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EXTRACTION AND CHARACTERIZATION OF ASTAXANTHIN FROM THE CRUSTACEAN SHELL WASTE FROM SHRIMP PROCESSING INDUSTRIES

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ABSTRACT: India having a large stretch of marine coastline is advantageous as a natural resource for marine aquaculture. But the waste produce from the marine processing industries fetch a greater environmental risk as there is accumulation of wastes in huge quantity. These wastes are a larger source of natural carotenoids that have a huge pharmaceutical potentials. So proper initiatives must be taken to recycle the waste into a product. Natural astaxanthin from crustacean shell waste is a better alternative to the synthetic orange-red pigment. In this present investigation astaxanthin was extracted from crustacean shell waste, by using organic solvents of different polarities (Hexane, methanol, ethanol, petroleum ether, chloroform and acetone). Characterization of the astaxanthin pigment were performed by TLC by comparing the Retardation factor (R_f) as indicated in Lorenz Todd standard chromatogram where three bands of Astaxanthin ($R_f=0.36$), Astaxanthin monoester ($R_f=0.60$) and Astaxanthin diester ($R_f=0.75$) were detected. The extraction yield of the natural carotenoid from shrimp waste was compared amongst all solvents spectrophotometrically at 470 nm and quantified using equation of Kelley and Harmon. Astaxanthin yield obtained from acetone extract was highest with 48.64 μ g/g compared to other solvent extracts. These extract were then subjected to the microbiological assay by against gram positive and gram negative bacteria, where the methanolic extract gave highest zone of inhibition as compared to the extracts with other organic solvents.

INTRODUCTION: Astaxanthin is a carotenoid that belongs to class of phytochemicals known as tetraterpenoids, which are built from eight isoprenes, C_5H_8 molecules; isopentenyl diphosphate (or IPP) and dimethylallyl diphosphate (or DMAPP) as the initial precursor. Like many carotenoids, astaxanthin is a colorful, lipid-soluble pigment. The colour is due to the extended chain of conjugated (alternating double and single) double bonds at the centre of the compound.

This chain of conjugated double bonds is also responsible for the antioxidant properties of astaxanthin (as well as other carotenoids) as it results in a region of decentralized electrons that can be donated to reduce a reactive oxidizing molecule.^{1,2}

Astaxanthin is found in microalgae, yeast, salmon, trout, krill, shrimp, crayfish, crustaceans, and the feathers of some birds. The red color of salmon meat and cooked shellfish is due to this pigment. Professor Basil Weedon's group was the first to prove the structure of astaxanthin by synthesis, in 1975³. Astaxanthin, unlike some other carotenoids, is not converted to vitamin A (retinol) in the human body⁴. Too much vitamin A is toxic for a human, animal, but astaxanthin has lower toxicity. It is an

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antioxidant with slightly lower antioxidant activity than other carotenoids⁵.

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. Crustacean waste could be the cheapest raw materials for carotenoid recovery, and later could be a better and cheaper alternative to synthetic carotenoid in aquaculture feed formulations and in surimi based products. The U.S. Food and Drug Administration (FDA) has approved astaxanthin as a food coloring (or color additive) for specific uses in animal and fish foods⁶. The European Commission considers it food dye and it is given the E number E161j⁷. Natural astaxanthin is considered generally recognized as safe (GRAS) by the FDA^{8,9}.

MATERIALS AND METHODS:

Collection of crustacean waste and raw material preparation:

The crustacean waste was collected from shrimp processing centre, East Godavari region of Andhra Pradesh, and transported to laboratory under iced condition. Samples were washed under running water and dried at 50°C in oven for 24 hours. They were packed in polyethylene bags and stored at -20°C until use. Dry samples were thawed in running water before use and homogenized in a laboratory mixer prior to extraction of astaxanthin and estimation in the shell.

Extraction of Astaxanthin:

Extraction of astaxanthin from crustacean shell waste was investigated by using different organic solvents such as Methanol, Ethanol, Petroleum ether, Chloroform, Hexane, and Acetone. 10 g of homogenized dry crustacean shell waste were extracted with 100ml of each solvent by stirring it for 1 hour on magnetic stirrer. Then the carotenoid extract were filtered by using Whatman No.42 filter paper. Recovered crustacean waste were repeatedly extracted with fresh solvent until the filtrate were colourless.

The extracts were pooled together and collected for further studies. In the case of acetone extraction the pooled extract was collected in a separating funnel,

12.5ml of petroleum ether (BP 40-600C) and 9.4ml of 0.73% (w/v) NaCl solution were added. After thorough mixing the epiphase was collected. To the lower phase an equal volume of water was added, mixed and the epiphase was collected, the pooled epiphase was evaporated to dryness.

Primary screening for phytochemical constituents:

The screening and study of six different solvent extracts for phytochemical constituents was performed using generally accepted laboratory technique for qualitative determinations. The constituents screened for tannins, saponins, phlobotannins, terpenoids, flavonoids, cardiac glycosides, combined anthraquinone, free-anthraquinone, carotenoids, steroids, reducing compounds and alkaloids. The distribution of these constituents in the crustacean extract specimens were assessed and compared.

Thin layer chromatography to determine the different compounds:

Astaxanthin was analysed by Thin layer chromatography, for this experiment 20 microliter of each extracts were spotted on the silica gel G plate along with standard astaxanthin. Spot development was done by using two different types of mobile phase such as; acetone : hexane (1:3) and methanol:chloroform (benzene : ethyl acetate (1:1)). The separated bands were identified using standard astaxanthin and internationally accepted Rf values for astaxanthin monoester and astaxanthin diester by Lorenz Todd, 1998¹².

Antimicrobial activity:

The antibacterial activity of the astaxanthin extracts was determined in accordance with agar-well diffusion method described by Rious et al. (1988)¹⁰. The bacterial isolates were first grown in a Beef Extract Peptone broth for 18 hours before use and standardized to 0.5 McFarland standards (106cfu/ml).

Two hundred microliter of standardized cell suspensions were spread on a Mueller-Hinton agar. Wells were then bored into the agar using a sterile 6mm diameter cork borer. Approximately 100µl of carotenoid extracts were introduced into the wells, allowed to stand at room temperature for about 2

hours and then incubated at 37°C. Controls were setup in parallel using the solvents that were extracted. The plates were observed for zone of inhibition after 24 hours.

Quantification of Astaxanthin:

The organic solvent extracted astaxanthin was quantified by measuring absorbance at 470 nm and using the equation of Kelley and Harmon ¹¹.

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

Where AST is astaxanthin concentration in $\mu\text{g/g}$; A is absorbance; D is volume of extract in hexane; 106 is dilution multiple; G is weight of sample in g; d is cuvette width (1cm); E is extinction coefficient, 2100.

TABLE 1: PHYTOCHEMICAL SCREENING FOR CRUSTACEAN SHELL EXTRACTS BY DIFFERENT ORGANIC SOLVENTS

Sl.no	Phytochemical compounds	Methanol	Chloroform	Ethanol	Acetone	Hexane	Petroleum ether
1.	Alkaloids	-	-	-	-	-	-
2.	Glycosidases	-	+	+	+	+	-
3.	Saponins	-	-	-	-	-	-
4.	Proteins	+	+	+	+	+	+
6.	Oils	-	+	+	+	+	-
7.	Fats	+	+	+	+	+	+
8.	Phenolic compounds	-	-	-	-	-	-
9.	Flavonoids	+	+	+	+	+	-
10.	Gum & Mucilage	+	+	+	+	+	+
11.	Terpenoid	+	+	+	+	+	+

Present: (+)

Absent: (-)

Identification of different carotenoids by TLC:

Purification and identification of this pigment were investigated by TLC. Results of TLC indicated that the Hexane extract and acetone extract highest number of carotenoid fractions were obtained followed by the mobile phase hexane: acetone (3:1) (Table 2) as compared to other organic solvent extracts followed by mobile phase benzene: ethyl acetate (1:1).

Three band were obtained in TLC (Fig.1) and R_f values for three bands were varied slightly using standards to confirm the carotenoids Table 3 (Lorenz Todd, 1998) ¹². The R_f values for three bands of acetic extract were 0.36, 0.60, 0.75 respectively (Table 2), which corresponded to astaxanthin, astaxanthin monoester, astaxanthin diester. The R_f values obtained for astaxanthin monester and astaxanthin diester are agreement

RESULTS AND DISCUSSION:

Extraction of Astaxanthin from crustacean shell wastes:

Extraction of astaxanthin esters from the crustacean shell wastes were done with different organic solvents. The solvent extracted carotenoid was in the form of an orange-red coloured solution.

Screening for phytochemical constituents

Investigations on the phytochemical screening of the present six different organic extract of crustacean shell waste were revealed the presence of protein, fats and terpenoids in all the extracts (Table 1). Carotenoids are the compounds belongs to major group of terpenoids.

with the results reported by Kobayashi and Sakamoto ¹³ which had R_f values indicated as 0.60 for astaxanthin monoester and 0.75 – 0.85 for astaxanthin diester.



FIG. 1: THIN LAYER CHROMATOGRAPHY OF ASTAXANTHIN

TABLE 2: Rf VALUES OF DIFFERENT CAROTENOIDS IN CRUSTACEAN SHELL EXTRACTS (MEAN OF THREE DETERMINATION) WITH SIX DIFFERENT ORGANIC SOLVENTS

Rf values	Hexane	Methanol	Chloroform	Acetone	Petroleum ether	Ethanol
Band 1	N.A.	N.A.	N.A.	0.36	N.A.	N.A.
Band 2	0.84	N.A.	N.A.	0.6	N.A.	N.A.
Band 3	0.95	0.92	0.97	0.75	0.84	0.95

N.A. = Not Adapted.

TABLE 3: Rf VALUES USING STANDARDS TO CONFIRM THE ASTAXANTHIN (CAROTENOIDS) (LORENZ, TODD, 1998) ¹²

Carotenoid	Typical Rf value
B-carotene	0.99
Echinenone	0.87
Astaxanthin Di-esters	0.75
Astaxanthin Monoesters	0.50
Canthaxanthin	0.40
Astaxanthin Free	0.33
Lutein	0.25

Antibacterial activity:

Antimicrobial activity was studied by agar well diffusion method. All six extracts were tested against both gram positive and gram negative

bacteria. Highest zone of inhibition were obtained from methanolic extract against all the microbes as compared to other extracts (**Table 4**).

TABLE 4: ANTIBACTERIAL ACTIVITY PROFILE FOR ALL SIX EXTRACTS OF ASTAXANTHIN AGAINST GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

Bacterial strains	Zone of inhibition in mm					
	Hexane	Methanol	Chloroform	Acetone	Petroleum ether	Ethanol
<i>E.coli</i>	15	45	ND	10	ND	16
<i>Bacillus</i>	12	30	ND	17	ND	20
<i>Staphylococcus</i>	10	36	ND	14	ND	21
<i>Pseudomonas</i>	20	42	ND	18	ND	36

ND: not detected

Quantification of Astaxanthin from crustacean shell wastes:

All six extracts of the crustacean shell extracts were shown varying extraction yield of carotenoids by using equation of Kelley and Harmon. The highest carotenoid yield of 48.64 μ g/g was obtained from acetic extracts, when compared to hexane, methanol, ethanol, petroleum ether and chloroform whose yield were 36.28 μ g/g, 30.47 μ g/g, 28.62 μ g/g, 11.9 μ g/g and 2.14 μ g/g respectively.

Table 5. Sachindra *et al.* ^{14, 15} reported that a 50:50 mixture of isopropyl alcohol and hexane gave the highest yield (43.91 μ g/g wet waste) of carotenoid compared to acetone (40.60 μ g/g wet waste). According to Sachindra and Mahendrakar ¹⁵ extraction of carotenoids from shrimp waste (*Penaeus indicus*) with vegetable oils and concluded that sunflower oil was the best oil for extraction, where the extraction yield was 26.3 μ g/g. Vimla and Paul ¹⁶ have reported that

maximum yield of carotenoids from *Penaeus monodon* waste was obtained with acetone, compared to other solvents used for extraction. Polar solvents are generally a good extraction media for xanthophylls whereas non polar solvents are not recommended as their penetration through the hydrophobic mass that surrounds the pigment is limited.

TABLE 5: EXTRACTION YIELD OF CAROTENOIDS WITH DIFFERENT ORGANIC SOLVENTS

Extraction solvent	Astaxanthin in μ g/g
Methanol	30.47
Ethanol	28.62
Chloroform	2.14
Petroleum ether	11.90
Acetone	48.64
Hexane	36.28

Several reports are available on the yield of astaxanthin from different species of deep sea shrimps. Of these, highest yield of 14.8mg/100 g

dry waste was reported by Shahidi and Synowiecki¹⁷ from the waste of *P. borealis* shrimp. In the present study we obtained a yield of 48.64µg/g crustacean shell waste (**Table 5**) thus reveals that, of the different species of deep sea shrimps *Aristeus alcocki* shell waste is an excellent source of astaxanthin.

In this study, the astaxanthin extracted from crustacean shell waste powder 48.64µg/g was calculated. A. Khanafari et al., (2007)¹⁸ reported that the carotenoid in the shrimp powder, was 23.123 mg/g. The carotenoid content in blue crab, *Callinectes sapidus*, was 4.63 mg/g according to Hinostroza GN et al, 2002¹⁹.

CONCLUSION: Crustacean shell wastes from industries can be utilized for the isolation of important bioactive compounds like natural carotenoid such as astaxanthin. From this above study acetone will be a good extraction media for astaxanthin among organic solvents. TLC separation confirmed that carotenoid extract from crustacean waste contained astaxanthin, astaxanthin mono and diester. Potent antimicrobial activity of crustacean waste extract may find application in pharmaceutical industries for treatment or prevention of pathogenic microbial infections.

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