthin standard	75
3.	*Portunus sanguinolentus* (Three Spotted Crab)	67
4.	*Callinectes sapidus* (Blue Crab)	79
5.	*Paralithodes brevipes* (Spiny King Crab)	67

DPPH is one of the stable free radical used for the assay of scavenging capacity of astaxanthin. The percentage of DPPH scavenging activity is formulated in Table 5. The DPPH activity of standard Gallic acid was found to be 88%. By comparing this percentage with the standard astaxanthin and astaxanthin isolated from three crab varieties, it is noticed that *Callinectus sapidus* (Blue crab) possess 79 %, a highest DPPH activity, where as other two crab such as *Portunus sanguinolentus* (Three spotted crab) and *Paralithodes brevipes*(Spiny king Crab) possess 67 %. Hence by over looking into the results, all the samples of astaxanthin possessed a good DPPH activity which is predicted that DPPH would have picked up the electron in the presence of a free radical scevenger which is reflected as the % of DPPH activity.

The present study was compared with Soundarapandian P *et al.,* 2014 where they reported the scavenging role of 59 % and 48 % in soft and hard crab shell of *C.lucifera*.

Table 6 Ferrous Metal Ion Chelating Activity

S.No	Content	% Scavenging
1.	Standard (Ascorbic acid)	88
2.	Astaxanthin standard	64
3.	*Portunus sanguinolentus* (Three Spotted Crab)	48
4.	*Callinectes sapidus* (Blue Crab)	52
5.	*Paralithodes brevipes* (Spiny King Crab)	40

The results of ferrous metal ion was depicted in Table 5. The standard ascorbic acid possessed 88 % which shows higher chelating activity compared to the other astaxanthin standard which shows 64 % and astaxanthin isolated from three varieties of crabs such as *Portunus sanguinolentus* (Three Spotted Crab), *Callinectus sapidus* (Blue Crab) and *Paralithodes brevipes* (Spiny King Crab) shows 48 %, 52 % and 40 % respectively. A good chelating % was gained with a prediction such that astaxanthin has reduced, .the concentration of metal ie. Fe^{2+} which is responsible for lipid peroxidation. Hence, astaxanthin isolated from crab varieties is a good antioxidant which reduces the lipid peroxidation property. Hence in food astaxanthin are added to minimize changes in flavours, aroma, color or nutritional values due to its antioxidant property. It is also used to protect the body against damages caused by free radicals and degenerative diseases (Schinella G R *et al.,* 2000).

Isolation of unknown organism from milk and rotten meat

Table 7 Biochemical tests against spoiled milk and rotten meat organism

Biochemical tests	Spoiled milk	Rotten Meat
Gram staining	Gram negative, Rod shaped	Gram negative, Rod shaped
Gelation liquefication	- VE	- VE
Starch hydrolysis	- VE	- VE
Lipid hydrolysis	- VE	- VE
Lactose, Dextrose, Sucrose	AG	AG
Hydrogen sulfide production	+ VE	+ VE
Nitrate reduction	+ VE	+ VE
Indole production	+ VE	+ VE
MR reaction	+ VE	+ VE
VP reaction	- VE	- VE
Citrate	- VE	- VE
Urease activity	+ VE	+ VE
Catalase activity	+VE	+VE
Oxidase activity	+VE	+VE

The unknown organism was isolated from spoiled milk and rotten meat and they were subjected to biochemical tests to find out the organism.By analysing the below biochemical tests represented in Table 7, the isolated organism from spoiled milk and meat was identified to be *E.coli*.

Antimicrobial activity of astaxanthin

Figure 1 Antimicrobial activity of astaxanthin standard against *E.coli* strains isolated from spoiled milk and rotten meat

Table 8 Zone of Inhibition against *E.coli* isolated from spoiled milk and rotten meat

Samples	Concentration 30 μg		Concentration 50 μg		Chloramphenicol + VE Control(30μg)
	Spoiled milk (mm)	Rotten meat (mm)	Spoiled milk (mm)	Rotten meat (mm)	
Standard astaxanthin	7.30 ± 0.64	6.23 ± 0.65	12.10 ± 0.98	12.11 ± 0.95	10.20 ± 0.53
Astaxanthin isolated from *Portunus sanguinolentus*	8.02 ± 0.74	8.13 ± 0.53	11.04 ± 0.63	12.06 ± 0.95	10.04 ± 0.54
Astaxanthin isolated from *Callinectes sapidus*	6.02 ± 0.23	7.06 ± 0.34	12.03 ± 0.33	12.06 ± 0.42	10.03 ± 0.55
Astaxanthin isolated from *Paralithodes brevipes*	5.05 ± 0.21	6.09 ± 0.67	11.07 ± 0.33	10.05 ± 0.53	10.06 ± 0.54

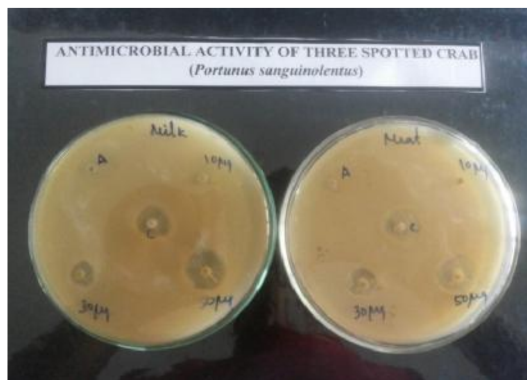

Figure 2 Antimicrobial activity of astaxanthin isolated from *Portunus sanginolentus*(Three Spotted Crab) against *E.coli* strains isolated from spoiled milk and rotten meat

Figure 3 Antimicrobial activity of astaxanthin isolated from *Callinectes sapidus* (Blue Crab) against *E.coli* strains isolated from spoiled milk and rotten meat

Figure 4 Antimicrobial activity of astaxanthin isolated from *Paralithodes brevipes* (Spiny King Crab) against *E.coli* strains isolated from spoiled milk and rotten meat

Figure 1 to 4 and Table 8 depicts the diameter of the zone of inhibition of extract against the *E.coli* strains isolated from the spoiled milk and rotten meat.

To evaluate the effect of astaxanthin pigment (standard, *Portunus sanguinolentus, Callinectes sapidus, Paralithodes brevipes*) against isolated strains, concentration ranging from 5 µg/ml to 50 µg/ ml was evaluated. But at concentration 10 µg and below no zone was visualized. For all astaxanthin samples, at concentration 50 µg, the diameter in the range of 10.05 ± 0.53 mm to 12.11 ± 0.95mm was gained, which was greater than the positive control chloramphenicol i.e. zone of diameter in the range of 10.03 ± 0.55 mm to 10.20 ± 0.53mm.

Uma Nath Usha Kumari and Ravi Ramanujan, 2013 reported that the astaxanthin pigment was found to be more significantly effective against all tested pathogenic species such as *S. typhi, P. aeruginosa, B. subtilis, and S. aureus*which produced 20mm, 24 mm, 18 mm and 16 mm diameter of zone which is interrelated with the present study.

The present investigation was also correlated with the study of Manimala M R aand Murugesan R, 2014 who reported that bacterial pathogen *E.coli* was highly inhibited by pigment extract of *Sporobolomycesspp.* with the inhibition zone of 2.8 cm.

Antimicrobial activity may be due to several factors, including charge density, structure of lipopolysaccharides and lipid composition of the cytoplasmic membrane in gram negative and gram positive bacteria (Devine D A and Hancock R E, 2002). The chemistry of the pigment has a significant influence on its antimicrobial activity (Manimala M R aand Murugesan R, 2014). By viewing the results, it is found that the natural pigment astaxanthin isolated from crab shells were highly effective against a common pathogen, than a standard antibiotic. Hence, the present investigation can be suggested for using astaxanthin as a drug against microbial infections with further range of studies.

CONCLUSION

Today, more prevalent pathogens are resistance to the prevailing antibiotics, which are available commercials. Findings of new antibiotics through research will result in more side effects. But the present investigation has shown that the natural pigment astaxanthin exhibited a good antioxidant and powerful antibacterial activity. Thus, with further expanded research astaxanthin can enter into the medical filed with success.

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