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Valorization of Freshwater Crab Derived Astaxanthin from *Spiralothelphusa hydrodroma* (Herbst, 1794) with Enhanced Antioxidant and Antibacterial Efficiency

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Abstract

Astaxanthin is a biologically important xanthophyll carotenoid known for its exceptional antioxidant activity and therapeutic potential. The present study focused on the extraction, characterization, antioxidant and antibacterial evaluation of astaxanthin obtained from the freshwater crab *Spiralothelphusa hydrodroma* collected from Kundrathur Lake, Tamil Nadu, India. The crab samples were separated into dry shell (DS), wet shell (WS), dry tissue (DT) and wet tissue (WT) fractions for comparative analysis. Proximate composition analysis revealed significantly higher moisture content in WT ($80.2\pm0.38\%$), whereas ash content was highest in DS ($70.87\pm0.96\%$), indicating the highly mineralized nature of the shell. Astaxanthin extraction was performed using acetone solvent extraction and quantified spectrophotometrically at 476 nm. The highest astaxanthin yield was recorded in DS ($218.01\pm0.18\ \mu\text{g/g}$), followed by WS ($193.94\pm0.26\ \mu\text{g/g}$), while tissue samples exhibited lower yields. UV–Visible spectroscopy showed a characteristic absorption maximum at 476 nm, confirming the presence of astaxanthin. FTIR and TLC analyses confirmed the presence of characteristic functional groups and different astaxanthin forms. Antioxidant assays including DPPH, ABTS and reducing power assays demonstrated concentration-dependent activity, with DS showing the highest antioxidant potential. Antibacterial activity against *Escherichia coli* revealed maximum inhibition in DS (15 mm), followed by WS (13 mm), WT (12 mm) and DT (11 mm). The findings suggest that freshwater crab shell waste is a promising natural source of astaxanthin for nutraceutical and pharmaceutical applications.

Keywords: Astaxanthin; *Spiralothelphusa hydrodroma*; FTIR analysis; Antioxidant activity; Antibacterial activity

Introduction

Carotenoids are naturally occurring lipophilic pigments widely distributed in plants, algae and microorganisms, where they impart yellow, orange and red coloration. Besides their pigmentation role, carotenoids possess strong antioxidant properties and are associated with protection against oxidative stress and chronic diseases [1,2]. Structurally, carotenoids consist of eight isoprene units with an extensive conjugated double-bond system responsible for their antioxidant activity. They are classified into carotenes and xanthophylls based on the presence or absence of oxygen-containing functional groups [3]. Among xanthophylls, astaxanthin has gained significant attention because of its exceptional antioxidant efficiency and diverse biological activities.

Astaxanthin is a red-orange ketocarotenoid containing hydroxyl and keto groups on each ionone ring, which enhance its polarity and biological activity [4]. Its conjugated polyene chain enables efficient scavenging of reactive oxygen species and prevention of oxidative damage [5]. Previous studies have reported antioxidant, anti-inflammatory, antibacterial, immunomodulatory, cardio protective and neuroprotective properties of astaxanthin, highlighting its importance in nutraceutical and biomedical applications [6,7]. In crustaceans, astaxanthin mainly occurs as carotenoprotein complexes associated with proteins and lipids [8]. Although synthetic astaxanthin dominates the market, concerns regarding its lower bioactivity have increased interest in natural sources such as microorganisms and aquatic organisms including shrimp, crabs and lobsters [9,10,11].

Crustacean shell waste generated during seafood processing is considered a valuable source of bioactive compounds. Nearly 50–70% of crustacean biomass is discarded as waste containing chitin, proteins, calcium carbonate and carotenoid pigments including astaxanthin [12,13]. In crustaceans, astaxanthin is commonly present as crustacyanin complexes, which release free astaxanthin upon denaturation [14]. However, extraction is challenging due to the rigid chitinous exoskeleton and strong pigment-protein interactions [15]. Conventional solvent extraction methods using acetone, hexane and ethyl acetate are commonly employed, followed by characterization techniques such as Fourier Transform Infrared (FTIR) spectroscopy and Thin Layer Chromatography (TLC) for confirmation of astaxanthin [16,17,18]. Among freshwater crabs, *Spiralothelphusa hydrodroma* has attracted attention because of its nutritional and industrial importance. This edible freshwater crab, belonging to the family Gecarcinucidae, is widely distributed in ponds, canals and paddy fields across India [19]. However, studies on astaxanthin extraction and biological activities from this species remain limited.

Oxidative stress caused by excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is associated with several chronic diseases including cancer and cardiovascular disorders [20,21]. Antioxidants protect cells by scavenging free radicals and inhibiting oxidative reactions [22]. Astaxanthin is recognized as one of the most potent natural antioxidants because of its conjugated double bonds and functional groups [23]. Antioxidant activity is commonly evaluated using multiple in vitro assays such as DPPH, ABTS and reducing power assays, which are based on hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms [24]. The DPPH assay measures free radical scavenging activity, whereas ABTS evaluates both hydrophilic and lipophilic antioxidant capacity [25,26]. The reducing power assay determines the electron donating ability of compounds through ferric ion reduction [27]. In addition to antioxidant activity, astaxanthin also exhibits significant antibacterial activity against both Gram-positive and Gram-negative pathogens by disrupting microbial

cell membrane integrity, causing leakage of intracellular components, and interfering with essential metabolic and enzymatic functions, thereby inhibiting microbial growth and survival [28,29]. Owing to its strong antioxidant potential, this dual bioactivity makes astaxanthin a promising natural compound for nutraceutical, pharmaceutical, and food preservation applications. In this context, the present study focused on the extraction and characterization of astaxanthin from the freshwater crab *Spiralothelphusa hydrodroma* using conventional solvent extraction, followed by FTIR and TLC analyses. Further, its antioxidant and antibacterial activities were evaluated to assess its potential biomedical and nutraceutical relevance.

Materials and methods

Collection and maintenance of experimental animals

Adult freshwater crabs were collected from Kundrathur Lake, Kanchipuram, Tamil Nadu, India. After being collected, the crabs were transported to the laboratory where they were maintained in tanks measuring 0.5×0.5 m. These tanks were filled with clean water ensuring the crabs were partially submerged. Given the tropical environment, the temperature in the tanks remained consistently moderate ranging from 27 to 30 °C. The crabs were allowed to acclimatize to the laboratory conditions for duration of two days before the experiment commenced. The crab sample was submitted to Zoology survey of India (ZSI) for identification of species.

Preparation of samples

The crabs were thoroughly washed with distilled water prior to processing. The killing procedure was performed in two steps. Initially, the crabs were subjected to ice slurry treatment (0–4 °C) for 10–15 minutes to induce desensitization. This was immediately followed by thermal killing through boiling at 100 °C for 8–12 minutes. After boiling, the samples were rapidly cooled by immersion in ice-cold water for 5–10 minutes to halt further thermal degradation. Subsequently, excess surface moisture was removed by allowing the samples to drain, followed by gentle blotting using clean tissue paper. The processed crabs were then dissected to separate tissues from the exoskeleton, with particular focus on the carapace and claws. During this process, non-target materials such as gills and gut contents were carefully removed and discarded.

The separated shells and tissues were subjected to homogenization, wherein the samples were ground using a mortar and pestle to obtain uniformity. The homogenized samples were then divided into two portions for wet and dry basis analyses. For dry basis preparation, the samples were dried in a hot air oven at 50–55 °C overnight until a constant weight was achieved and subsequently ground into a fine powder. For wet basis analysis, the freshly homogenized paste was directly used for further experimental procedures without drying. The samples prepared were categorized as dry shell (DS), wet shell (WS), dry tissue (DT) and wet tissue (WT).

Proximate analysis

The estimation of moisture content, ash content and solubility of crab shells and tissues were observed. Moisture and ash contents were determined using standard methods following (AOAC 930.15 and AOAC 938.08), respectively. The solubility of shell and tissue samples was assessed using a standardized protocol [30]. Briefly, 0.1 g of the sample was accurately weighed and transferred into a centrifuge tube, to which 10 mL of 40% acetic acid was added.

The mixture was allowed to stand for 2 hours to facilitate dissolution of soluble components and was subsequently heated in a boiling water bath for 10 minutes. The samples were then cooled to room temperature and centrifuged at 10,000 rpm for 10 minutes. The supernatant was carefully decanted and the residual pellet was washed with distilled water and centrifuged again under the same conditions to remove any remaining soluble matter. The final insoluble residue was dried at 60 °C for 24 hours and weighed. The percentage solubility was calculated based on the difference between the initial sample weight and the weight of the dried insoluble fraction using an appropriate formula.

$$\% \text{ of solubility} = \frac{(\text{Initial weight of tube + sample}) - (\text{Final weight of tube + sample})}{(\text{Initial weight of tube + sample}) - (\text{Initial weight of the tube})}$$

Astaxanthin extraction

For the extraction of astaxanthin, 1 g of each sample was accurately weighed and dissolved in 10 mL of acetone and followed by continuous stirring for 1 hour using a magnetic stirrer. The extraction process was carried out under aluminum foil covering to prevent photo-oxidative degradation of the pigment. The mixture was then filtered through Whatman filter paper and the filtrate was collected. This extraction procedure was repeated until the filtrate became colorless, ensuring maximum recovery of astaxanthin. The pooled acetone extracts were subsequently transferred into a separatory funnel to which 12.5 mL of petroleum ether and 9.4 mL of 0.73% NaCl solution were added. The mixture was gently shaken to facilitate phase separation resulting in two distinct layers: an upper petroleum ether phase containing dissolved astaxanthin and a lower aqueous phase comprising acetone and NaCl solution. The upper layer was carefully collected and subjected to solvent evaporation using a rotary evaporator. The concentrated residue containing astaxanthin was then transferred into an amber vial and stored at -20 °C to prevent degradation.

Astaxanthin Quantification

Following the extraction procedure, the resulting four samples were transferred into a cuvette, for the quantification process and the absorbance was measured at the maximum wavelength ($\lambda_{\text{max}} = 476 \text{ nm}$) using a spectrophotometer (Shimadzu UV 2500).

Fourier Transform Infrared Spectrometry (FTIR) Analysis

Fourier Transform Infrared (FTIR) spectroscopy was performed to identify the functional groups and structural characteristics of astaxanthin in both crude and extracted samples. The spectra were recorded over a wavenumber range of 4000–400 cm^{-1} using an FTIR spectrometer (Nicolet iS50). A background scan was performed prior to sample measurement to minimize atmospheric interference.

Thin Layer Chromatography

Thin Layer Chromatography (TLC) was performed to confirm the presence of astaxanthin and its esterified derivatives in the extract. A small volume of the isolated astaxanthin extract was applied as a spot onto a pre-coated silica gel TLC plate. The plate was developed in a solvent system consisting of acetone and hexane in the ratio of 3:7 (v/v). The separated bands were observed and the retention factor (R_f) values were calculated.

$$R_f = \frac{\text{Distance Travelled by Solute}}{\text{Distance Travelled by Solvent}}$$

Antioxidant assay

DPPH Radical Scavenging Assay

The antioxidant activity of the sample was evaluated using the DPPH radical scavenging assay followed [31]. 0.1 M DPPH solution was prepared by dissolving 10 mg of DPPH in 250 mL of methanol. For the assay, 100 μ L of the DPPH solution was mixed with 100 μ L of sample solutions at different concentrations such as 20 and 50 μ L. The reaction mixture was incubated in the dark at room temperature for 30 minutes. Following incubation, the decrease in color intensity from deep purple to pale yellow was observed and the absorbance was measured at 517 nm using a spectrophotometer. DPPH solution without sample served as the blank.

$$\text{DPPH radical scavenging (\%)} = \frac{[\text{Control} - (\text{Sample} - \text{Blank})]}{\text{Control}} \times 100$$

ABTS Radical Scavenging Assay

The antioxidant activity of astaxanthin was determined using the ABTS radical cation scavenging assay followed [32]. The ABTS radical cation reagent was prepared by dissolving 7 mM ABTS in 10 mL of double-distilled water and 2 mM potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$) in 10 mL of double-distilled water. Equal volumes of both solutions were mixed and allowed to react in the dark at room temperature for 16 hours to generate the ABTS radical cation. Prior to the assay, the ABTS solution was diluted with double-distilled water until an absorbance of 0.8 ± 0.02 was obtained at 734 nm. For the assay, 150 μ L of the ABTS reagent was mixed with 20 and 50 μ L concentrations of astaxanthin samples. The reaction mixture was incubated for 6 minutes at room temperature and the absorbance was measured at 734 nm using a spectrophotometer. The ABTS reagent without a sample served as the blank.

$$\text{ABTS scavenging activity (\%)} = \frac{[\text{Control} - (\text{Sample} - \text{Blank})]}{\text{Control}} \times 100$$

Reducing Power Assay

The reducing power of the samples was determined using the standard ferric reducing power method [31]. 0.2 M phosphate buffer (pH 6.6) was prepared and used as the reaction medium. Add 0.5 mL of sample solution at different concentrations was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$). The reaction mixture was incubated at 50°C for 20 minutes to facilitate the reduction of Fe^{3+} to Fe^{2+} . After incubation, the tubes were cooled to room temperature and the reaction was terminated by adding 2.5 mL of 10% trichloroacetic acid (TCA). The mixture was then centrifuged at 4,000 rpm for 10 minutes. Subsequently, 2.5 mL of the upper supernatant was transferred to a fresh tube, mixed with 2.5 mL of double-distilled water and 0.5 mL of 0.1% ferric chloride (FeCl_3) solution was added. The mixture was incubated at room temperature for 10 minutes and the absorbance was measured at 700 nm using a spectrophotometer.

Antibacterial Assay using Agar Well Diffusion Method [33]

The antibacterial potential of astaxanthin extracted from freshwater crab samples, including dry shell (DS), wet shell (WS), dry tissue (DT) and wet tissue (WT) was evaluated using the agar well diffusion assay against selected gram-

negative bacteria. Mueller–Hinton Agar (MHA) plates were prepared under sterile conditions. Fresh bacterial cultures were grown in Mueller–Hinton Broth and sterile cotton swabs were used to uniformly spread each microbial suspension over the surface of the agar plates to obtain a confluent lawn culture. Wells of uniform diameter were made in the agar using a sterile cork-borer. The bottoms of the wells were sealed with a thin layer of molten agar to prevent leakage of the sample solution.

To determine the antibacterial efficacy of the astaxanthin, the wells were loaded with distilled water as the negative control and different samples of astaxanthin at concentration of 20µg/mL. After sample loading, the plates were incubated at 37°C for 24hrs. The antibacterial activity was assessed by measuring the diameter of the zone of inhibition around each well using a calibration scale and the results were recorded in millimeters.

Statistical analysis

The data were expressed as mean $\pm$ SD of triplicate experimental trials.

Results

Proximate Analysis

Significant variations in moisture, ash and solubility contents were observed between shell and tissue samples of *Spiralothelphusa hydrodroma*. Moisture content on a wet basis was markedly higher in wet tissue ($80.2 \pm 0.38\%$) than in wet shell samples ($20.7 \pm 0.83\%$). In contrast, ash content was substantially greater in shell samples, with dry shell exhibiting $70.87 \pm 0.96\%$ ash compared to $2.02 \pm 0.43\%$ in dry tissue. Similarly, wet shell showed $51.87 \pm 1.29\%$ ash, whereas wet tissue contained only $0.73 \pm 0.75\%$. The elevated ash content of shells is attributed to high mineral deposition, predominantly calcium carbonate, which forms the structural framework of the exoskeleton. Solubility analysis revealed the highest value in wet tissue ($1.941 \pm 0.004\%$) and the lowest in dry tissue ($0.631 \pm 0.006\%$) (Table 1). Dry shell and wet shell exhibited moderate solubility, with slightly higher values in wet shell samples. The greater solubility of tissue samples is associated with the presence of soluble proteins and organic compounds, whereas the shell contains insoluble structural components such as chitin and calcium carbonate. Overall, the results indicate that the shell of *S. hydrodroma* is highly mineralized with low moisture and solubility, while the tissue is characterized by high moisture content, greater solubility and lower inorganic matter.

Astaxanthin Quantification

Astaxanthin was extracted and quantified from the freshwater crab *S. hydrodroma* using acetone as the primary extraction solvent, owing to its high polarity and proven efficiency in solubilizing xanthophyll carotenoids. The results revealed considerable variation in astaxanthin yield among the four experimental groups: dry shell (DS), wet shell (WS), dry tissue (DT) and wet tissue (WT).

Among these, the dry shell sample exhibited the highest astaxanthin concentration, recording 218.01 ± 0.18 µg/g. The wet shell sample followed closely, yielding 193.94 ± 0.26 µg/g. In contrast, tissue samples showed markedly lower pigment concentrations, with dry tissue yielding 28.03 ± 0.32 µg/g and wet tissue showing the lowest value of 15.20 ± 0.60 µg/g (Table 2).

UV–Vis Absorption Spectrum Analysis

The UV–Visible absorption spectra of the pigments extracted from all four crab samples (DS, WS, DT and WT) showed a characteristic carotenoid absorption pattern with a single, well-defined absorption maximum (λ_{max}) observed at approximately 476 nm. This peak falls within the typical range of carotenoids (450–500 nm), confirming the presence of astaxanthin in all samples (Figure 1). The consistent appearance of a single prominent peak across all extracts suggests the presence of relatively pure carotenoid fractions with minimal interference from other pigments.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis revealed characteristic functional groups associated with astaxanthin in both crude and extracted samples. Carbonyl-associated peaks in crude samples were observed at 1624.55 cm^{-1} (WT), 1622.52 cm^{-1} (DT), 1641.21 cm^{-1} (WS) and 1621.87 cm^{-1} (DS), whereas extracted samples exhibited prominent C=O stretching bands at 1714.78 cm^{-1} (AWT), 1744.16 cm^{-1} (ADT), 1745.42 cm^{-1} (AWS) and 1742.83 cm^{-1} (ADS), within the characteristic range of 1650–1750 cm^{-1} . Aliphatic C–H stretching peaks were observed in both crude and extracted samples around 2921–2957 cm^{-1} . Broad hydroxyl (O–H) stretching bands were detected in crude samples between 3264.97–3427.77 cm^{-1} and in extracted samples between 3347.17–3449.79 cm^{-1} . Methyl group bending vibrations (C–CH₃) were identified in crude samples at 1363.53–1454.43 cm^{-1} and in extracted samples at 1459.45–1462.30 cm^{-1} . Overall, crude samples exhibited broadened and overlapping absorption bands, indicating the presence of complex biological matrices, whereas extracted samples showed sharper and well-defined peaks, suggesting improved purity and reduced biomolecular interference (Figure 2A, B).

Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) was performed using acetone:hexane (3:7) as the mobile phase for Dry Shell (DS), Wet Shell (WS), Dry Tissue (DT) and Wet Tissue (WT) samples. TLC analysis revealed three distinct orange bands in all samples, indicating the presence of three carotenoid fractions (Figure 3). The calculated R_f values corresponding to diester, monoester and free astaxanthin fractions were DS (0.82:0.55:0.22), WS (0.84:0.64:0.26), DT (0.84:0.66:0.37) and WT (0.77:0.62:0.37), respectively (Table 3). Slight variations in R_f values among the samples may be attributed to differences in pigment composition and polarity.

Antioxidant Assay

DPPH Free Radical Scavenging Activity

The DPPH assay was performed to evaluate the free radical scavenging activity of astaxanthin extracts at 20 μl and 50 μl concentrations. At 20 μl , the scavenging activity was 48% for DS, 23% for WS, 15% for DT and 8% for WT. At 50 μl , the activity increased to 52% for DS, 29% for WS, 21% for DT and 12% for WT (Figure 4). In both concentrations, DS exhibited the highest scavenging activity followed by WS, DT and WT. The concentration-dependent increase in scavenging activity indicates the antioxidant potential of the astaxanthin extracts.

ABTS Radical Scavenging Activity

The ABTS radical scavenging activity of astaxanthin extracts was evaluated at 20 µl and 50 µl concentrations. At 20 µl, scavenging activity was 69% for DS, 52% for WS, 26% for DT and 10% for WT. At 50 µl, the activity increased to 93% for DS, 81% for WS, 49% for DT and 41% for WT (Figure 5). DS consistently showed the highest scavenging activity followed by WS, DT and WT. The increase in activity with concentration reflects the strong antioxidant potential of the extracts.

Reducing Power Activity

The reducing power of astaxanthin extracts was determined at 20 µl and 50 µl concentrations. At 20 µl, absorbance values were 0.364 for DS, 0.175 for WS, 0.118 for DT and 0.121 for WT. At 50 µl, absorbance values increased to 0.438 for DS, 0.211 for WS, 0.158 for DT and 0.134 for WT (Figure 6). DS exhibited the highest reducing power at both concentrations, followed by WS, DT and WT. The increased absorbance values indicate greater electron-donating capacity and reducing ability of the astaxanthin extracts.

Antibacterial activity

The antibacterial activity of the different samples against *Escherichia coli* demonstrated varying degrees of inhibitory effects. Among the tested samples, DS exhibited the highest antibacterial activity with a zone of inhibition measuring 15 mm, indicating its strong effectiveness against *E. coli*. In comparison, WS showed moderate antibacterial activity with a zone of inhibition of 13 mm. Similarly, WT and DT exhibited inhibitory zones of 12 mm and 11 mm, respectively (Figure 7), suggesting comparatively lower antibacterial potential. The results indicate that all the tested samples possessed antibacterial activity against *E. coli*, although the extent of inhibition varied depending on the sample type. Overall, the findings suggest that the tested samples possess promising antibacterial potential against gram-negative bacteria such as *E. coli*.

Discussion

The present investigation demonstrates the efficient recovery and extraction of astaxanthin from the freshwater crab *Spirothelphusa hydrodroma*, emphasizing its potential as a valuable natural carotenoid source. In addition, the physicochemical characteristics of the raw material were evaluated to support extraction efficiency; specifically, moisture analysis revealed a significantly higher content in wet tissue (80%) compared to wet shell (20%), reflecting inherent physiological differences. This elevated level in tissues is primarily associated with their muscular composition and higher water-holding capacity, whereas the rigid and mineralized nature of shells results in comparatively lower retention[34]. Similar observations have been reported in *Pandalus borealis* (74–76%) and *Chionoecetes opilio* shells (45–66%) [35,36], supporting the distribution pattern observed in the present study. In contrast, ash content exhibited an inverse trend, with higher values recorded in shell samples, particularly in dry shells (70%) and wet shells (51%), while tissue samples showed considerably lower levels of 2% in dry tissue and 0.73% in wet tissue. This reflects the mineral-rich composition of crustacean exoskeletons. Comparable findings have been documented, where cooked shells exhibited 26% dry basis; 6% wet basis contained higher ash than cooked meat 8.54% dry; 2.15% wet [35]. However, the values obtained in this study are notably higher than those reported for *Astacus leptodactylus* (0.64–0.97%) suggesting possible species-specific or environmental influences

[36]. In addition, the solubility profile further indicated variation among sample types, with the highest value observed in wet tissue ($1.941 \pm 0.004\%$) and the lowest in dry tissue ($0.631 \pm 0.006\%$). When compared with *Portunus trituberculatus* [37], which showed values ranging from $1.12 \pm 0.49\%$ to 0.56 ± 0.46 , the present findings demonstrate slightly higher solubility, particularly in wet tissue samples.

A distinct variation in astaxanthin yield was observed between shell and tissue fractions. The highest concentration was recorded in dry shell ($218.01 \mu\text{g/g}$), followed by wet shell ($193.94 \mu\text{g/g}$), whereas tissue samples exhibited significantly lower values ($28.03 \mu\text{g/g}$ in dry tissue and $15.20 \mu\text{g/g}$ in wet tissue). This distribution indicates preferential accumulation within the exoskeletal matrix. Prior studies have similarly reported higher yields in shell fractions, with cooked shells reaching $270.04 \mu\text{g/g}$ compared to $33.52 \mu\text{g/g}$ in cooked meat [35]. Additionally, yields reported as $35.26 \mu\text{g/g}$ in *Callinectes sapidus* and $19.46 \mu\text{g/g}$ in *Portunus pelagicus* [14] are considerably lower than those obtained in the present study. The enhanced recovery in shell samples may be attributed to the presence of astaxanthin in protein–lipid complexes, while the higher values in dry samples reflect reduced moisture interference [38].

Spectral analysis using UV–Visible spectroscopy revealed a well-defined absorption maximum (λ_{max}) at 476 nm, which lies within the characteristic range of carotenoids (450–500 nm). Comparable absorption peaks at 472.5–475 nm [39] and around 480 nm in *Procambarus clarkia* [40] have been reported, confirming the identity of the extracted pigment. The presence of a single prominent peak suggests minimal interference from other compounds, indicating efficient extraction and relative purity. This behavior is attributed to the extended conjugated double-bond system of astaxanthin, which enables absorption in the visible region [41,42]. Further, a comparative evaluation of the FTIR spectra obtained in the current study with those reported for standard astaxanthin confirms the preservation of its structural integrity following extraction. Astaxanthin is characterized by the presence of key functional groups, including hydroxyl (O–H), carbonyl (C=O), aliphatic (C–H) and methyl (C–CH₃) groups, which define its chemical structure. According to standard astaxanthin, hydroxyl stretching vibrations in the range of $3275\text{--}3450 \text{ cm}^{-1}$, aliphatic C–H stretching within $2852\text{--}2923 \text{ cm}^{-1}$ and carbonyl (C=O) groups around $1650\text{--}1750 \text{ cm}^{-1}$ [43].

In the current investigation, the crude samples displayed hydroxyl group peaks within $3270\text{--}3430 \text{ cm}^{-1}$, while the extracted samples showed peaks in the range of $3414\text{--}3449 \text{ cm}^{-1}$, which closely align with the reported standard values. These findings reported a hydroxyl peak at 3427 cm^{-1} , demonstrating strong agreement with the current results are further supported by [44]. Similarly, the aliphatic regions in the crude samples were observed within $2920\text{--}2960 \text{ cm}^{-1}$, whereas the extracted samples exhibited peaks in the range of $2853\text{--}2925 \text{ cm}^{-1}$, again consistent with previously reported literature values. Existing literature shows an aliphatic peak at 2930.96 cm^{-1} , [45] which closely corresponds to the present study, particularly in dry shell (DS) samples. The carbonyl group, another characteristic feature of astaxanthin, was observed in the extracted samples within the range of $1622\text{--}1641 \text{ cm}^{-1}$, which falls close to the standard range of $1650\text{--}1750 \text{ cm}^{-1}$ reported in the literature. Supporting this, a carbonyl peak at 1651.53 cm^{-1} which is in close agreement with the present findings. In addition, methyl (C–CH₃) stretching vibrations in the current study were observed within $1363\text{--}1454 \text{ cm}^{-1}$ in crude samples and $1459\text{--}1462$

cm⁻¹ in extracted samples. These values show strong similarity with the study showed methyl group peaks within the range of 1459–1462 cm⁻¹ [46] and a comparable peak at 1452.51 cm⁻¹ [45].

Thin-layer chromatographic (TLC) analysis in the current study revealed three well-defined bands corresponding to free astaxanthin, astaxanthin monoesters and astaxanthin diesters in both shell and tissue extracts of *Spiralothelphusa hydrodroma*. The observed R_f values ranged from 0.22–0.37 for free astaxanthin, 0.55–0.66 for monoesters and 0.77–0.84 for diesters across all sample types (DS, WS, DT and WT). These results demonstrate a clear separation pattern and are in strong agreement with previously reported findings. The study reported the R_f values of 0.33 for free astaxanthin, 0.60 for monoesters and 0.78 for diesters in carotenoids extracted from *Aristeus alcocki* [47]. Similarly, the reported R_f values of approximately 0.25 (free astaxanthin), 0.50 (monoester) and 0.75 (diester) from *Hematococcus pluvialis* [48]. The resulted R_f values 0.32 (free astaxanthin), 0.73 (diester) from *Carcinus maenas* is closely matched [49] values confirms the reliability of the separation and identification of astaxanthin and its esterified forms. Although slight variations were observed in the R_f values of free astaxanthin (0.22–0.37), these differences can be attributed to variations in solvent systems, sample composition and extraction conditions. The progressive increase in R_f values from free astaxanthin to monoesters and diesters reflects a decrease in polarity with increasing esterification, resulting in greater mobility of esterified forms on the TLC plate [46]. Furthermore, the comparatively higher R_f values and greater band intensity observed for diesters in the present study indicate their predominance, which aligns with the reported ratio of 1:1:2 (free: monoester: diester) [47].

In addition, astaxanthin is recognized as a potent carotenoid antioxidant capable of efficiently neutralizing reactive oxygen species and inhibiting lipid peroxidation induced by ultraviolet radiation [50]. Its exceptional antioxidant performance is primarily attributed to its unique chemical structure, characterized by an extended system of conjugated double bonds (polyene chain), which plays a crucial role in quenching singlet oxygen and scavenging free radicals [51]. The antioxidant activity of these astaxanthin samples was evaluated using DPPH free radical scavenging [40], ABTS radical scavenging [52] and reducing power assays [31]. A clear concentration-dependent enhancement in DPPH radical scavenging activity was observed across all tested samples, indicating a progressive increase in antioxidant potential with rising extract concentration [53]. At 20 µg, the dry shell (DS) fraction exhibited the highest scavenging activity 48.9%, followed by wet shell (WS) 23.9%, dry tissue (DT) 15.2% and wet tissue (WT) 8.7%. This trend remained consistent at 50 µg, where DS again showed superior activity 52.2%, with subsequent values recorded for (WS) 29.3%, (DT) 21.5% and (WT) 12.4%. The persistence of this pattern across concentrations highlights the comparatively stronger antioxidant capacity of shell-derived extracts, particularly the dry shell fraction. Similarly, DPPH scavenging efficiencies have been documented in astaxanthin extracted from crab species, including 79% in *Callinectes sapidus* and 67% in both *Portunus sanguinolentus* and *Paralithodes brevipes* [31]. In addition, research on *Procambarus clarkii* reported a DPPH activity of 72.1% with an EC₅₀ value of 131 µg/mL [40]. Further supporting this observation, EC₅₀ values of 50.93 µg/mL and 39.73 µg/mL were reported for shell extracts of *Callinectes sapidus* and *Portunus pelagicus*, respectively [14].

The antioxidant potential of astaxanthin was further assessed using the ABTS assay, which revealed a distinct concentration-dependent increase in radical scavenging activity across all samples [54]. At 20 µg, the highest activity was recorded in the dry shell (DS) fraction 69.2%, followed by wet shell (WS) 52%, dry tissue (DT)

26.3% and wet tissue (WT) 10%. This trend persisted at 50 µg, where DS exhibited the maximum activity 93.3%, followed by (WS) 81%, (DT) 49% and (WT) 41.6%. Additionally, an ABTS scavenging activity of 84% was reported from shrimp waste, which is comparable to the activity observed in the DS sample of the current reports [55]. Furthermore, a higher ABTS activity of 94.02% has been documented in mantis shrimp [56]. The consistency of this pattern across concentrations clearly indicates a comparatively stronger antioxidant capacity in shell-derived samples, particularly in the dry shell fraction. In comparison with prior investigations, antioxidant activity has frequently been expressed in terms of EC₅₀ values. For instance, ABTS scavenging activity in the shells of *Callinectes sapidus* and *Portunus pelagicus* showed EC₅₀ values of 17.56 µg/mL and 28.46 µg/mL, respectively [14]. In another investigation involving the shells of *Palaemon serratus* and *Palaemon varians*, ABTS [57] activity was expressed as EC₅₀ values ranging from 0.3–0.6 mg/mL.

The reducing power of astaxanthin was assessed based on its ability to reduce ferric ions, as evidenced by an increase in absorbance [58]. The results demonstrated a clear concentration-dependent enhancement in reducing activity across all samples [59]. At 20 µg, the highest absorbance was recorded in the DS fraction (0.364), followed by WS (0.175), WT (0.121) and DT (0.118). Similar pattern was observed at 50 µg, where DS exhibited the maximum absorbance (0.438), followed by WS (0.211), DT (0.158) and WT (0.134), indicating a comparatively stronger reducing potential in shell-derived samples. When compared with existing literature, reducing power activity in *Charybdis lucifer* was reported to reach a maximum of 59% in soft shells [60]. Similarly, a comparative investigation demonstrated that *Portunus sanguinolentus* exhibited greater reducing activity than *Callinectes sapidus* and *Paralithodes brevipes* under identical experimental conditions [31]. In another study, astaxanthin extract showed an absorbance value of 0.29 ± 0.01 at 0.25 mg/mL, where increased absorbance corresponds to enhanced antioxidant activity [59]. Notably, the DS sample in the present study at 50 µg demonstrated a higher absorbance value (0.438), suggesting a comparatively greater reducing potential. In addition to the anti-oxidant assays, the present study also involves the antibacterial activity of the different extracts against *Escherichia coli* showed marked variation in efficacy. Among them, DS exhibited the highest zone of inhibition (15 mm), followed by WS (13 mm), WT (12 mm), and DT (11 mm) (Figure 7), indicating that all samples possessed antibacterial activity, with DS being the most effective. These results are in agreement with the previously studies reported a comparable inhibition zones (10.05–12.11 mm) for crab-derived astaxanthin against *E. coli*, with variations likely influenced by differences in extraction efficiency, pigment concentration and associated bioactive constituents [31]. Similarly, acetone-extracted astaxanthin from *Haematococcus pluvialis* showed antibacterial activity against several pathogens, including *E. coli*, *Salmonella typhi*, *Vibrio cholerae* and *Staphylococcus aureus* [61]. Another study further demonstrated the broad-spectrum antibacterial potential of acetone-extracted astaxanthin, reporting inhibition zones of 10 mm against *E. coli*, 17 mm against *Bacillus* sp., 14 mm against *Staphylococcus* sp., and 18 mm against *Pseudomonas* sp., [62]. Compared with previous reports, the higher inhibition zone observed in the DS extract indicates enhanced antibacterial efficacy, highlighting shell-derived astaxanthin as a promising natural antibacterial agent for pharmaceutical, nutraceutical and food preservation applications.

Conclusion

The present investigation demonstrated that shell biomass of *Spiralothelphusa hydrodroma* is a rich source of astaxanthin with significant antioxidant and antibacterial properties. Among the tested samples, the dry shell fraction exhibited the highest astaxanthin yield and superior radical scavenging activity, indicating its enhanced bioactive potential. UV–Vis, FTIR and TLC analyses further confirmed the successful extraction and characterization of astaxanthin from crab-derived biomass. The high mineral content and bioactive profile of shell waste suggest its potential application as a value-added raw material in nutraceutical, pharmaceutical and functional food industries. Overall, the study highlights the feasibility of utilizing freshwater crab processing waste for sustainable biomass valorization and supports its role in promoting circular bio economy and waste to resource approaches.

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Author Contributions

Rangasamy Shanthi: Guidance, design and laboratory providing. Sundararajan Sowmiya: Execution, writing-original draft of the manuscript and analysis of all the experimental works. Prakash Monica: writing-original draft of the manuscript, Reviewing and experimental analysis. Mullaivanam Ramasamy Sivakumar: Visualization, Editing and software. All authors read and approved the final manuscript.

Ethics approval

Not applicable.

Data availability

Data will be made available upon request.

Conflict of interest

The authors declare that they have no known conflict of financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure and Table legends

Figure 1: UV–Visible absorption spectrum of astaxanthin extracted from freshwater crab *S. hydrodroma* shells and tissues showing a maximum absorbance peak at 476 nm.

Figure 2a: FTIR spectra of crude samples A (DS), B (WS), C (DT), D (WT).

Figure 2b: FTIR spectra of Extracted astaxanthin A (DS), B (WS), C (DT), D (WT).

Figure 3: Thin layer chromatography image of extracted astaxanthin from freshwater crab *S. hydrodroma* shells and tissues on wet and dry weight basis.

Figure 4: DPPH radical scavenging activity of astaxanthin extracted from freshwater crab *S. hydrodroma* shells and tissues at different concentrations, expressed on wet and dry weight basis.

Figure 5: ABTS scavenging activity of astaxanthin extracted from freshwater crab *S. hydrodroma* shells and tissues at different concentrations, expressed on wet and dry weight basis.

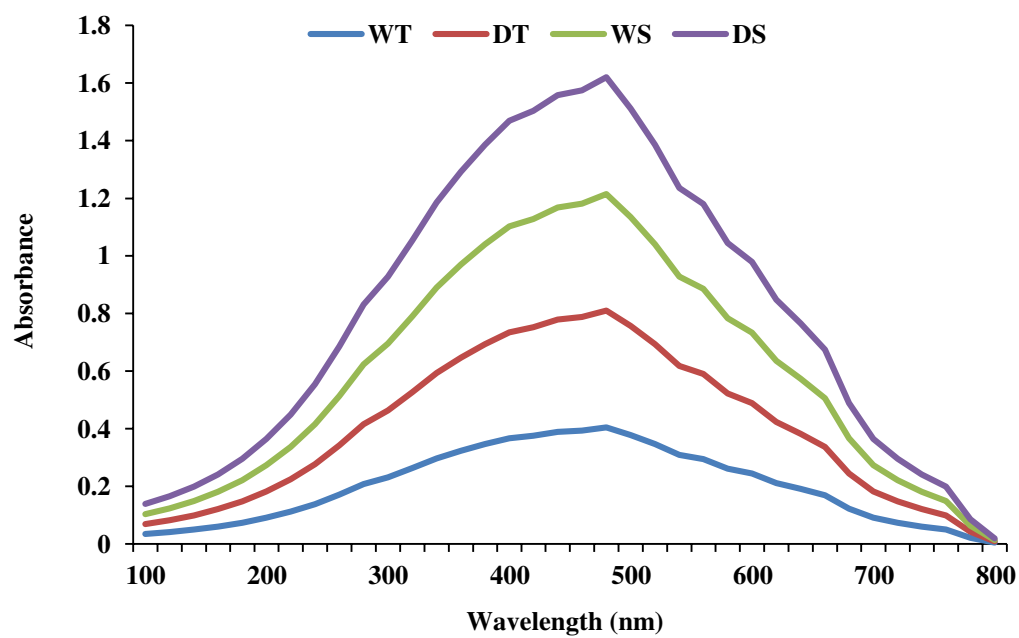
Figure 6: Reducing power activity of astaxanthin extracted from freshwater crab *S. hydrodroma* shells and tissues at different concentrations, expressed on wet and dry weight basis.

Figure 7: Antibacterial activity of astaxanthin extracted from freshwater crab *S. hydrodroma* shells and tissues at 20µg/ml, expressed on wet and dry weight basis.

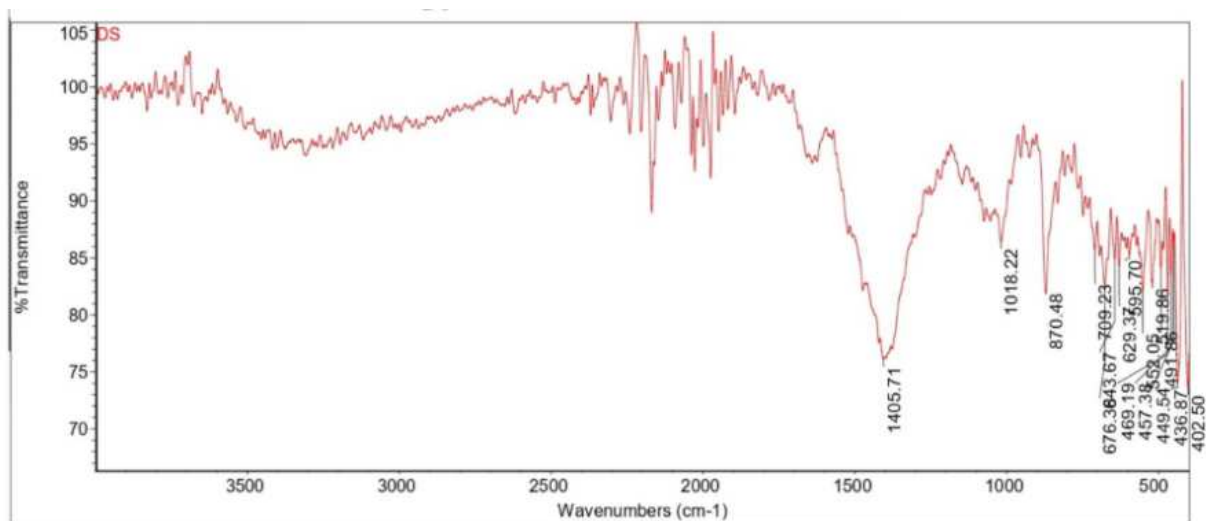
Table 1: Proximate composition of freshwater crab *Spiralothelphusa hydrodroma* shells and tissues on wet and dry weight basis (%).

Table 2: Extracted Astaxanthin yield of freshwater crab *S. hydrodroma* shells and tissues on wet and dry weight basis (µg/g).

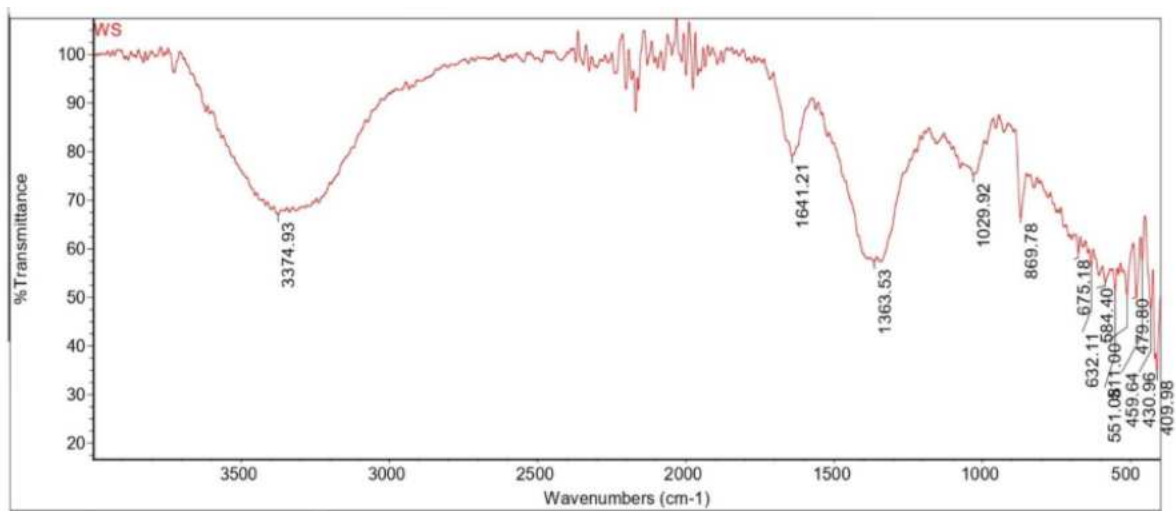
Table 3: Rf values of extracted astaxanthin from freshwater crab *S. hydrodroma* shells and tissues on both wet and dry weight basis.



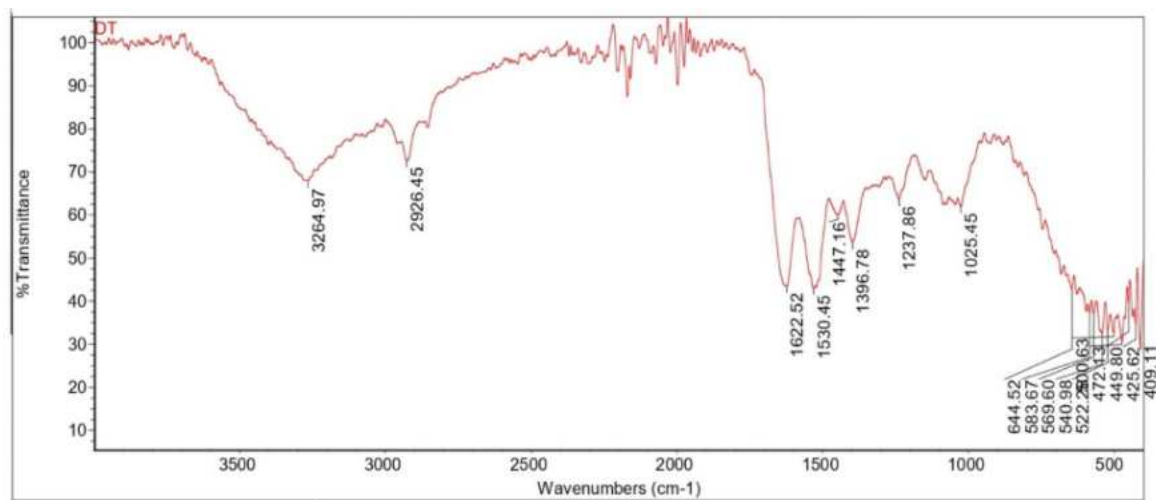
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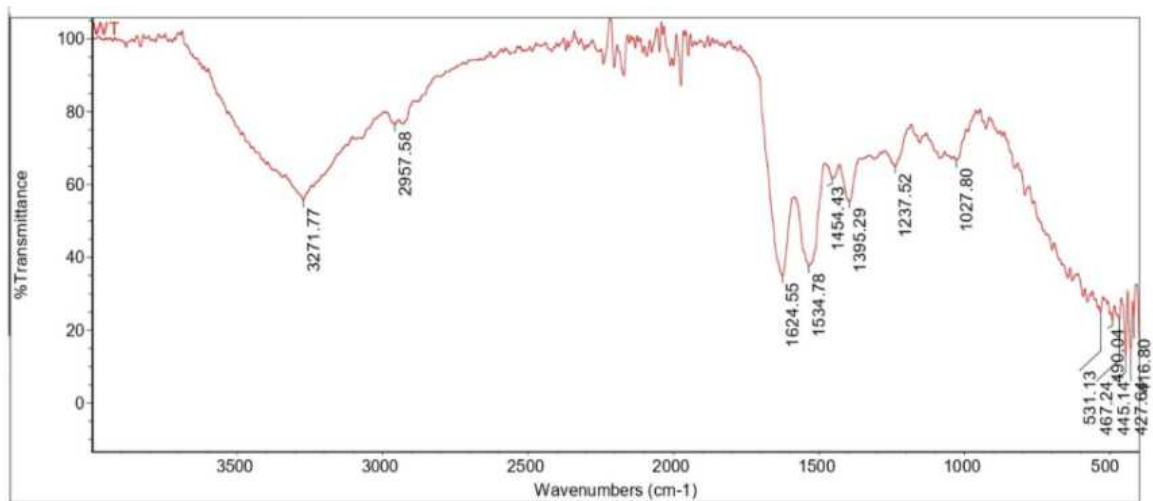
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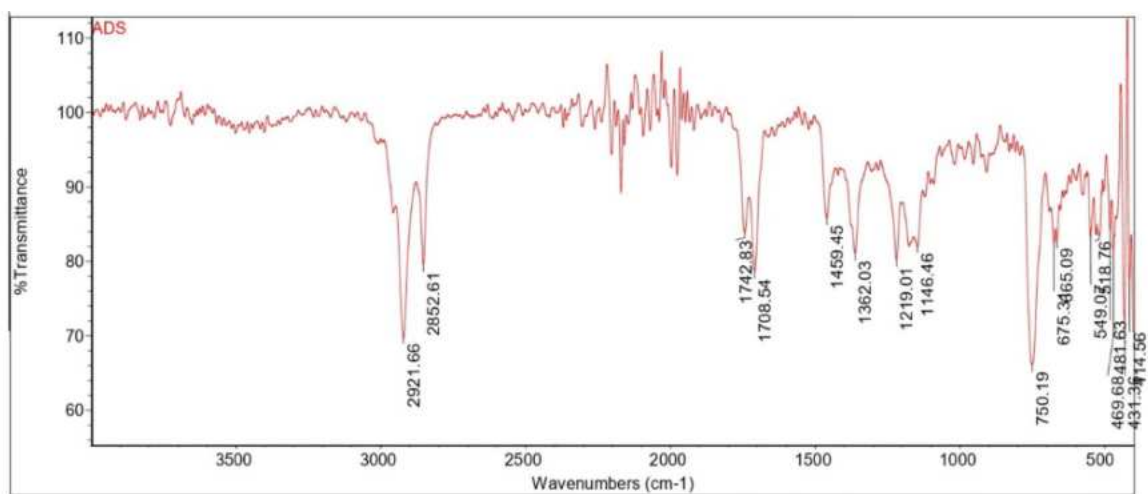
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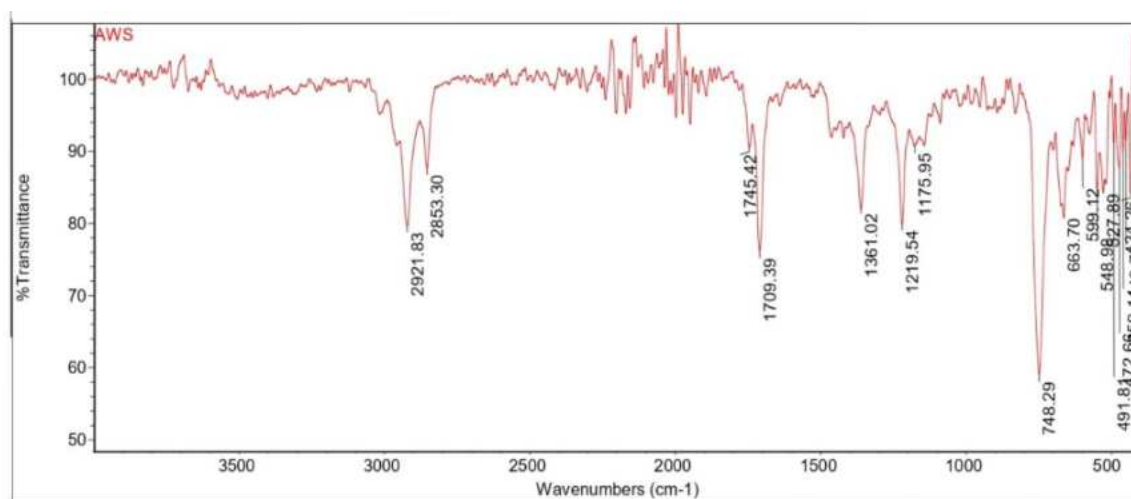
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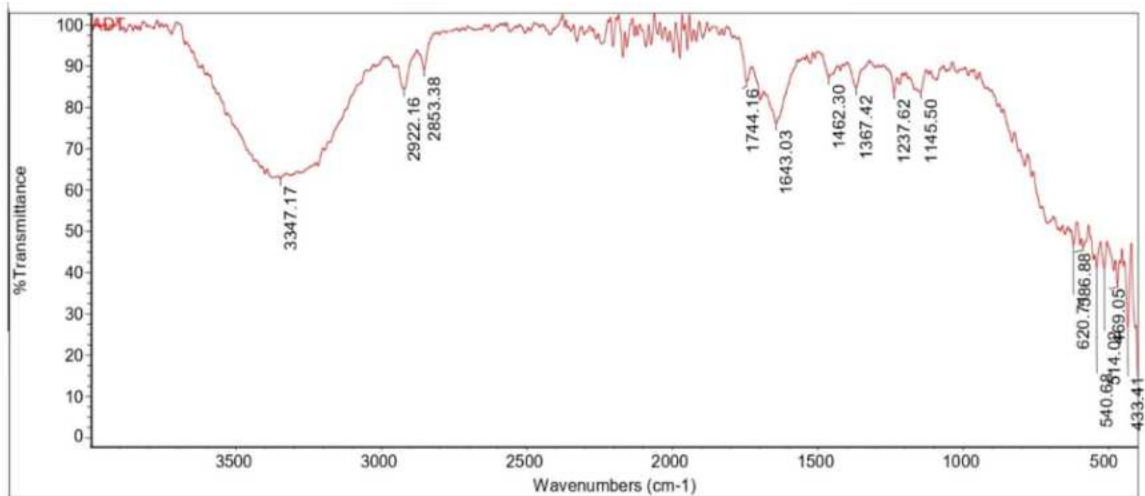
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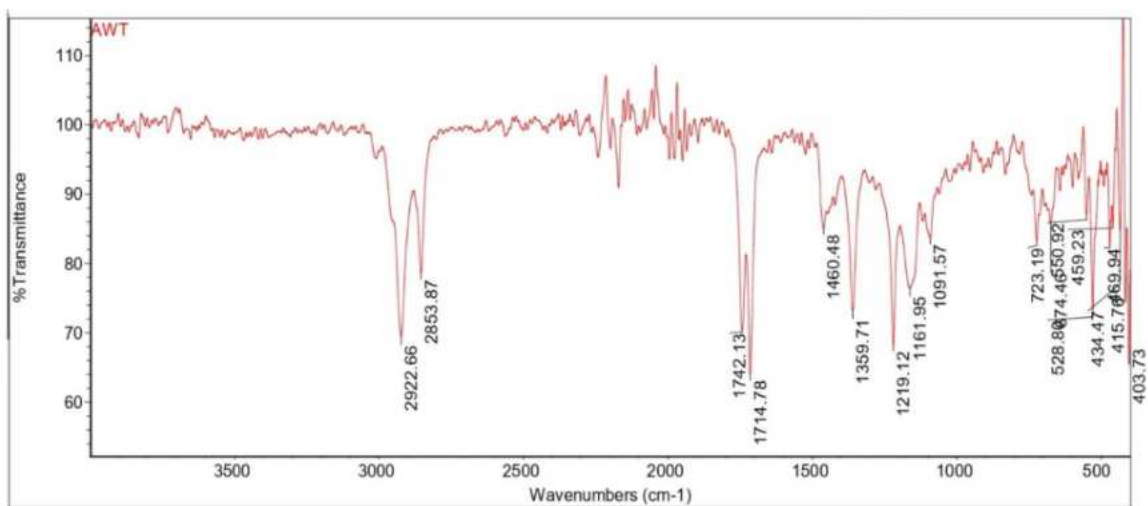
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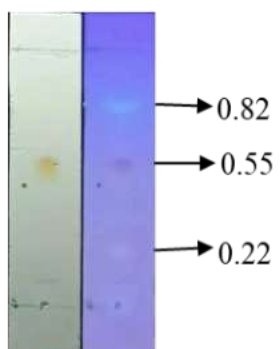
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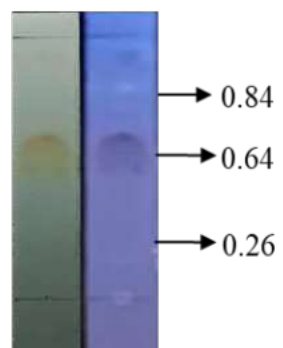
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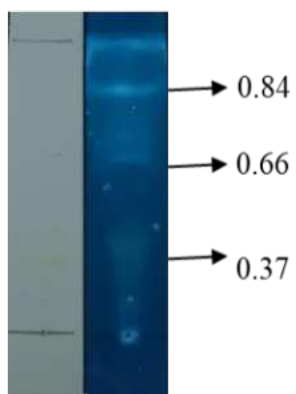
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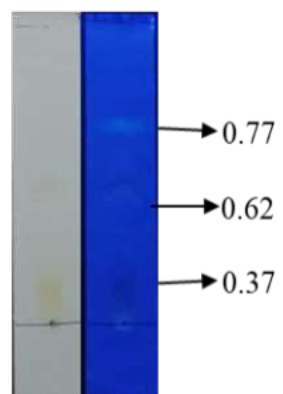
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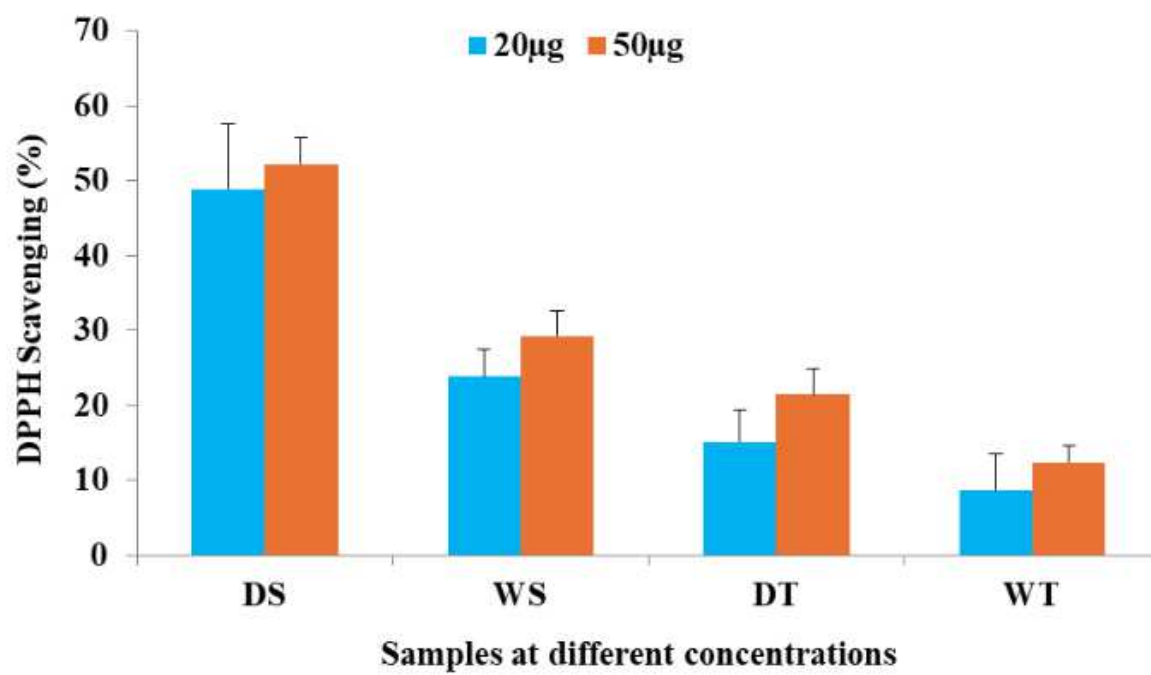


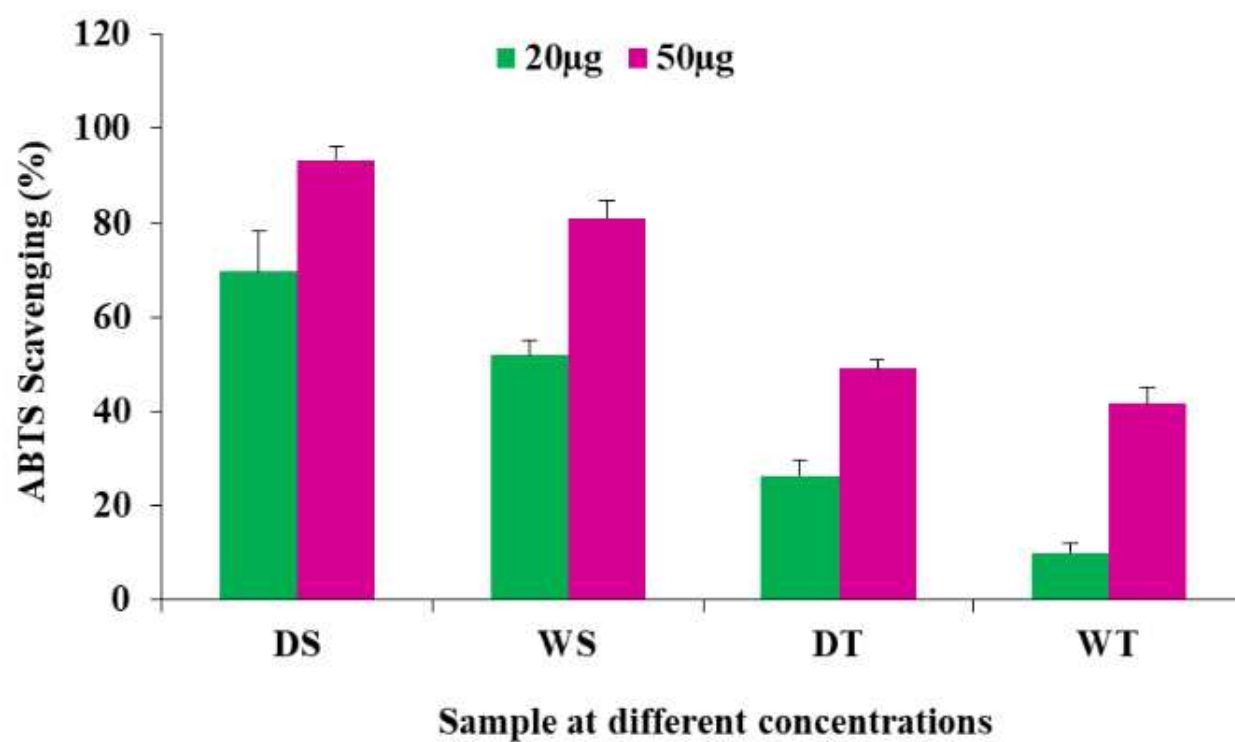
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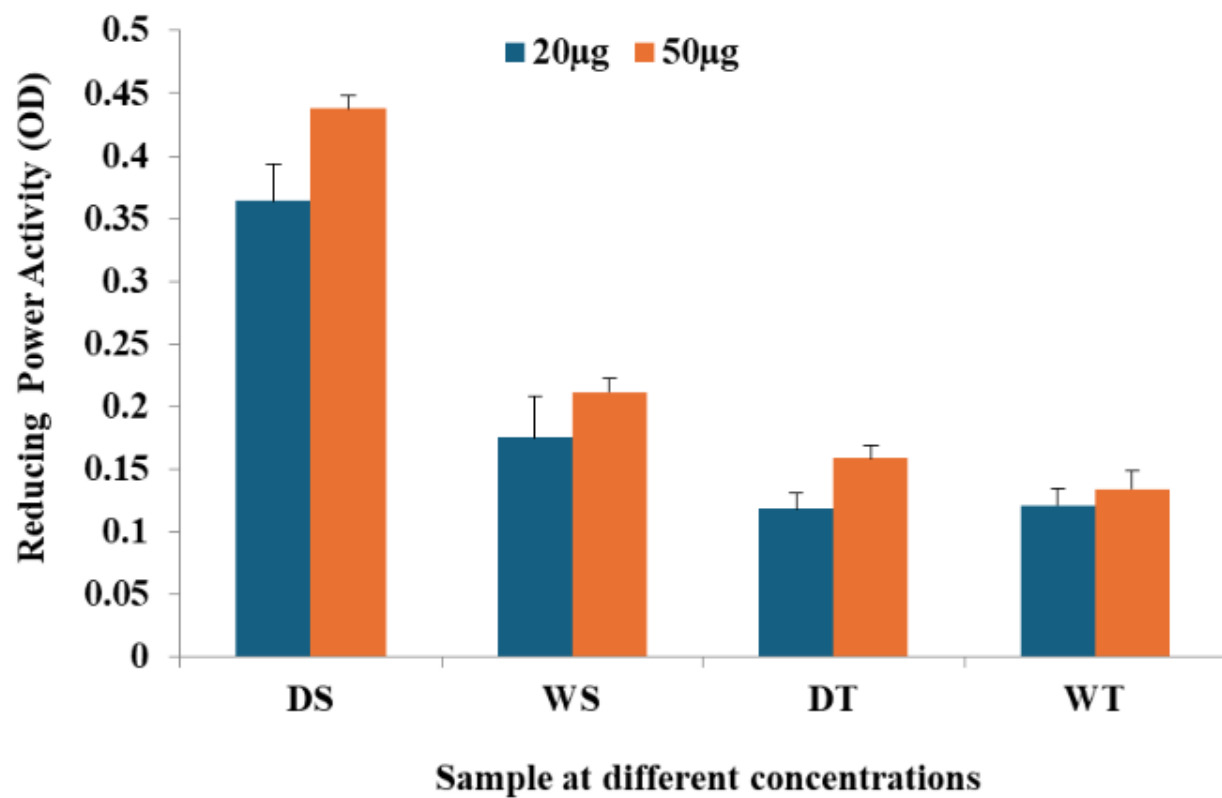


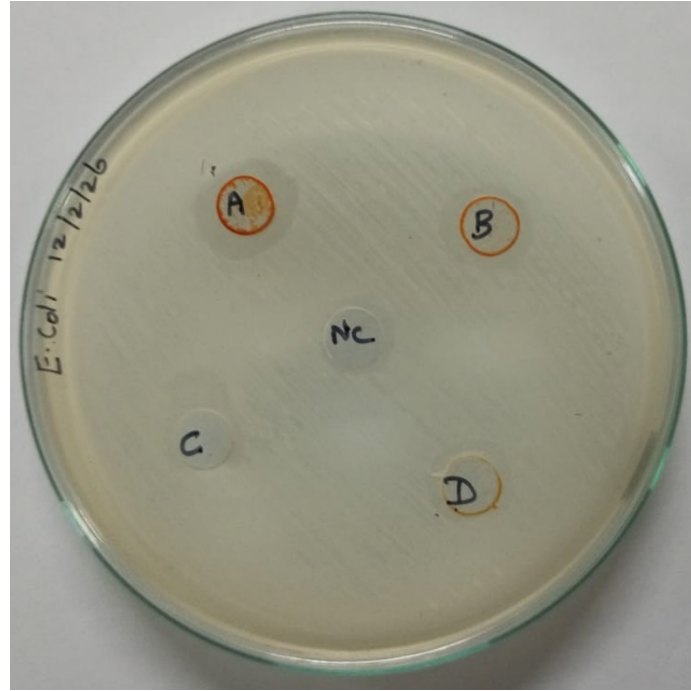
WT











Sample components	Moisture content	Ash content	Solubility content
Dry shell (DS)	-	70.87±0.96	0.877±0.02
Dry tissue (DT)	-	2.02±0.43	0.631±0.006
Wet shell (WS)	20.7 ± 0.83	51.87±1.29	0.928± 0.005
Wet tissue (WT)	80.2±0.38	0.73±0.75	1.941±0.004

Samples	Astaxanthin yield
DS	218.01±0.18
DT	28.03±0.32
WS	193.94±0.26
WT	15.20±0.60

Carotenoids	DS	WS	DT	WT
Astaxanthin Monoester	0.82	0.84	0.84	0.77
Astaxanthin Diester	0.55	0.64	0.66	0.62
Free Astaxanthin	0.22	0.26	0.37	0.37

Supplementary Files

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