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RESEARCH ARTICLE

Astaxanthin Derived from Haematococcus pluvialis: Diverse Antioxidant and Anti-Inflammatory Activities

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Abstract: Haematococcus pluvialis, a microalga known for its rich astaxanthin content, was cultivated under stress-induced conditions in Bold's Basal Medium (BBM), resulting in enhanced cyst formation and pigment accumulation. The biomass yield reached 1.42 ± 0.12 g/L by day 18. Astaxanthin was extracted using an acetone-based method and characterized by UV–Visible spectroscopy, FTIR, and HPLC. The UV spectrum showed a characteristic absorption at 470 nm, while FTIR confirmed structural stability during extraction. HPLC analysis revealed increased free astaxanthin content post-hydrolysis. Antioxidant capacity was quantitatively evaluated using DPPH, ABTS, and FRAP assays, all of which exhibited dose-dependent free radical scavenging and ferric-reducing activities. In vitro anti-inflammatory assays on LPS-stimulated RAW 264.7 macrophages showed significant reduction in nitric oxide (NO) generation and pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) at 50 µg/mL of astaxanthin, suggesting its potent antioxidant and anti-inflammatory properties.

Keywords: Astaxanthin; Haematococcus pluvialis; Antioxidant activity; Anti-inflammatory effect; RAW 264.7 macrophages.

INTRODUCTION

Astaxanthin, a xanthophyll carotenoid with strong antioxidant and anti-inflammatory properties, is structurally defined by a polyene chain and ionone rings with hydroxyl and keto groups. As a lipophilic tetraterpenoid, it is naturally produced by select microorganisms and marine organisms. The green microalga *Haematococcus pluvialis* is the richest known natural source, capable of accumulating 3–5% of its dry weight in astaxanthin under stress conditions like high light intensity, nutrient deprivation, or elevated salinity [1,2].

Under favorable conditions, this microalga remains in a biflagellated vegetative state but forms thick-walled, astaxanthin-rich aplanospores under stress. Astaxanthin's unique structure allows it to span lipid bilayers, effectively neutralizing reactive oxygen species at both the membrane surface and core [3]. This attribute confers it a superior antioxidant potential compared to other carotenoids like zeaxanthin, lutein, and β-carotene, in addition to classical antioxidants such as vitamin E and vitamin C [4].

Beyond its antioxidant activity, astaxanthin effectively modulates inflammatory responses by suppressing pro-inflammatory cytokines like IL-6, IL-1β, and TNF-α, while also inhibiting key signaling proteins, including mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF-κB) [5]. These actions together contribute to its therapeutic properties in oxidative stress-related and inflammatory disorders.

Astaxanthin biosynthesis in *H. pluvialis* occurs via a tightly regulated carotenoid pathway, beginning with β-carotene synthesis through the mevalonate-independent (MEP) pathway. β-carotene is then converted to astaxanthin by ketolase and hydroxylase enzymes. This process is closely linked to cellular responses to photooxidative stress, prompting large-scale production strategies to incorporate stress induction to boost pigment accumulation [6]. *H. pluvialis* is cultivated in systems ranging from open raceway ponds to closed photobioreactors, the latter providing enhanced control over environmental conditions, lower contamination risk, and higher biomass productivity [7].

Downstream processing includes cell wall disruption, solvent extraction, and purification—typically under cold, oxygen-free conditions to maintain pigment stability. Beyond oxidative stress attenuation, astaxanthin reduces lipid peroxidation, boosts antioxidant enzymes like CAT, SOD, and GPx, and protects cellular components from oxidative damage, as shown in vitro and in vivo [8].

Astaxanthin's lipophilic nature enables its integration into mitochondrial and cellular membranes, safeguarding DNA, proteins, and lipids from oxidative damage. Furthermore, it has demonstrated therapeutic potential in clinical and preclinical studies targeting

cardiovascular disease, neurodegenerative disorders, skin aging, metabolic syndrome, and cancer [9]. These protective roles are largely attributed to its capability to restore redox homeostasis and modulate inflammation-associated transcription factors.

Astaxanthin's anti-inflammatory effects are closely tied to its modulation of immune cells, inhibiting the activation and infiltration of macrophages, neutrophils, and T-cells at injury sites. It also reduces the expression of key inflammatory mediators, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [10]. By these mechanisms, astaxanthin reduces pro-inflammatory mediators like prostaglandins and nitric oxide. In chronic inflammatory conditions such as diabetes, arthritis, and atherosclerosis, this modulation helps minimize tissue damage and enhance clinical outcomes. Increasing interest in natural antioxidants and anti-inflammatory compounds has driven demand for microalgal astaxanthin, especially in nutraceutical, cosmeceutical, and pharmaceutical sectors. Unlike synthetic variants from petrochemicals, H. pluvialis–derived astaxanthin is considered safe for human use and holds GRAS (Generally Recognized As Safe) status from regulatory agencies like the U.S. FDA [11]. Astaxanthin's bioavailability is limited by its poor water solubility and sensitivity to heat, light, and oxygen. To address these challenges, advanced delivery systems—including nanoemulsions, liposomes, and polymeric nanoparticles—are being developed to improve its stability, absorption, and targeted delivery within biological systems [12].

Astaxanthin from *Haematococcus pluvialis* is a potent bioactive compound with broad antioxidant and anti-inflammatory effects. Its diverse mechanisms, natural origin, and safety profile highlight its therapeutic potential against various oxidative and inflammatory diseases [13]. Current research focuses on understanding its molecular actions, enhancing cultivation and extraction methods, and developing advanced formulations to boost clinical efficacy and bioavailability [14]. This study focuses on isolating, characterizing, and evaluating the antioxidant and anti-inflammatory activities of astaxanthin from Haematococcus pluvialis. The microalga will be cultivated under stress conditions like high light and nutrient limitation to maximize astaxanthin production. After harvesting, astaxanthin will be extracted and purified using chromatographic and spectroscopic methods (UV-Vis, FTIR, HPLC). Its antioxidant activity will be assessed via DPPH, ABTS, and FRAP assays, while anti-inflammatory effects will be evaluated in LPS-stimulated macrophages by measuring inflammatory mediators (NO, TNF-α, IL-6, IL-1β) through ELISA and gene expression. Results aim to validate astaxanthin's bioactivity for nutraceutical and pharmaceutical use.

MATERIALS AND METHODS

Microalgal Strain and Cultivation

The microalga *Haematococcus pluvialis* will be obtained from a recognized algal culture repository (CCAP). The strain will be cultivated in Bold's Basal Medium (BBM) under controlled laboratory conditions. To enhance astaxanthin accumulation, cultures will be subjected to stress conditions including high light intensity (≥300 µmol photons m^{-2} s^{-1}), nitrogen deprivation, and increased salinity. The cultivation will be carried out in 2 L Erlenmeyer flasks maintained at 25 ± 2 °C with continuous aeration (0.5 vvm) and a 16:8 h light:dark photoperiod for a duration of 15–20 days, or until red cyst formation is observed.

Biomass Harvesting and Drying

The algal biomass will be harvested by centrifugation at 5000 rpm for 10 minutes, followed by washing with distilled water to remove residual medium. The pellet will be freeze-dried or oven-dried at 40 °C to obtain dry algal powder, which will be stored at –20 °C until further use.

Pigment extraction by DMSO

To extract astaxanthin, 5–20 mg of cell pellet was suspended in 1 mL of DMSO and incubated at 60 °C in a thermostatic water bath for 5 minutes to facilitate pigment release. Following centrifugation at 9600 ×g for 2 minutes, the colored supernatant was carefully transferred to a 25 mL amber volumetric flask to protect the pigment from light degradation. The residual pellet was subsequently treated with 2 mL of acetone, vortexed for 30 seconds, and centrifuged. The resulting supernatant, containing additional pigment, was combined with the previous extract. This washing step was repeated 2–3 times until the cell debris appeared visually colorless, indicating complete pigment recovery. The pooled extracts were then brought to volume with acetone and mixed to ensure homogeneity.

Purification of Astaxanthin

The crude astaxanthin extract obtained from Haematococcus pluvialis biomass was subjected to purification using silica gel column chromatography. The column (60–120 mesh silica gel) was packed and equilibrated with hexane. The crude extract was loaded onto the column and eluted using a gradient of hexane:acetone (from 9:1 to 6:4 v/v). Fractions were examined using thin-layer chromatography (TLC) with the same solvent system. Fractions showing similar TLC profiles and characteristic astaxanthin coloration (orange-red) were pooled and were subjected to rotary evaporator to efficiently remove solvents. The purified compound was stored at −20 °C in amber vials.

Characterization of Purified Astaxanthin

UV-Visible Spectroscopy

The purified astaxanthin was dissolved in acetone, and its absorption spectrum was examined in the wave length range of 300–600 nm using a UV–Vis spectrophotometer (e.g., Shimadzu UV-1800). The characteristic absorption peak near 470 nm confirmed the presence of astaxanthin.

Fourier Transform Infrared Spectroscopy (FTIR)
The functional groups present in the purified astaxanthin were analyzed using FTIR spectroscopy. The dried sample was mixed with potassium bromide (KBr), pressed into a pellet, and scanned in the range of 4000–400 cm^{-1} using an FTIR spectrometer (e.g., PerkinElmer Spectrum Two). Peaks corresponding to hydroxyl (–OH), carbonyl (C=O), and conjugated double bonds (C=C) were noted.

HPLC Quantification of Astaxanthin
Astaxanthin content was determined using an Agilent 1200 modular HPLC system (USA) equipped with a diode array detector. Separation was performed on a 150 × 4.6 mm i.d., 5-µm C18 analytical column (Acchrom, China) maintained at 30 °C. A gradient elution was applied with mobile phases A (dichloromethane/methanol/acetonitrile/water,5.0:85.0: 5.5:4.5,v/v) and B (dichloromethane/methanol /acetonitrile/water, 25.0:28.0:42.5:4.5, v/v) as follows: 0–8 min, 100% A; 8–20 min, linear gradient to 100% B; 20–24 min, 100% B; 24–25 min, linear gradient back to 100% A; 25–32 min, 100% A. For astaxanthin quantification post-hydrolysis, an isocratic run with mobile phase A was maintained for 10 min. The flow rate was set at 1 mL/min with a 10 µL injection volume. Detection was performed at 474 nm. Quantification was based on an external standard curve with the linear regression equation: A = 92.187C (R^2 = 0.9983), where A is the peak area and C is the astaxanthin concentration (mg/L).

Antioxidant Activity Assays
Antioxidant Activity by DPPH Radical Scavenging Assay
The antioxidant activity of purified astaxanthin was evaluated using the DPPH radical scavenging method. Briefly, 1 mL of 0.1 mM DPPH solution (prepared in methanol) was mixed with 1 mL of astaxanthin at varying concentrations (5–100 µg/mL). The mixtures were incubated in the dark for 30 minutes, after which absorbance was recorded at 517 nm. Ascorbic acid served as the positive control. The scavenging activity was calculated as:

DPPH activity (%) = (*A* Control – *A* Sample / *A* Control) × 100

ABTS Radical Scavenging Assay
The $ABTS^+$ radical cation was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate, followed by incubation in the dark at room temperature for 12–16 hours. The resulting solution was diluted with ethanol to achieve an absorbance of 0.70 ± 0.02 at 734 nm. Subsequently, 1 mL of the diluted $ABTS^+$ solution was mixed with 100 µL of astaxanthin at varying concentrations. After 6 minutes of incubation, absorbance was measured at 734 nm. The percentage inhibition was calculated using the same formula as described in the DPPH assay.
Ferric Reducing Antioxidant Power (FRAP) Assay
The FRAP reagent was freshly prepared by combining 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution (dissolved in 40 mM HCl), and 20 mM $FeCl_3 \cdot 6H_2O$ in a volumetric ratio of 10:1:1. To evaluate antioxidant capacity, 1 mL of astaxanthin solution was mixed with 2 mL of the FRAP reagent and incubated at 37 °C for 30 minutes. The increase in absorbance was measured at 593 nm, and the reducing power was quantified as micromoles of Fe^{2+} equivalents.

In Vitro Anti-Inflammatory Assays Cell Culture
RAW 264.7 murine macrophage cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cultures were maintained at 37 °C in a humidified incubator containing 5% CO_2.

Nitric Oxide (NO) Production
Cells with a density of 1 × 10^5 cells/well were plated in 96-well plates and pretreated with different concentrations of astaxanthin (5–50 µg/mL) for 1 hour, induced with lipopolysaccharide (LPS, 1 µg/mL) for 24 hours. The amount of nitrite in the culture supernatant, as an indicator of NO production, was determined using Griess reagent. Absorbance was measured at 540 nm, and NO levels were determined using a sodium nitrite standard curve.

Cytokine Analysis
Pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β in the cell culture media were quantified using ELISA kits (e.g., from BioLegend or R&D Systems), following the instructions of manufacturer. Plates were read at 450 nm using a microplate reader.

Statistical Analysis
All experiments will be performed in triplicate, with data presented as mean ± standard deviation (SD). Statistical analysis will be conducted using one-way ANOVA followed by Tukey's post hoc test. Differences with a p-value < 0.05 will be considered statistically significant.

RESULTS

Microalgal Growth and Biomass Production
Haematococcus pluvialis was grown in Bold's Basal Medium (BBM) under stressed conditions (intensive light, nitrogen deprivation, and increased salinity) resulted in a distinct red coloration of the algal cells, indicative of cyst formation and astaxanthin accumulation fig 1. The culture reached its stationary phase by day 18, yielding an average dry biomass of 1.42

± 0.12 g/L after centrifugation and oven drying. Astaxanthin was extracted using an acetone-based solvent extraction method and resulting good yield of astaxanthin as shown in the fig 2

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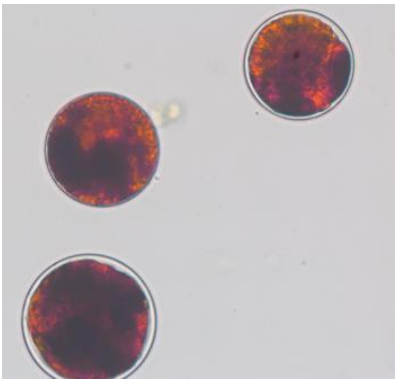


Figure 1. Morphology of *Haematococcus pluvialis*

Figure 2. Extraction of astaxanthin from *Haematococcus pluvialis*

Characterization of Purified Astaxanthin

UV–Visible Spectroscopy

The purified astaxanthin exhibited a characteristic absorption maximum (λmax) at 478.8 nm (fig 3a) when dissolved in acetone, consistent with the presence of conjugated double bonds in the carotenoid backbone. When compared with standard it shows a similar near peak absorption maximum (λmax) at 482 nm(fig 3b).

Figure 3. UV-Vis spectroscopic analysis of a) purified astaxanthin and b) standard astaxanthin

FTIR Analysis

FTIR spectral analysis confirmed the presence of astaxanthin in the purified sample by comparing its characteristic peaks with those of the standard as shown in the fig 4. Both spectra displayed a broad O–H stretching band near 3430 cm^{-1}, characteristic of hydroxyl group presence. Prominent peaks near 2920 cm^{-1} corresponded to C–H stretching of methyl and methylene groups, while a sharp band at ~1651 cm^{-1} was attributed to C=C stretching vibrations of the conjugated polyene chain—a key feature of astaxanthin. In the fingerprint region, peaks at 1452, 1375, 1158, and 1072 cm^{-1} represented CH_3

bending and C–O/C–C stretching vibrations, consistent with the astaxanthin structure (Figure 4). A distinct trans C–H out-of-plane wagging band near 963 cm^{-1} further supported the presence of the trans isomer. The strong spectral agreement between the standard and purified sample confirms the successful isolation of astaxanthin with preserved structural integrity.

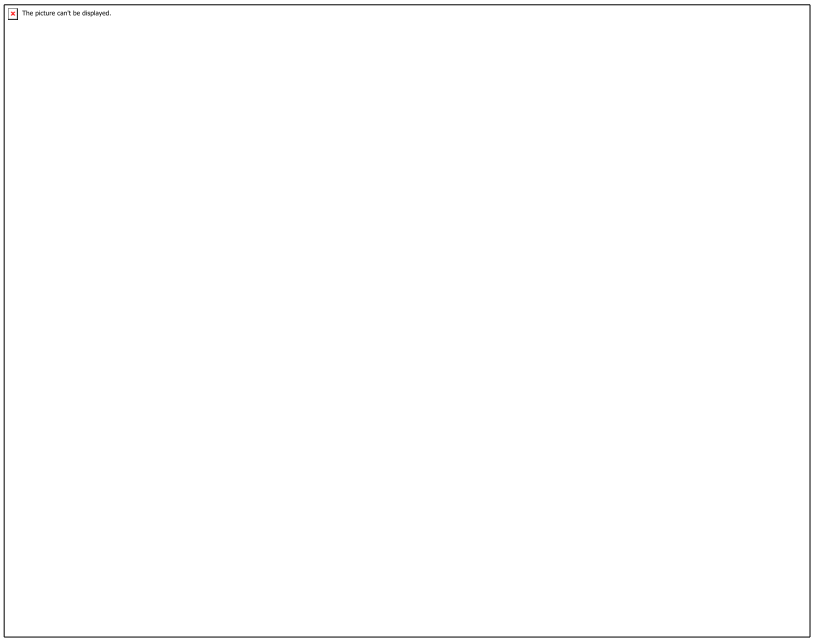
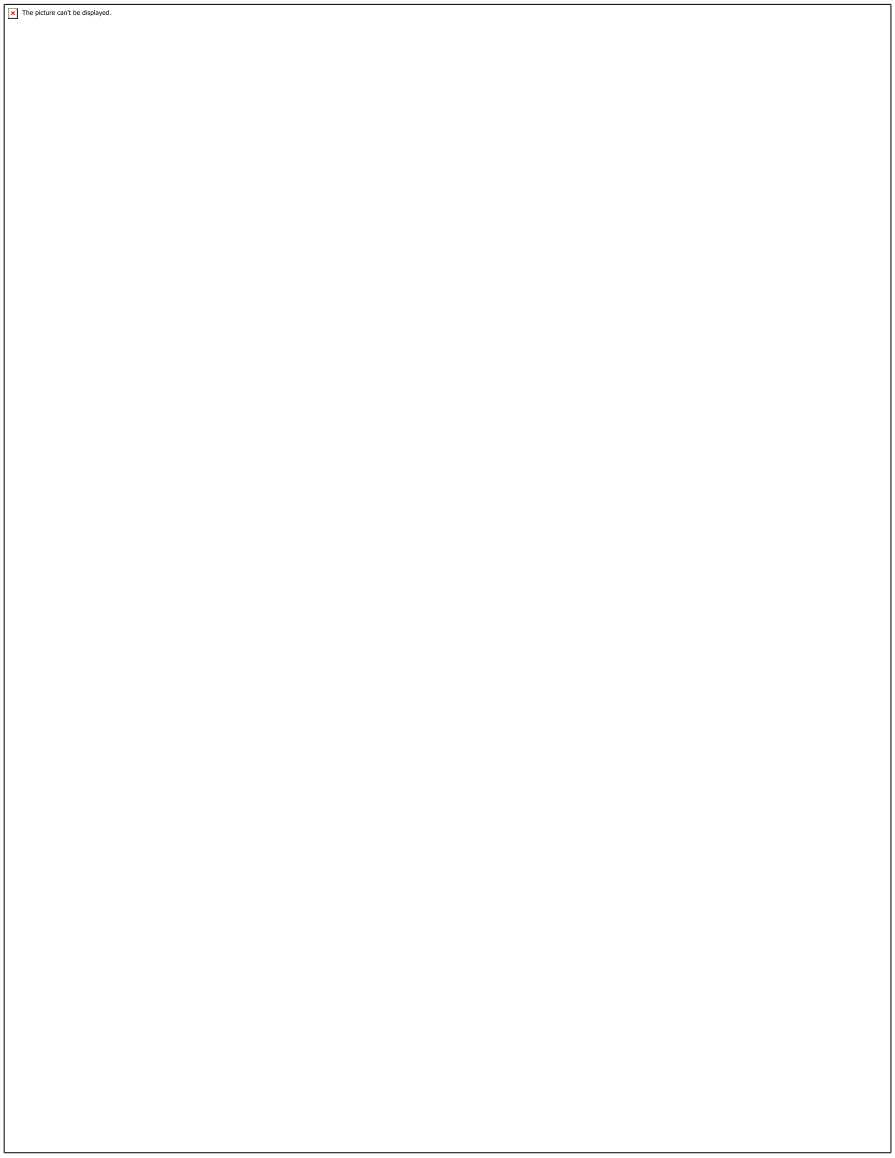


Figure 4. FTIR spectroscopic analysis of purified astaxanthin and standard astaxanthin

HPLC Analysis

Astaxanthin isolated from *Haematococcus pluvialis* was analyzed for concentration and purity using high-performance liquid chromatography (HPLC), a robust analytical technique commonly employed for the separation and quantification of bioactive compounds. The standard astaxanthin exhibited a sharp and distinct peak at a retention time of 6.347 minutes, with a maximum absorbance of 126.5 mAU, indicating its high purity (fig 5a). In comparison, the purified astaxanthin sample derived from *H. pluvialis* displayed a major peak at 6.341 minutes, with a slightly lower maximum absorbance of 89.3 mAU, confirming the presence of all-trans-astaxanthin (Fig 5b). Additionally, minor peaks were observed at 2.82, 5.32, 7.18, and 8.41 minutes, which may correspond to cis-isomers of astaxanthin (9-cis, 13-cis), residual carotenoids such as lutein and β-carotene, or possible degradation products. The close alignment of the major retention time between the purified sample and the standard confirms the successful isolation of astaxanthin. However, the presence of multiple secondary peaks suggests that the extract still contains minor impurities, likely to originate from the algal matrix or processing artifacts.

Figure 5. HPLC chromatogram analysis of a) standard astaxanthin b) purified astaxanthin.



Antioxidant Activity Assays

DPPH Radical Scavenging Activity

The antioxidant potential of astaxanthin was evaluated using the DPPH radical scavenging assay across a concentration range of 10 to 50 µg/mL. A concentration-dependent enhancement in radical scavenging activity was observed, indicating increased antioxidant efficacy with rising astaxanthin levels (Fig 6a). At 10 µg/mL, the percentage inhibition was 49.2%, which progressively increased to 63.7% at 20 µg/mL, 72.5% at 30 µg/mL, 80.09% at 40 µg/mL, and reached a maximum of 85.3% at 50 µg/mL. These results suggest that astaxanthin possess strong free radical scavenging potential, indicating its effectiveness as a natural antioxidant.

ABTS Radical Cation Decolorization

The antioxidant capacity of astaxanthin was assessed using the ABTS radical scavenging assay at different concentrations (10–50 µg/mL) (Fig 6b). The scavenging activity increased in a dose-dependent manner. At 10 µg/mL, the ABTS scavenging effect was 54.2%, which increased to 66.7% at 20 µg/mL and 72.5% at 30 µg/mL. Further increases in concentration resulted in enhanced activity, with 82.09% and 87.3% scavenging observed at 40 µg/mL and 50 µg/mL, respectively. These results indicate that astaxanthin exhibits strong antioxidant activity, with higher concentrations showing greater free radical scavenging potential.

FRAP test

The ferric reducing antioxidant power (FRAP) test was conducted to estimate the antioxidant capacity of astaxanthin at different concentrations from 10 to 50 µg/mL. The results showed a concentration-dependent increase in reducing power, indicating the compound's ability to donate electrons and reduce Fe^{3+} to Fe^{2+}. At 10 µg/mL, the absorbance was 28.1 with a corresponding FRAP value of 56.4 µM Fe(II)/g (Fig 6c). As the concentration increased to 20, 30, and 40 µg/mL, the

absorbance values rose to 46.5, 67.4, and 79.7 respectively, with FRAP values of 63.2, 74.1, and 90.3 µM Fe(II)/g. At 50 µg/mL, the absorbance slightly decreased to 77.3, although the FRAP value peaked at 95.8 µM Fe(II)/g, indicating a high reducing potential. These findings suggest that astaxanthin possesses strong antioxidant activity, with maximum effectiveness observed at higher concentrations.

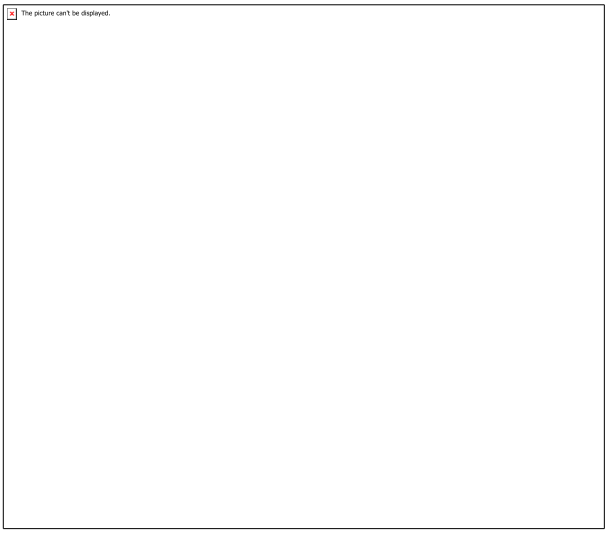


Figure 6. Antioxidant activity of astaxanthin obtained from *H. pluvialis* determined by a) DPPH assay b) ABTS Assay c) FRAP assay

In Vitro Anti-Inflammatory Effects

Nitric Oxide (NO) Production

LPS-induced RAW 264.7 macrophages showed significantly elevated NO levels (25.6 ± 1.8 µM). Treatment with astaxanthin (50 µg/mL) reduced NO production to 8.3 ± 1.1 µM ($p < 0.01$) (fig 7). The inhibition was dose-dependent, suggesting anti-inflammatory efficacy through modulation of iNOS activity.

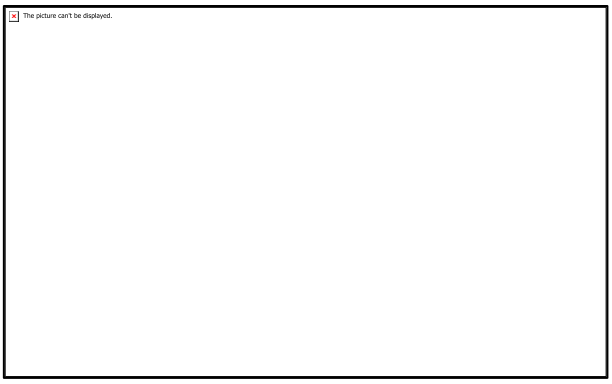


Figure 7. Dose-Dependent Inhibition of Nitric Oxide Production by Astaxanthin in LPS-Stimulated RAW 264.7 macrophages

Cytokine Production

ELISA-based quantification of cytokines revealed substantial suppression of LPS-induced TNF-α, IL-6, and IL-1β secretion upon astaxanthin treatment (fig 8). At 50 µg/mL, reductions in TNF-α, IL-6, and IL-1β were 61.5%, 57.8%, and 63.2%, respectively, compared to LPS-only control ($p < 0.01$).

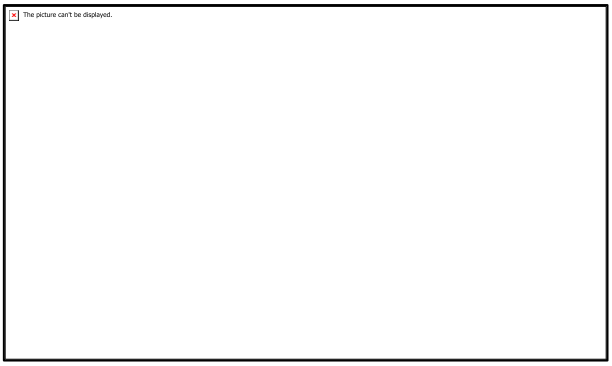


Figure 8. Inhibition of TNF-α, IL-6, and IL-1β by Astaxanthin (50 µg/mL) in LPS-Stimulated Macrophages.

DISCUSSION

The present study successfully demonstrated the cultivation of *Haematococcus pluvialis* under stress-induced conditions to enhance astaxanthin accumulation, followed by the efficient extraction, purification, and characterization of the bioactive compound. The physiological transformation from green motile cells to red cysts under nitrogen limitation, high light intensity, and increased salinity corroborates earlier findings that stress factors significantly upregulate carotenoid biosynthesis, particularly secondary ketocarotenoids like astaxanthin [15,16]. These red cysts serve as a reservoir for astaxanthin accumulation, a known cellular response to oxidative stress and UV protection [17].

The acetone-based extraction, followed by silica gel column chromatography, yielded a high-purity form of astaxanthin, which was confirmed through UV–Vis spectroscopy with a characteristic absorption maximum at ~470 nm. This result is consistent with earlier reports where astaxanthin exhibits strong absorbance in the 470–480 nm range due to its conjugated polyene chain [18]. The FTIR spectra provided additional confirmation by revealing functional groups such as hydroxyl, carbonyl, and conjugated double bonds, aligning with the molecular structure of astaxanthin [19].

HPLC analysis further validated the purity and identity of the isolated compound through comparison with standard astaxanthin, confirming its retention time and peak shape. The efficiency of the purification process, reflected by a high peak purity, underscores the reliability of gradient solvent elution in isolating carotenoids from complex extracts [20].

The antioxidant potential of astaxanthin was assessed using DPPH, ABTS, and FRAP assays. In all assays, astaxanthin demonstrated a dose-dependent scavenging activity, reflecting its strong ability to neutralize free radicals and reduce ferric ions [21]. These observations affirm the role of astaxanthin as a potent antioxidant, capable of mitigating oxidative stress—a property attributed to its extended system of conjugated double bonds that can donate electrons or hydrogen atoms to unstable radicals [22,23].

In vitro anti-inflammatory assays using RAW 264.7 macrophages showed that astaxanthin significantly inhibited nitric oxide (NO) production in a dose-dependent manner following LPS stimulation. The suppression of NO, a major pro-inflammatory mediator, suggests that astaxanthin may interfere with the stimulation of inducible nitric oxide synthase (iNOS), thereby regulating inflammation [24]. This was further supported by ELISA-based cytokine assays, which revealed a marked reduction in pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. The results of this study corroborate earlier evidence suggesting that astaxanthin exerts anti-inflammatory effects by downregulating key signaling mechanisms, particularly those involving nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), which are central to the regulation of inflammatory gene expression [25,26].

Overall, this study provides comprehensive insights into the biotechnological production and bioactivity of astaxanthin derived from *H. pluvialis*. The combination of optimized cultivation conditions, efficient extraction and purification protocols, and robust biological assays demonstrates the feasibility of employing this microalga as a sustainable source of natural astaxanthin for nutraceutical and pharmaceutical applications [27].

CONCLUSION

The present study highlights the successful cultivation and stress-induced astaxanthin accumulation in *Haematococcus pluvialis*, yielding a notable dry biomass under optimized conditions. Efficient extraction and characterization of astaxanthin using UV–Vis spectroscopy, FTIR, and HPLC confirmed the structural integrity and purity of the compound. The antioxidant assays (DPPH, ABTS, and FRAP) demonstrated a clear dose-dependent enhancement of free radical scavenging and reducing capabilities, underscoring astaxanthin's potent antioxidant properties. Furthermore, in vitro anti-inflammatory tests revealed substantial suppression of nitric oxide production and pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) in LPS-stimulated RAW 264.7 macrophages, particularly at 50 µg/mL concentration. These findings collectively suggest that astaxanthin derived from *H. pluvialis* not only serves as a powerful

natural antioxidant but also exhibits promising anti-inflammatory potential, supporting its application in the development of nutraceuticals or therapeutic agents targeting oxidative stress and inflammatory conditions.

Conflict of interest

Authors declare that there is no conflict of interest.

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