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Extraction and Characterization of Astaxanthin from Prawn Shell Waste

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Abstract: *Astaxanthin is a colored, lipid soluble pigment which is an effective antioxidant. Natural Astaxanthin from prawn shell waste could be a better alternative for synthetic orange red pigment. In India, 3 percent of exports are marine products. The wastes produced from marine processing industries possess a great environmental threat which can be recycled into a better alternative product. These wastes are a larger source of natural carotenoids that has varied pharmaceutical potentials. Astaxanthin finds its application in food additive and pharmacology mainly in cosmetology and anti-diabetic activity. It was extracted from shell waste by using organic solvent extraction method. Astaxanthin yield obtained using acetone solvent extraction was 12.5mg/g. The extracted Astaxanthin was confirmed using TLC which proved the presence of astaxanthin. FTIR studies revealed the presence of the functional groups. NMR indicated the protons present in the molecule. The antioxidant studies revealed the perfect antioxidant activity of astaxnthin. Hence this studies shows that astaxanthin from shrimp had better alpha amylase inhibition activity.*

Keywords: *Astaxanthin, Proximate Analysis , UV-vis spectra analysis, TLC , NMR Analysis , FT-IR Analysis, Antibacterial activity , Antioxidant activity.*

INTRODUCTION

Astaxanthin is a carotenoid of xanthophyll found in different micro-organisms and marine animals. It is a red fat-soluble pigment that has no pro-Vitamin A activity in the human body, though some studies have indicated that astaxanthin has more potent biological activity than other carotenoids. The United States Food and Drug Administration (USFDA) has approved the use of astaxanthin in animal and fish feeds as a food colorant. The European Commission considers human astaxanthin as a food dye. *Haematococcuspluvialis*a green micro algae that gathers high levels of astaxanthin under stress conditions like high salinity, deficiency in nitrogen, high temperature and light. Astaxanthin is naturally exist in three forms, monoester, diester and free form based on the reaction with fatty acid. Astaxanthin is localized in cellular membranes vertically in order to involve in both intracellular and extracellular activity, which is found in different microorganisms and marine animals. Natural Astaxanthin may complexes with protein as carotenoid protein or in esterified form whereas synthetic Astaxanthin is not in esterified form, it always produced in free form, which is unstable and susceptible to oxidation. Naturally isolated esterified Astaxanthin has more stability and greater biological function than other Carotenoids and nutrients.

MATERIALS & METHODS

Materials used

The astaxathin extraction process was carried out by collecting the shrimp waste from local fish market in Trichy. Chemicals required for the process were acetone, petoleum ether, sodium chloride, chloform-methanol mixture, conc.

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H2SO4, phosphor- vanillian reagent, sodium hydroxide, orthophosphoric acid, carbonate, copper sulphate, potassium sodium tartarate, folin-ciocalten reagent, 0.001% bovine serum albumin, sodium hydroxidetartarate, DNS, NaOH, glucose, acetone, hexane, benzene, ethyl acetate, dichloromethane. All chemicals were purchased from Precision and Co in Trsichy. All of the chemicals were of analytical quality and used for the experiments as obtained and used without further purification. For the preparation of solutions, double distilled water was used.

Sample collection

Collection of sample (shrimp waste) plays animportant role in extraction of astaxanthin. Once the shrimp waste was collected was cleaned with normal tap water. After washing, it should be allowed to dry under sun shade for 5-6 days. The dried samples were collected and it was homogenized using mortar and pestle. Now the dried homogenized sample was ready for proximate analysis.

Proximate Analysis

Dried samples were collected and proximate analysis has been done. Various proximate analysis test include determination of lipid content, protein content and carbohydrate content was carried out.

Evaluation of lipid content

NaCl was added to remove non-lipid contaminants and to release the bound acid lipids. The Samples were centrifuged to 0.5 mg of sample, 2.5ml of choloroform–methanol mixture was added and homogenized. 0.4ml of and the upper and middle phase proteins are removed. The lower phase was made up to 1ml with chloroform-methanol mixture. The samples were added about 0.5 ml of sulphuric acid and kept in the water bath for 10 minutes. (Folchet al., 1957)

Estimation of protein content

5ml of the reagent mixture was added to 1 ml of sample, and incubated for 10 minutes. Folin -ciocalten reagent was added to 0.5 ml after incubation. Simultaneously, blank and standard were prepared by using 1N sodium hydroxide and BSA respectively. The protein concentration was calculated using UV spectrophotometer at 740nm. (Lowry et al., 1951)

Evaluation of carbohydrate

1 ml of the sample, blank (Distilled water) and the Standard glucose were taken in three different test tubes. The three test tubes had been incubated in a water bath or incubator at 650C for 15 min. Then, 3 ml of DNS was added to all three tubes and held for 15 minutes in boiling water-bath and chilled to room temperature.**(**Miller, 1959**)**23.

Extraction of Astaxanthin

Extraction of astaxanthin from crustacean shell was examined with the use of various organic solvents (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. The carotenoid extract was then filtered using filter paper Whatman No.42.(Dalei and Sahoo, 2015).

Quantification of Astaxanthin

The organic solvent extracted astaxanthin was quantified using the Sachindra and Mahendraker equation and by measuring absorbance at 470 nm. AST (μg /g) = (A x D x 106) / (100 x G x d x E) .

Thin layer chromatography

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 benzene: ethyl 24 acetate (1:1). The separated bands were identified using standard astaxanthin and internationally accepted Rf values for astaxanthin monoester and astaxanthin diester (Todd et al., 1998).

Characterisation of Astaxanthin

The characterization of Astaxanthin was performed by UV-Vis, NMR and FT-IR studies.

UV-vis spectra analysis

Samples (1 mL) of the suspension were gathered periodically to track the completion of the bioreduction of TiO_2 in aqueous solution, pursued by sample dilution with 2 ml of deionized water and subsequent scanning in UV-visible (vis) spectra, between 200 to 700 nm wavelengths in a spectrophotometer (Mapada UV-1800 china) having a resolution of 1 nm.

NMR Analysis

Astaxanthin extracted was subjected to NMR analysis along with the standard. DMSO was used as a solvent in NMR analysis. (Rao et al., 2005).

FT-IR Analysis

Astaxanthin extracted using acetone: petroleum ether was subjected to FTIR along with the standard in the range of 200 to 4500 cm-1. The range spectrum was evaluated to determine the functional groups present in different samples along with the standard.

Antioxidant property

DPPH Assay

0.1mM DPPH solution was prepared by dissolving 4mg of DPPH in 100ml of ethanol. The reagent was prepared. Different volumes of the extract were made up to 40µl with DMSO and 2.96ml DPPH (0.1mM) solution was added. The Reaction mixture was incubated in dark condition at room temperature for 20 min.After 20 min, the absorbance of the mixture was read at 517 nm. 3ml of DPPH was taken as control. The % radical scavenging activity of the plant extracts was calculated using the following formula, 25 %RSA = $Abscontrol – AbssampleAbscontrol$ 100Where, RSA is the Radical Scavenging Activity; Abs control is the absorbance of DPPH radical + ethanol; Abs sample is the absorbance of DPPH radical + plant extract.(Alam et al., 2012).

FRAP Assay

This technique measures antioxidants capability to reduce ferric iron. It is based on decrease of the ferric iron complex and 2,3,5-triphenyl-1,3,4- triaza-2-azoniacyclopenta-1,4- diene chloride to the low pH ferrous form. This reduction is tracked using a diode-array spectrophotometer, by calculating the difference in absorption at 593 nm. Antioxidant assay may be conducted by using the Benzie and Strain (1999) method. Three milliliters of prepared FRAP reagent is combined with a diluted sample of 100 µl; the absorbance at 593 nm is recorded at 370C after a 30 min incubation.The FRAP values can be obtained by comparing the absorption difference in the test combination with those obtained from increased Fe3 + concentrations and expressed as mM of Fe2 + equivalents per kg (solid food) or per L (beverages) of the sample (NurAlam et al.,2012).

ANTIBACTERIAL ACTIVITY

Inoculum preparation

The nutrient broth was prepared and sterilized for 15 minutes in an autoclave at a pressure of 15 pounds. All the bacterial strains in the sterilized nutrient broth were inoculated individually and incubated at 37° C for 24 hours.

Evaluation of antimicrobial activity

Antibacterial activity of the chitosan (extracted) and chitosan silver nanoparticles were analysed by following the technique of Bauer *et al*., 1996. Three various concentrations of samples (10 µg/ml, 25 µg/ml, and 50µg/ml) were tested for antibacterial activity using agar disc diffusion assay. Media were ready using nutrient Agar (Himedia), poured on petridishes and inoculated using cotton swabs with the test organisms from the seeded broth. Sterile discs of six millimeter width had been impregnated with different concentrations of sample and introduced onto the upper layer of the seeded agar plate. The plates were incubated overnight at 37^0 C. Antibacterial activity was assessed by measuring millimeters of the inhibition zone formed around the discs. The experiment was done three times, and it provided the mean values. Tetracycline and erythromycin were used as positive controls for *Staphylococcus aureus* and *E.Coli*, respectively.

In-vitro Anti diabetic α-Amylase inhibitory activity

α-amylase inhibitory activity of extract and fractions was carried out according to the standard method with minor modification. 50 µl phosphate buffer (100 mM, pH = 6.8), 10 µl α-amylase (2 U/ml), and 20 µl of varying concentrations of extract and fractions (0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml) was preincubated at 37°C for 20 min. Then, the 20 µl of 1% soluble starch (100 mM phosphate buffer pH 6.8) was added as a substrate and incubated further at 37°C for 30 min; 100 µl of the DNS colour reagent was then added and boiled for 10 min. The absorbance of the resulting mixture was measured at 540 nm. Acarbose at various concentrations (0.1-0.5 mg/ml) was used as a standard. The results were expressed

as percentage inhibition, which was calculated using the formula, Inhibitory activity (%) = (1 − As/Ac) ×100 Where, As is the absorbance in the presence of test substance and Ac is the absorbance of control.

RESULTS AND DISCUSSION

Extraction of Astaxanthin

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

Confirmation using Thin Layer Chromatography

Analysis of astaxanthin and its esters in the extract was confirmed using Thin Layer Chromatography (TLC). A small volume of the isolated astaxanthin was spotted on silica gel plate along with standard astaxanthin. Spot development was done by using two different types of mobile phase such as; acetone: hexane(1:3) and benzene: ethyl acetate (1:1). The separated bands were identified using standard astaxanthin and internationally accepted Rf values for astaxanthinastaxanthin monoester and astaxanthin diester (Todd, 1998). The sample was found to contain free astaxanthin and astaxnthin diesters which was crossed checked with the international Rf value.

Characterization of Astaxanthin

The extracted astaxanthin was taken for various characterisation to check forits chemical structure and concentration.

Characterization by UV Visible Spectroscopy

The sample was analysed from UV to visible range. The peak for the samplewas observed between 460 to 500 nm, indicating the absorbance range of astaxanthin. The molecule had its λmax at 470nm which was in coherence with theprevious obtained results(Chen and Meyers ,1984). The λmax was at high range duethe double bonds present in it.

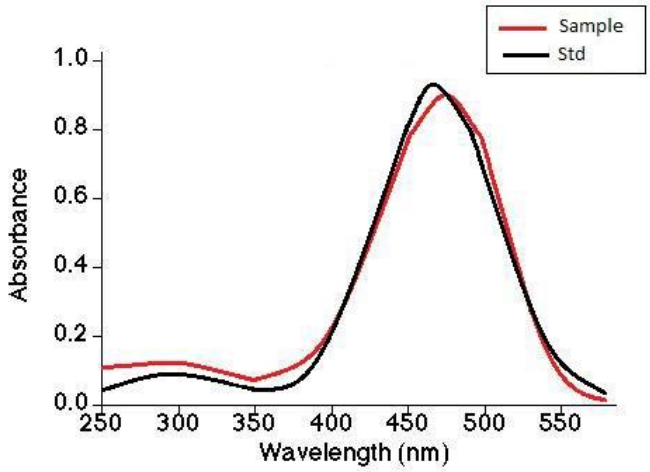

Figure 1: Absorption curve of Astaxanthin

Characterization by FT-IR Spectral Analysis

The shape, shift, and intensity variation of the peaks of IR absorption provides enough information for ASTX to occur. In agreement with the earlier report, astaxanthin's IR spectrum showed its characteristic bands(Subramanium et al., 2015, Chen et al., 2005). The band absorbed at 3402 cm−1was the O-H stretching vibration. The bands between 2850-3000 cm−1 denoted theC-H stretching. There was a very strong absorption band at 1621.71 cm−1 for C=Ostretching vibration. Absorption band at 1410.72 cm−1 was denoted for methyl insymmetric deformation. Absorption band at 1067.66 cm−1 denoted the CHstretching which was out of plane.The prominent peak at 990 cm−1 was forabsorption band of C-H in C-C conjugate system. All these bands prove the structural data for Asataxanthin.

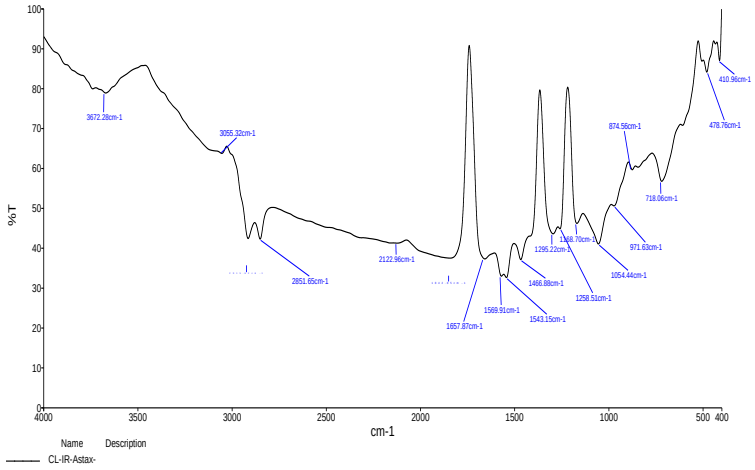

Figure 2: IR Spectra of Astaxanthin

Characterization using 1H NMR

1H NMR was used to predict the structure of the extracted astaxanthin molecule. Signals between 7.0 and 8.5 ppm represent the methine protons on the ASTX backbone. These signals appear as two sets in the monoesterified compounds due to the loss of symmetry. At 2.47 ppm, a multiplet integrating for four protons corresponds to the methylene protons and α to the carbonyl. Singletsat 2.04, 1.92 and 1.82 ppm, correspond to the methyl moieties. A large signal corresponding to the methylene protons on the fatty acid moiety appears between 1.25 and 1.4 ppm. Overlapping peaks appearing around 1.85 and 2.0 ppm correspond to the methylene protons. A broad signal at 3.89 ppm corresponds to theOH moiety on the ASTX molecule, which correlated with the value reported by (Subramanium et al., 2015).

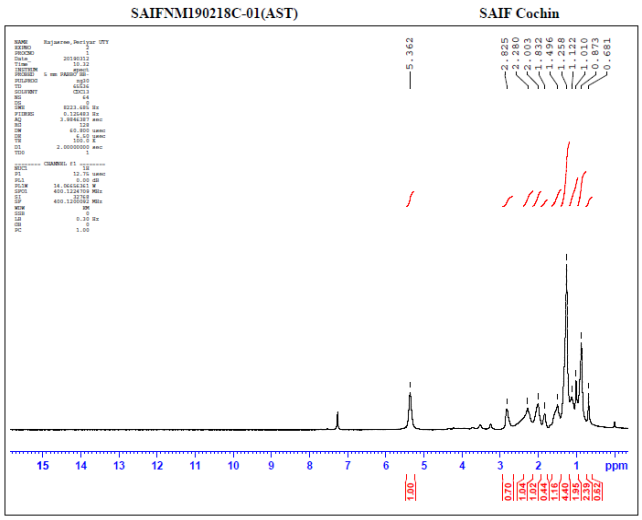

Figure 3: [1]H NMR Spectra of Astaxanthin

Estimation of Astaxanthin

From the data obtained from UV-Vis spectroscopy the amount of astaxanthin present in the shrimp waste was calculated using the formula stated by (Sachindra and Mahendrakar, 2004). Thus the amount of carotenoid as astaxanthin found was to be 12.5mg/g waste. This correlates the fact that astaxanthin is the main composition of shrimp waste.

Anti-oxidant Studies

Free radicals are responsible for a huge number of diseases, including cancer (Kinnula and Crapo, 2004). A large intake of dietary antioxidants can compromise defence against free radicals.

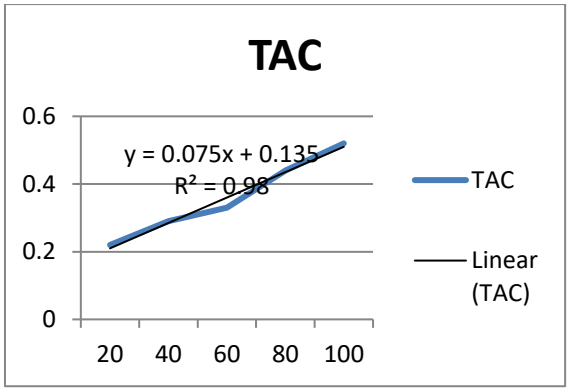

Figure 4: Percentage inhibition of TAC

DPPH Scavenging Assay

The stable free radical present on the DPPH dye is delocalized by astaxanthin which let to the formation of a dark colour. It proves that ASTX acted as a hydrogendonor for the dye to delocalize the electron in its own molecule. The percentage of DPPH scavenging was stated below.

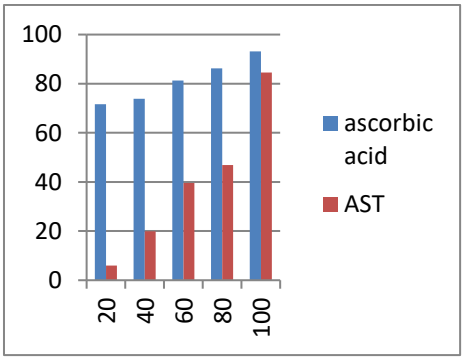

Figure 5: Percentage inhibition of DPPH

FRAP Assay

The ferric reducing antioxidant power ASTX was done using the FRAP reagent. The color change of the reagent was considered as the increase in percentage of inhibition. Astaxanthin was found to very effective against ferricradical.

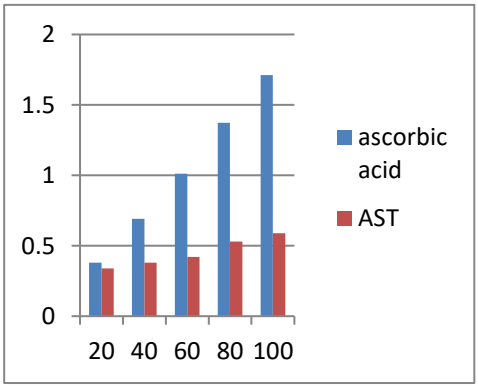

Figure 6: Percentage inhibition of FRAP

TOTAL ANTIOXIDANT CAPACITY (TAC) ASSAY

Total antioxidant capacity (TAC) was based on decrease of Mo(VI) to Mo(V) by extract and subsequent creation of green phosphate/Mo(V) complex at acid pH. It assesses both water-soluble and fat-soluble antioxidants (total antioxidant capacity). The total antioxidant activity of astaxanthin showed consistent increase in antioxidant activity with increased concentration (figure 19, graph 4). We used as normal

ascorbic acid.TAC of chitosan and chitosan silver nanoparticles were calculated as 318 ±99.37 mg/g and 555 ±251.23 mg/g ascorbic acid respectively.

The results indicate higher TACs of chitosan and chitosan nanoparticles, respectively, at low concentration. This indicates their potential antioxidant constituents because ascorbic acid's antioxidant capacity was used as a reference standard with which chitosan and chitosan silver nanoparticles were compared with potential antioxidants (Kahkonen*et al*., 1999).

ANTI MICROBIAL ACTIVITY

The synthesized nanoparticles' antibacterial activities were analyzed by a qualitative well-diffusion assay on both Gram-positive and Gram-negative bacteria. The diameter of the zone of inhibition (ZOI) for each organism. After 18 h of incubation at 37°C, Staphylococcus aerues 2,4,10 12,and *Escherichia coli* showed zones of inhibition of 3,6,12 and15 mm respectively, at 20 µg/ml of Astaxanthin, 21, 32, and 38 mm respectively, at 50 µg/ml of and Bacillessubtence 3,4,,10 and 12mm zones of inhibition respectively, at 100 µg/ml of Astaxanthin. The results showed that the nanoparticles are most effective against Gram-negative *Escherichia coli* compared to another two Gram-positive bacterial strains.

Table 1: Anti-Microbial Activity

Species	PC	NC	50	100	150	200
Escheria .coli	32	-	3	6	12	15
Streptococcus aureus	28	-	2	4	10	12
Pseudomonas .aerogenosa	22	-	-	-	9	10
Bacillus .Subtitles	20	-	3	4	10	12

In-vitro Anti diabetic α-amylase inhibitory activity

The results of the present study indicated astaxanthin shows higher antidiabetic activity in DNS method. Test samples possess the best activity when compared to standard drug acarbose.

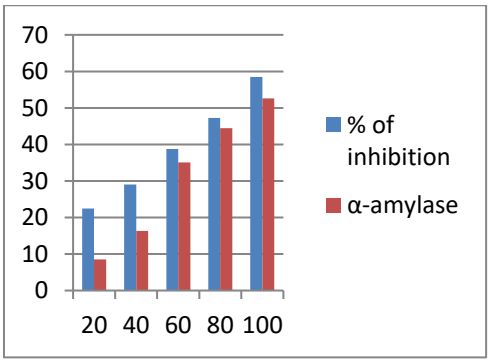

Figure 7:Percentage inhibition of α-Amylase

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