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RESEARCH ARTICLE

Potential of Astaxanthin from Asian Tiger Shrimp (*Penaeus Monodon*) Shell Extract as an Antibacterial and Anti-inflammatory

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Abstract

Asian tiger shrimp (*Penaeus monodon*) shell is an industrial waste which becomes one of the sources of carotenoids in the form of astaxanthin (3.3'-dihydroxy-ß, ß'-carotene-4.4'-dione) which has a very potential biological activity because it can work on target molecules. Astaxanthin in Asian tiger shrimp shell is well known to be lipophilic, being insoluble in water but soluble in organic solvents, one of which acetonitrile. The characteristics of astaxanthin were obtained through qualitative and quantitative analyses using FTIR (Fourier Transform Infra-Red) and HPLC (High Performance Liquid Chromatography). Furthermore, antibacterial and anti-inflammatory activities had been tested. Based on the research results done on the inhibition of Asian tiger shrimp (*Penaeus monodon*) shell extract on the 5th growth of the test bacteria, the tiger shrimp shell extract had antibacterial activity on *Eschericia coli., Streptococcus mutans, Pseudomans auriginosa, Salmonella typhi, Staphylococcus aureus* which was characterized by the presence of clear zone / inhibition zone / Oligodynamic zone which showed the effect of tiger shrimp shell extract in killing and inhibiting the growth of test bacteria. Besides, An anti-inflammatory testing through extracting chemical components with acetonitrile solvent with several concentrations showed that the anti-inflammatory activity with a concentration of 1000 ppm with % stability on erythrocyte membrane obtained 60.516%, indicating that the higher the concentration used, the better was the anti-inflammatory effect.

Keywords: *Astaxanthin, Asian tiger shrimp, Antibacterial, Anti-inflammatory*.

Introduction

Shrimps are animals that live in waters, especially rivers and seas or lakes. Shrimps are classified into phylum *Arthropoda*, subfilum *crustacea*, class *Malacostraca* and order *Decapoda* [1]. Shrimp farming has grown rapidly because it is a reliable export commodity in increasing non-oil exports. Shrimps are one type of marine biota with high economic value. Indonesia is one of the largest shrimp exporting countries in the world with an export value reaching 1 billion USD per year (The Indonesian Ministry of Marine Affairs and Fisheries, 2006).

In general, shrimp shell and head are underutilized and become waste that can cause environmental problems [2]. Therefore, it is expected that this shrimp waste can be recycled into something useful. Through various studies that have been carried out, shrimp shell waste has great potential as a chitin producer.

Chitin is the main polysaccharide found in shrimp shell. At present the utilization of shrimp shell waste is still limited to sources of chitin and chitosan which are used as anti-oxidant [1] and anti-cholesterol [4]. In many studies the development of chitin and chitosan has also reached the stages of isolation of glucosamine HCl compound which plays a role in joint flexibility [5]. Nevertheless, there are still chemical components of carotenoids in Asian tiger shell known as astaxanthin [6]. Astaxanthin is a carotenoid xanthophyll that has an antioxidant activity, like other carotenoid compounds.

Antioxidant activity of astaxanthin is stronger than other carotenoids in reducing free radical activity as a trigger for the emergence of degenerative diseases such as cancer and heart diseases [7].

Astaxanthin has an anti-inflammatory effect by inhibiting cytokines and chemokines [1].

Astaxanthin is a group of xanthophyll as it does not only consist of carbon and hydrogen, but also has oxygen atoms.

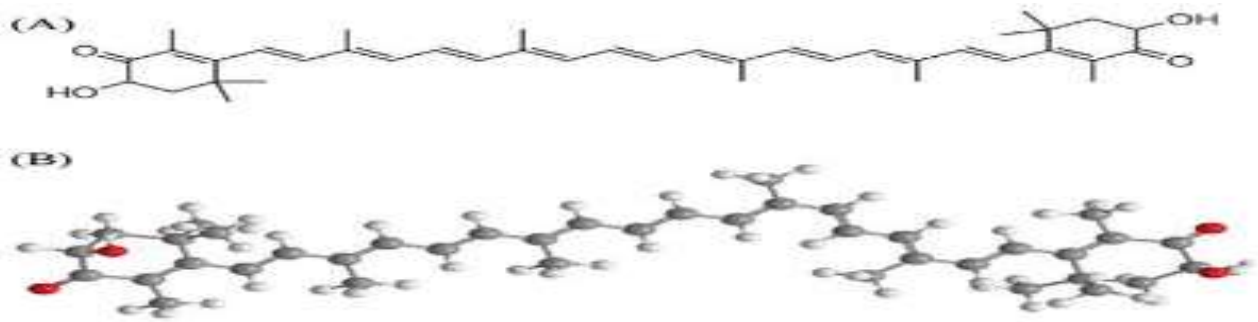

Figure 1: Chemical structure of astaxanthin. (A) Molecular structure.(B) Stick and ball model[8]

Materials and Methods

Preparation of Samples and Extraction

The shrimp shells were taken from waste in Makassar. Firstly, the shrimp waste was washed with water to remove the attached dirt then dried in the sun for approximately two days. After dried, it was mashed up and sifted to make powder with smaller particle size. These results of the sieve were used as the samples. The shrimp shells were extracted by mixing 5 grams of shell powder, adding 50 mL of methanol and boiling stones.

Afterwards, it was vortexed for 30 seconds then placed in a water bath at 50°C for 10 minutes. The liquid and residue were separated using a centrifuge at a speed of 3000 rpm for 5 minutes. This procedure was repeated twice, and then the supernatant was concentrated until a thick extract was obtained.

Qualitative Analysis

Identification and characterization of astaxanthin were done by comparing the spectrum produced from the standard astaxanthin and the tiger shrimp shell extract. Astaxanthin was made into a pellet with KBr to form a transparently thin layer. Furthermore, absorption was measured using the FTIR spectrophotometer in the wave range at 4000-800 cm^{-1}.

Determination of astaxanthin levels was carried out by dissolving 3 mg of standard astaxanthin with 10 ml of acetonitrile, then homogenized over the water bath and the volume was made sufficient with setonitrile to 100 ml (stock solution). Concentration series made were 1.5, 3.0, 4.5, and 6.0 µg/ml, using acetonitrile solvents. The same treatment was for the samples of Asian tiger shrimp shell extract. After that, it was detected by using HPLC silica 60 column with a flow rate of 1.2 ml/min, room temperature, methanol mobile phase: acetonitril (82:18 v / v), injection volume of 20 µl.

Antibacterial Test

Prior the antibacterial testing, the test bacteria and rejuvenation used in this research were prepared, including *Eschericia coli, Pseudomonas aeroginosa, Salmonella typhi, Staphylococcus aureus, Streptococcus mutans* bacteria. Each of them was taken one ose then inoculated by scratching on a sloping NA medium and incubated for 1 x 24 hours at 37°C.

Preparation of bacterial suspension test: the rejuvenated bacteria were suspended with 0.9% physiological NaCl solution to obtain 25% transmittance for bacteria and 75% transmittance for fungi using a spectrophotometer with a wavelength of 580 nm, and as a blank the 0.9% physiological NaCl solution was used [9].The testing was done using agar diffusion method.

The antibacterial samples were active compounds from the extraction process. Each of the bacteria that had been inoculated into the growth media (NB) was inserted into the sterile soft-NA medium (0.7%). Subsequently the soft agar medium containing the test bacteria was poured over the sterile solid medium in a compacted petri dish. The medium became two layers and left at room temperature to make it solid.

In the upper layer there were 4 holes with a diameter of 4 mm. into the holes were inserted the extracts of *P cruentum* microalgae with concentrations of 10,000,

8,000, 7,500 and 6,000 ppm. Then the petri dish was incubated at 37^{O}C for 24 hours. The inhibition zone measurement formed around the hole using a caliper (mm) was also done.

Anti-inflammatory Test

To prepare phosphate buffer pH 7.4 (0.15 M), 2,671 grams of disodium hydrogen phosphate ($NaH_2PO_4.H_2O$) was dissolved in distilled water up to 100 mL (0.15 M). 2,070 grams of sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$) was dissolved in distilled water up to 100 ml (0.15 M). Then 81 ml of $NaH_2PO_4.h_2O$ solution (0.15 M) was mixed with 19 mL of $NaH_2PO_4.H_2O$ (0.15 M) at room temperature. Next, the pH was checked using a pH meter. Then it was sterilized by autoclaving at 121^{O}C for 2 hours. The next was preparing isosaline and hyposaline. Isosaline and hyposaline solutions were made by dissolving 0.85 grams and 0.25 grams of NaCl in phosphate buffer pH 7.4 (0.15 M) to a volume of 100 mL at room temperature, then sterilized using an autoclave at 121^{O}C for 2 hours.

To prepare extract concentrations and diclofenac sodium, 50 mg of extract was dissolved in isosaline to 50 mL (1000 ppm) at room temperature, then diluted into several series of concentrations (25, 50, 100, 200, 400, and 800 ppm). Similarly, to make diclofenac sodium, 50 mg of diclofenac Na was dissolved in 50 mL of isosaline (1000 ppm) at room temperature, and then diluted to a concentration of 100 ppm.

The next step was preparation of red blood cell suspension. This method was delineated by Gandhisan, 1991 in Kumar et al., 2012 and modified with Sadique et al.'s method, 1989 in Oyedapo et al., 2010. 10 mL of blood was centrifuged at 3000 rpm for 10 minutes at 27^{O}C. The supernatant formed was separated using a sterile pipette. The remaining sediment of blood cells were then washed with an isosaline solution and re-centrifuged. The process was repeated 4 times until the isosaline was clear. Blood cell volume was measured and re-suspended with isosaline to obtain red blood cell suspension at a concentration of 10% (v / v). The blood cell suspension was stored at 4^{O}C if it was not used yet.

Then the solution for testing was made. The test solution (4.5 mL) consisted of 1 mL phosphate buffer pH 7.4 (0.15 M), 0.5 mL red blood cell suspension, 1 mL sample solution, and 2 mL hyposaline. The positive control solution consisted of 1 mL phosphate buffer pH 7.4 (0.15 M), 0.5 mL red blood cell suspension, 1 mL diclofenac Na solution, and 2 mL hyposaline.

Meanwhile, the control solution of test solution consisted of 1 mL phosphate buffer pH 7.4 (0.15 M), 0.5 mL isosaline solution as a substitute for red blood cell suspension, 1 mL sample solution, and 2 mL hyposaline. The negative control solution consisted of 1 mL phosphate buffer pH 7.4 (0.15 M), 0.5 mL red blood cell suspension, 1 mL isosaline solution as a substitute for the sample solution, and 2 mL hyposaline.

Analysis of Anti-inflammatory Activity

Each solution above was then incubated at 37^{O}C for 30 minutes and centrifuged at 5000 rpm for 10 minutes. The supernatant liquid obtained was taken and the hemoglobin content was calculated using a UV spectrophotometer at a wavelength of 560 nm. The percent stability of the red blood cell membrane was calculated by the following formula:

$$\% \text{ stability} = 100 - \frac{(\text{abs. test solution} - \text{abs control solution of test solution}) \times 100\ \%}{\text{Abs negative control solution}}$$

Results

Extraction

The shrimp shell was taken from waste in Makassar. The shrimp waste was washed with water to clean the dirt then dried in the sun for approximately two days. After dried, they were mashed and sifted to obtain powder with smaller particle size. The results of the sieve were used as the samples. Methanol is good in dissolving the metabolites of the samples, which can break down the walls and the cell membrane, due to differences in pressure inside and outside the cell. Thus, the metabolite found in the cytoplasm was dissolved in methanol and extracted perfectly [11].

Then they were vortexed during 30 seconds and then placed in a water bath with a temperature of 50 ° C for 10 minutes. The liquid and residue were separated using a centrifuge at 3000 rpm for 5 minutes. This procedure was repeated twice, and then the supernatant was concentrated to obtain the thick extract of ± 3.4 grams.

Qualitative Analysis

The FTIR spectroscopy of the test with Asian tiger shrimp shell samples obtained infrared spectrum as shown in the figure below:

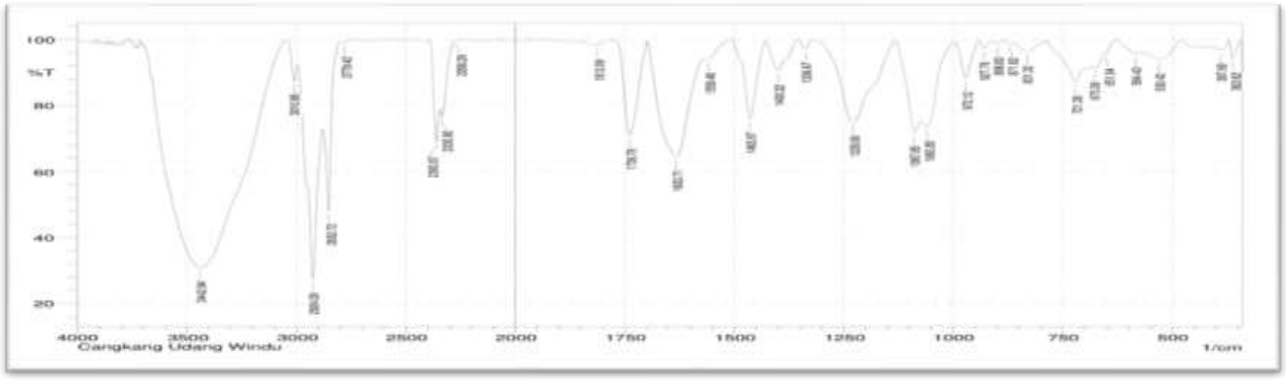

Figure 2: Results of astaxanthin measurement on Asian tiger shrimp shell samples

Data based on Figure 2 were then analyzed and adjusted to the tables of functional groups, obtained the groups C-H and N-H in the area of spectrum between 2000-3000 cm-1 with the highest intensity of 27 359. The structure function that existed in the samples also contained astaxanthin. Hence, it can be summed up that the Asian tiger shrimp shells used as the samples were material part of the astaxanthin. Analysis of astaxanthin level determination using the HPLC method showed the following results:

Table 1: Results of astaxanthin level determination

PeakTable

Detector A Ch1 450nm

Peak#	Ret. Time	Area	Height	Area %	Conc.
1	2.044	2183	371	0.292	0.000
2	2.440	8898	1000	1.188	0.000
3	2.768	3070	231	0.410	0.000
4	3.276	734683	36775	98.110	0.000
Total		748834	38377	100.000	

Table 2: Results of Asian tiger shrimp shell sample level determination

PeakTable

Detector A Ch1 450nm

Peak#	Ret. Time	Area	Height	Area %	Conc.
1	2.200	2183	198	1.953	0.000
2	2.508	4718	461	4.221	0.000
3	2.677	6864	1157	6.142	0.000
4	2.833	1532	267	1.371	0.000
5	3.040	2306	300	2.064	0.000
6	3.246	50464	4389	45.156	0.000
7	3.589	9745	1072	8.720	0.000
8	3.752	7143	943	6.392	0.000
9	3.857	10285	892	9.203	0.000
10	4.167	3454	545	3.091	0.000
11	4.254	6745	589	6.036	0.000
12	4.736	4779	291	4.276	0.000
13	5.028	1537	131	1.375	0.000
Total		111754	11234	100.000	

Results of Antibacterial Test

Based on these research results on the inhibition of Asian tiger shrimp shell extract (*Penaeus monodon*) on the 5th growth of the test bacteria where the tiger shrimp shell extract had antibacterial activity against *Eschericia colii* (E. Coli), *Streptococcus mutans* (BC), *Pseudomans auriginosa* (PA),

Salmonella typhi (ST) and *Staphylococcus aureus* (SA) which was characterized by the presence of clear zone / inhibition zone / oligodynamic zone which showed the effect of tiger shrimp shell extract in killing and inhibiting the growth of test bacteria. The results of the activity test can be seen in Table 3 below:

Table 3: Antibacterial Test Results

No.	Replication	[ ] Asian tiger shrimp samples	Average inhibitory power in Bacteria				
			SA	E. Coli	PA	BC	ST
1	I	10000 ppm	15.67	18.67	13.67	12.67	20.00
	II		15.33	18.67	15.67	17.33	17.00
	III		15.67	**23.67**	16.33	17.00	20.33
2.	I	8000 ppm	14.33	14.33	13.00	10.67	15.66
	II		14.00	12.67	13.67	11.67	12.00
	III		15.00	10.00	15.00	13.00	23.00
3.	I	7500 ppm	13.67	10.33	13.00	12.00	12.00
	II		14.33	13.33	13.00	12.33	13.67
	III		14.33	12.33	13,33	8.67	13.33
4.	I	6000 ppm	14.33	10.00	14.00	12.00	10.67
	II		13.00	12.23	14.33	10.67	13.00
	III		15.00	10.33	11.00	10.67	12.67

Results of Anti-inflammatory Test

Results of the stabilization power of tiger shrimp shell extract on hypotonic solution induction at the concentrations of 200, 400, 600, 800 and 1,000 ppm can be seen in Table 4 below:

Table 4: Anti-inflammatory Test Results

	Concentration (ppm)	Absorbance		% Stability
		Control EMDT	EMDT	
1.	Negative Control	0.000	2.211	0
2.	200	0.005	2.054	7.328
3.	400	0.002	1.882	14.971
4.	600	0.009	1.542	30.665
4.	800	0.004	1.378	37.857
6.	1000	0.000	0.873	60.516

From these data, it can be seen that at the concentration of 200 ppm the tiger shrimp shell extract was able to inhibit hemolysis in the red blood cell membrane by 7.328% while at the concentration of 600 ppm the obtained stabilization power was 30.665 %.

The highest anti-inflammatory activity was shown at a concentration of 1,000 ppm by 60,516 %. These data showed that the increased stabilization power of the extract against the red blood cell membrane was in line with the increased extract concentration.

Conclusion

Based on the results of this research, it can be concluded that the higher the concentration of acetonitrile extract, the greater is the extract stabilization power as an anti-inflammatory and antibacterial.

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