Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 26(2): 95 – 106 (2022)
www.ejabf.journals.ekb.eg

In vitro Antimicrobial Activity of Astaxanthin Crude Extract from *Haematococcus pluvialis*

Entesar A. Ahmed[1], Hager E. Mohamed[2] and Hemat S. Abd El-Salam[3]

[1]Botany and Microbiology Department, Faculty of Science, Al-Azhar University (Girls Branch), Cairo, Egypt.

[2] Al Shorouk Academy for Science and Technology, Egypt.

[3]Medical Analysis Specialist at EL-Zeny Laboratory, Egypt.

***Corresponding author: entesar18@yahoo.com**

ARTICLE INFO

Article History:
Received: Jan. 24, 2022
Accepted: Feb. 25, 2022
Online: March 11, 2022

Keywords:
Astaxanthin,
Antibacterial,
Antifungal,
Antioxidant,
Anticancer

ABSTRACT

Haematococcus pluvialis is green microalgae well known for its ability of astaxanthin accumulation. In the present investigation, *H. pluvialis* was culture for 12 days under salinity stress and astaxanthin was extracted from the encysted cells with acetone, methanol, DMSO, and hexane. HPLC analyses showed that astaxanthin was the major content of pigment, the crude extract was tested with the agar well diffusion method for their antibacterial against two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), two Gram-negative bacteria (*Escherichia coli* and *P. aeruginosa*) as well as for their antifungal activity against (*Aspergillus fumigates* and *Candida albicans*. The highest antibacterial activity was observed as 11 mm on *S.aureus* while as least antibacterial activity was found as 9 mm on *P. aeruginosa* and no antifungal activity. Astaxanthin crude extract was tested for its effectiveness as antioxidant activity. Also, the extract was screened for their anticancer activities against HepG-2 (hepatocellular carcinoma cells) . The results indicate that the HepG-2 was sensitive at (IC50) with 123.01 ± 2.97 µg/ml.

INTRODUCTION

Microalgae play important role of ecosystems have been used as source of nutrient rich food, feed and health promoting compounds. Astaxanthin has important applications in the nutraceuticals, cosmetics, food and aquaculture industries. Astaxanthin is a xanthophyll carotenoid which is found in various microorganisms and marine environment i.e. in microalgae, plankton, krill seafood and also present in yeast, fungi, complex plants and as well as in the feathers the feathers of some birds (flamingos and quail) **(Hussein *et al.*, 2006)**. *Haematococcus pluvialis* is one of the best sources of natural astaxanthin **(Ranga *et al.*, 2010)**. *H. pluvialis* is a green microalga, which accumulates high amounts of a complex mixture of secondary carotenoids and ketocarotenoids, especially astaxanthin content under unfavorable and stress conditions such as high salinity **(Tjahjono *et al.*, 1994)** nitrogen deficiency, high temperature **(Cordero *et al.*, 1996)** and light **Rather and Singh (2018)**.

H.pluvialis can accumulate up to5% Dry weight of astaxanthin and is considered as the best natural source of this high- value carotenoid pigment **(Wayama *et al*., 2013**).

Santoyo *et al*. (2009) found highest antimicrobial activities of astaxanthin ethanolic extract from microalgae *Haematococcus pluvialis*. **Shanmugapriya *et al*. (2018)** reported that Minimum inhibition concentration (MIC) for astaxanthin extract with values for Gram-positive and Gram-negative species were 500–4000 µg/mL .In particular, natural astaxanthin has significantly greater antioxidant capacity than the synthetic one. **Kobayashi *et al*. (1993)** reported that astaxanthin production by *Haematococcus pluvialis* is enhanced by oxidative stress, due to its role in oxygen free radicals removal.

Antioxidant activity astaxanthin is 65 times more powerful than vitamin C, 54 times stronger than β-carotene, 10 times more potent than β-carotene, canthaxantin, zeaxanthin, and lutein and 100 times more effective than α- tocopherol ((**Miki, 1991 and Cyanotech, 2015)** and consequently has important nutraceutical properties including enhancement of immune responses and protection against various diseases, among which are certain types of cancer(**Kobayashi *et al*., 1993).** Astaxanthin also had an anti-inflammatory effect reducing gastric inflammation and cytokine production by aplenocytes **(Bennedsen *et al*., 2000). Park *et al*. (2010)** reported that astaxanthin reduced the DNA oxidative damage biomarker inflammation, thus enhancing immune response in young healthy adult female human subjects. Also, astaxanthin is a promising molecule for the treatment of ocular inflammation in eyes as reported by the Japanese researchers **(Ohgami *et al*., 2003 and Suzuki *et al*., 2006)**

The aim of the present work was to study the effect of salt stress on production of astaxanthin from *H. pluvialis* study antimicrobial activity of crude astaxanthin extract.

MATERIALS AND METHODS

1. Algal Cultivation

H. pluvialis was obtained from Water Pollution Research Department, National Research Centre, Cairo, Egypt. Cultures were incubated at 25-26°C, in Erlenmeyer flasks containing 2 L Bold's Basal Medium (BBM) **(Stein, 1973)**, under 16:8 h light/dark photoperiods, lit by cool-white fluorescent lamps with continuous bubbling of air (500–700 cm^3/min).

2. Salt concentration:

H. pluvialis culture was grown at different concentration of NaCl (0.12 0.25, 0.5, 1.0 and 2.0 % w/v). After 12 days of culture, *H. pluvialis* cells were collected by centrifugation, freeze-dried and subsequently analyzed for the pigment content.

3. Chlorophyll content:

For pigment analyses, 10-mL samples were centrifuged at 6000 × g for 10 min, and the pellet extracted with 5 mL acetone. The extracts were centrifuged again and chlorophyll a, chlorophyll b and total carotenoids were determined spectrophotometrically (Shimadzu 160A), recording the absorption at 661.6, 644.8 and 470 nm and using the equations of **Lichtenthaler (1987**). Astaxanthin was determined at 480 nm using an absorption coefficient, A1% of 2500 by the method of **Davies, 1976**.

4. Extraction of Astaxanthin:

20 mg biomass of *H. pluvialis* was mixed in mortar and the paste extracted with acetone, methanol, DMSO and hexane,, centrifuged at 3000x g for 10 min at 4^{o} C, the pellet was discarded and the supernatant was taken for the estimation of astaxanthin **(Davies, 1976)**. Solvent was evaporated in the rotary evaporator and the crude astaxanthin extract was individually tested for antimicrobial, antioxidant and antitumor activity.

5. Pigment analysis by HPLC:

The general procedure for HPLC pigment analysis, identification and quantification has been described by (**Claustre *et al*., 1994 a,b**). With the separation system used (RP-C18), a partial resolution of divinely chlorophyll a (DV Chl a) from chlorophyll a (Chl a) has been achieved. The algal extract was analyzed for photosynthetic pigments in a HPLC system (Agilent-1100) analysis was determined at the Desert Research Center

6. Antimicrobial activity Assay

The antimicrobial activity was investigated on the tested compound to determine the activity towards test microorganisms. All microbial strains were provided from culture collection of the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt.

The antimicrobial profile was tested against Gram-positive bacterial species (*Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* RCMB 015 (1) NRRL B-543, Gram negative bacterial species (*Escherichia coli* (RCMB 010052) ATCC 25955and *P. aeruginosa* ATCC 27853), as well as against fungi including one filamentous fungus (*Aspergillus fumigatus*(RCMB 002008) and one yeast species (*Candida albicans* RCMB 005003 (1) ATCC 10231) using a modified well diffusion method. gentamycin was used as standard for antibacterial drugs and ketoconazole as standard antifungal drug. The extract was tested at different concentration of 10 mg/ml against both bacterial and fungal strains. The test was done using the diffusion agar technique, Well diameter: 6.0 mm (100 µl was tested),

7. Antioxidant Assay:

The antioxidant activity of extract was determined by DPPH free radical scavenging assay in triplicate and average values were considered by **Yen and Duh (1994)**.

8. Antitumor activity assay:

Antitumor activity assay of extract was determined by Human hepatocellular carcinoma (HepG2 Cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI- 1640 medium supplemented with 10% inactivated fetal calf serum and 50µg/ml gentamycin. The cells were maintained at 37°C in a humidified with 5% CO_2 and were subcultures two to three times a week according to **Mosmann(1983**). All previous analysis was determined at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University. in triplicate and average values were considered.

RESULTS AND DISCUSSION

Chlorophyll content (mg/l)

The effect of different concentration of salinity on chlorophyll a, carotenoid and astaxanthin content in *H.pluvialis* cultures grown for 12 days was shown in **Table 1**. The results revealed that the highest total astaxanthin content was detected 2.5mg/l at 1% of NaCl while carotenoid detected 2.2mg/l, on the other hand chlorophyll a content recorded 0.8mg/l at the same concentration. These results agree with **Sarada *et al*. (2002)** who illustrated that the chlorophyll a content was significantly lower in stress induced cultures compared to control cultures. However, relatively high concentrations of NaCl (0.5-0.2%, w/w) decrease of chlorophyll a content.

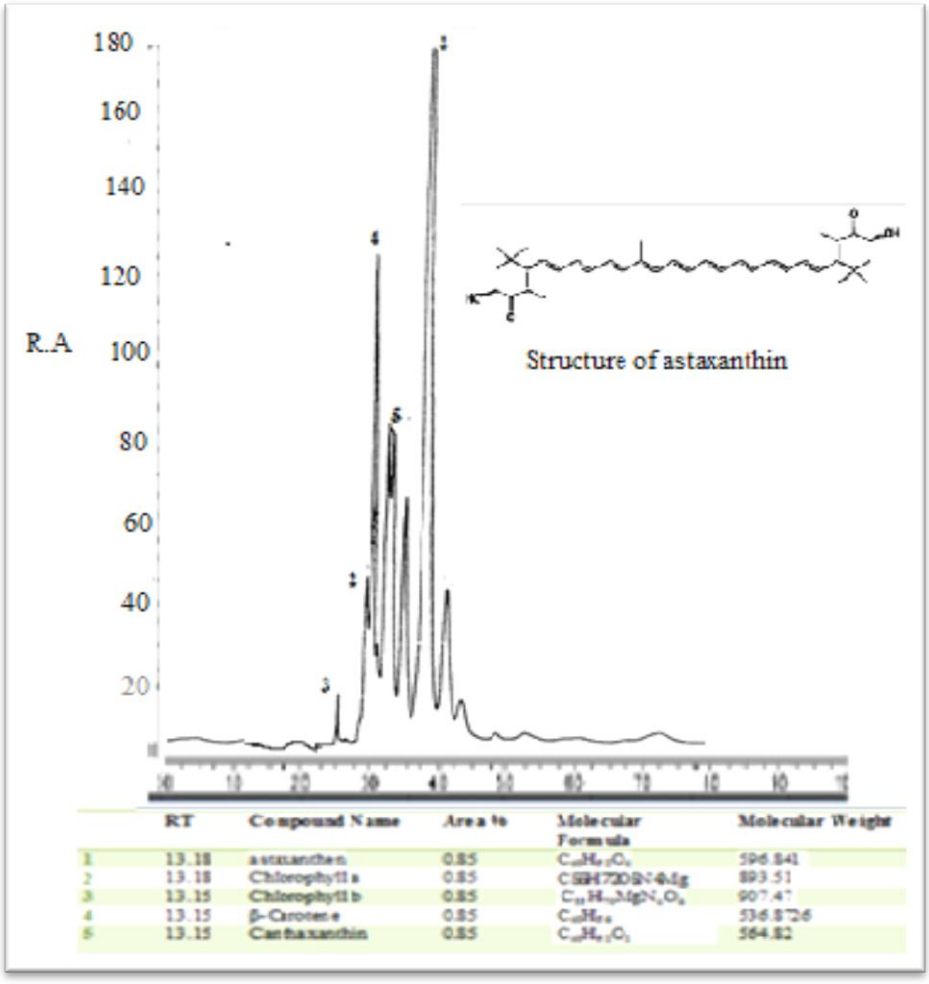
Table (1). Total Chlorophyll a , Carotenoid and Astaxanthin content in *H. pluvialis* cultures grown for 12 days at different concentrations of NaCl

Conc. of salt (%)	Chlorophyll a (mg/l)	Cartenoid (mg/l)	Astaxanthin (mg/l)
Control	**2.62**	**1.4**	**0.1**
0.12	**2.61**	**1.9**	**0.5**
0.5	**1.9**	**2.52**	**1.6**
1	**0.8**	**2.2**	**2.5**
2	**0.56**	**1.4**	**2.2**

These results come in harmony with those reported by **Harker *et al*. (1996) and Sarada *et al*. (2002)**who recorded the astaxanthin synthesis in *H. pluvialis* can induced by salinity stress. NaCl (0.1-0.5%, w/w) was used to increase astaxanthin accumulation in laboratory cultures. Also, high concentrations of NaCl may cause severe cell mortality, in particularly for flagellated zoospores, which limit the

implementation of this strategy in large-scale *Haematococcus* culture **(Harker *et al*., 1996; Sarada *et al*., 2002 and Cifuentes *et al*., 2003).**

Pigment analysis by HPLC of *H. pluvialis* after 12 days under 1% NaCl was illustrated in **Figure 1.** The major content of pigments analysis of crude extract of *H. pluvialis* under stress cultures recorded that high content of astaxanthin followed by β -carotene and chlorophyll a .The results obtained in the present study clearly indicated enhanced astaxanthin production under stress conditions and chlorophyll degradation. This result was agreement with the **Sarada *et al*. (2002)** who assumed that astaxanthin productivity and improved by salinity.



	RT	Compound Name	Area %	Molecular Formula	Molecular Weight
1	13.18	astaxanthin	0.85	$C_{40}H_{52}O_4$	596.841
2	13.18	Chlorophyll a	0.85	$C_{55}H_{72}O_5N_4Mg$	893.51
3	13.15	Chlorophyll b	0.85	$C_{55}H_{70}MgN_4O_6$	907.47
4	13.15	β-Carotene	0.85	$C_{40}H_{56}$	536.8726
5	13.15	Canthaxanthin	0.85	$C_{40}H_{52}O_2$	564.82

Figure (1): Pigment analysis by HpLC of *Haematococcus pluvialis* after 12 days under 1% NaCl

Antimicrobial activity

Antimicrobial activity of astaxanthin crude extract (10µl) was tested against two strains of fungi and four strains of bacteria two Gram (+) and two Gram (-), were evaluated in **Table 2**. The result showed that astaxanthin extract has not record antifungal activity against *Aspergillus fumigatus and Candida albicans*. On the other

hand crude extract possess highest antibacterial activity against *Staphylococcus aureus* with the inhibition zones (11mm) but not record antibacterial activity against *Bacillus subtilis*. While crude extract showed (10 and 9mm) respectively against *Escherichia coli* and *Pseudomonas aeruginosa* These result agree with **Rather *et al.* (2021)** found that the acetone crude astaxanthin extract (10µl) possess highest antibacterial activity against *Escherichia coli* with 10.2 ±0.20 mm and 8.7 ±0.12 mm on *Staphylococcus aureus* on methanol extract.

Table (2). Antibacterial and antifungal activity of astaxanthin crude extract against pathogenic bacteria and fungal (Mean zone of inhibition in mm beyond well diameter (6 mm)

Tested microorganism		**Sample**	**Standar treatment**	
***Aspergillus fumigatus* (RCMB 002008)**	**Fungi**	**NA**	**17**	***Ketoconazole* 100 µg/ml**
***Candida albicans* RCMB 005003 (1) ATCC 10231**		**NA**	**20**	
***Staphylococcus aureus* ATCC 25923**	**Gram (+) Bacteria**	**11**	**24**	***Gentamycin* 4 µg/ml**
***Bacillus subtilis* RCMB 015 (1) NRRL B-543**		**NA**	**26**	
***Escherichia coli* (RCMB 010052) ATCC 25955**	**Gram (-) Bacteria**	**10**	**30**	
***Pseudomonas aeruginosa* ATCC 27853**		**9**	**27**	

*NA: No activity.

Kumari and Ramanujan (2013) reported that the astaxanthin pigment was found to be more significantly effective against pathogenic species such as *S. typhi, P. aeruginosa, B. subtilis, and S. aureus* which produced 20mm, 24 mm, 18 mm and 16 mm diameter of zone which is interrelated with the present study

Inhibition of bacteria may be due to astaxanthin can act directly on bacterial cell and membrane, leading to damage of the cell wall , membrane and leakage of cell content. **Mahizan *et al.* (2019) and Seukep *et al.* (2020)** evaluated that caroteniod and terpenoids can lead to the accumulation of toxic compounds inside bacteria and can have an impact on ATP hydrolysis, leading to disturbance of efflux pump activation. In Gram-negative bacteria, these compounds can increase the permeability of the outer membrane and can change the conformation of efflux protein structures .

Antimicrobial activity may be due to several factors, including charge density, structure of lipopolysaccharides and lipid composition of the cytoplasmic membrane in Gram negative and Gram positive bacteria **(Devine and Hancock, 2002)**

Antioxidant activity

Antioxidant activity of the crude extract of astaxanthin was determined in terms of IC50 value based on the percentage of free radical scavenging activity **Table 3**. IC50 value for the crude extract of astaxanthin determined by DPPH assay was (63.20 ± 2.64 µg/ml).

Table (3): Evaluation of Antioxidant Activity crude extract of astaxanthin (µg/ml) using DPPH scavenging

Sample conc. (µg/ml)	DPPH scavenging %	(±) SD
1280	**95.64**	**0.82**
640	**93.25**	**0.79**
320	**88.13**	**1.05**
160	**76.41**	**1.43**
80	**57.62**	**1.84**
40	**39.47**	**1.91**
20	**25.08**	**1.34**
10	**11.64**	**0.52**
5	**7.82**	**0.67**
2.5	**4.09**	**0.35**
0	**0**	

Singh *et al*. (2021) evaluated that astaxanthin is known as "King of antioxidant" or "Super vitamin E" because of its potent antioxidant property. Antioxidant property of astaxanthin known to modulate different biological activity relating to antioxidant defense system, inflammation, immunity and ameliorating adverse effect of oxidative stress during summer season. Astaxanthin has two oxygenated groups on each ring structure that are responsible for its increased antioxidant characteristics **(Guerin *et al*., 2003).**

Astaxanthin in *H. pluvialis* offered the best protection from free radicals in rats followed by β-carotene and lutein **(Ranga Rao *et al.*, 2013).** Astaxanthin contains a unique molecular structure in the presence of hydroxyl and keto moieties on each ionone ring, which are responsible for the high antioxidant properties (**Hussein *et al.*, 2006 and Liu *et al.*, 2009**). **Sowmya and Sachindra (2012)** evaluated that the crude extract of astaxanthin showed strong antioxidant activity as indicated by radical scavenging

Astaxanthin binds with free radical to form unreactive compound and help in quenching electron out of membrane and resisting it from converting into pro-oxidant molecule **Singh *et al.*, 2021.**
Carotenoids are pigment that plays an essential role in mitigating oxidative processes. They are potent antioxidants that can scavenge mono-molecular oxygen and peroxyl radicals. They influence cellular signaling and activate redox sensitive controlling pathways **(Stahl and Sies, 2005)**

Antitumor activity
The cytotoxicity of astaxanthin crude extract showed 50% inhibition (IC50) of Hepatocellular carcinoma cells (HepG- 2) at 123.01 ± 2.97 µg/ml **Figure 3**.

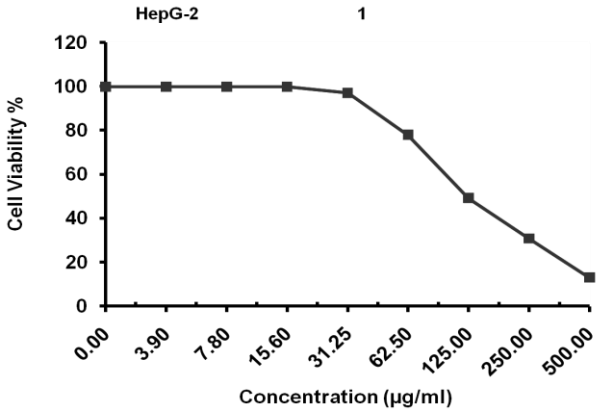


Figure 3: Antitumor activity of astaxanthin crude extract (µg/ml) against HepG-2 cell line

Chew *et al* . (1999) and Chew &Park (2004) stated that: astaxanthin significant antitumor activity when compared to other carotenoids like canthaxanthin and β-carotene. Astaxanthin inhibited cell death, cell proliferation and mammary tumors in chemically induced male/female rats and mice **(Tanaka *et al.*, 1995 and Nakao *et al.*, 2010)**. *H. pluvialis* extract inhibited the growth of human colon cancer, breast, and prostate cells by arresting cell cycle progression and promoting apoptosis reported by **(Palozza *et al.*, 2009).**

Speranza *et al.* (2012) recorded that treatment with astaxanthin helps in reducing the secretion of pro-inflammatory cytokines from NF- κB transcription factor by ROS induced production in H_2O_2- stimulated mononuclear U937 cells

Finally, the results give an indication *H. pluvialis* accumulates high amounts of astaxanthin content under salinity stress and astaxanthin showed potential biological activity *in vitro*, give an indication to the presence of promising antimicrobial compounds in methanol crude extract of astaxanthin ,also source of antitumor effect, cancer chemoprevention properties, anti-inflammatory and antioxidant.

REFERENCES

Bennedsen, M.; Wang, X.; Willén, R.; Wadström, T. and Andersen, L.P. (2000). Treatment of H. pylori infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. Immunol. Lett. 2000, 70: 185–189

Chew, B.P. and Park, J.S. (2004). Carotenoid action on the immune response. J. Nutr. 134: 257S–261S.

Chew, B.P.; Park, J.S.; Wong, M.W.and Wong, T.S.A. (1999). comparison of the anticancer activities of dietary β-carotene, canthaxanthin and astaxanthin in mice *in vivo*. Anticancer Res.19: 1849–1853.

Cifuentes, A. S.; González, M. A.; Vargas, S.; Hoeneisen, M. and González, N. (2003). Optimization of biomass, total carotenoids and astaxanthin production in *Haematococcus pluvialis* Flotow strain Steptoe (Nevada, USA) under laboratory conditions. Biol. Res. 36:343-357.

Claustre, H.; Kerhervé, P.; Marty, J.C.; Prieur, L.; Videau, C. and Hecq, J.H., (1994a). Phytoplankton dynamics associated with a geostrophic front: ecological and biogeochemical implications. Journal of Marine Research 52: 711-742.

Claustre, H.; Kerhervé, P.; Marty, J.C.and Prieur, L. (1994b). Phytoplankton photoadaptation in relation to some frontal physical processes. Journal of Marine Systems 5: 251-265.

Cordero, B.; Otero, A.; Patino, M.; Arredondo, B.O. and Fabregas J. (1996). Astaxanthin production from the green alga *H. pluialis* with different stress conditions. Bio.tech. Lett18:213–8.

Cyanotech (2015). BioAstin - Natural Astaxanthin. Available online at: http://www.cyanotech.com/bioastin.html

Davies, B.H. (1976) .Carotenoids. In: Goodwin TW, editor. Chemistry and biochemistry of plant pigments, vol. 2. London: Academ Press, 1976:38–166.

Devine, D.A. and Hancock, R.E .(2002). Cationic peptides distribution and mechanism of resistance. Curr Pharma. 8: 703-714

Guerin, M.; Huntley, M.E. and Olaizola, M. (2003). Haematococcus astaxanthin: applications for human health and nutrition. Trends Biotechnol 21:210-216

Harker, M.; Tsavalos, A. J. and Young, A. J. (1996). Autotrophic growth and carotenoid production of *Haematococcuspluvialis* in a 30 liter air-lift photobioreactor. J. Ferment. Bioeng. 82:113-118.

Hussein, G.; Sankawa, U.; Goto, H.; Matsumoto, K. and Watanabe, H.(2006). Astaxanthin, a carotenoid with potential in human health and nutrition. J Nat. Prod. 69:443–449

Kobayashi, M.; Kakizono, T. and Nagai, S. (1993). Enhanced carotenoid biosynthesis by oxidative stress in acetate induced cyst cells of the green algae *Haematococcus pluvialis.* Appl . Environ. Microbiol 59(3): 867-873.

Kumari,U.N. and Ramanujan, R. (2013). Isolation of astaxanthin from shrimp Metapenaeus Dobsoni and study of its pharmacological activity. J Curr Chem Pharma Sci, 3 (1): 60-63.

Lichtenthaler, H.K. (1987). Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. In: Packer L, Douce R, editors. Methods in enzymology, vol. 148. San Diego, California: Academic Press, 1976:350–382

Liu, X. B.; Shibata, T.; Hisaka, S. and Osawa, T. (2009). Astaxanthin inhibits reactive oxygen species-mediated cellular toxicity in dopaminergic SH-SY5Y cells via mitochondria-targeted protective mechanism. Brain Res.1254: 18–27.

Mahizan, N.A.; Yang, S.-K.; Moo, C.L.; Song, A.A.-L.; Chong, C.-M. and Chong, C.W. (2019). Terpene Derivatives as a Potential Agent against Antimicrobial Resistance (AMR) Pathogens. Molecules 24, E2631.

Miki, W. (1991). Biological functions and activities of animal carotenoids. Pure Appl. Chem. 63: 141–146. doi: 10.1351

Mosmann, T. (1983): Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods;* 65: 55-63

Nakao, R.; Nelson, O.L.; Park, J.S.; Mathison, B.D.; Thompson, P.A. and Chew, B.P. (2010): Effect of dietary astaxanthin at different stages of mammary tumor initiation in BALB/c mice. Anticancer Res, 30: 2171–2175

Ohgami, K.; Shiratori, K.;Kotake, S.; Nishida, T.; Mizuki, N.; Yazawa, K. and Ohno, S. (2003). Effects of astaxanthin on lipopolysaccharide-induced inflammation *in vitro* and *in vivo*. Invest. Ophthalmol. Vis. Sci., 44: 2694–2701.

Palozza, P.; Torelli, C.; Boninsegna, A.; Simone, R.; Catalano, A.; Mele, M.C.; Picci, N. (2009). Growth-inhibitory effects of the astaxanthin-rich alga *Haematococcus pluvialis* in human colon cancer cells. Cancer Lett., 283: 108–117.

Park, J.S.; Chyun, J.H.; Kim, Y.K.; Line, L.L.and Chew, B.P. (2010). Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans. Nutr. Metab7: 1–10.

Ranga, R. A.; Baskaran, V.; Sarada, R.and Ravishankar, G.A. (2013).*In vivo* bioavailability and antioxidant activity of carotenoids from micro algal biomass—A repeated dose study. Food Res. Int., 54: 711–717.

Ranga ,R. A.; Raghunath, R. R.L.; Baskaran, V.; Sarada, R. and Ravishankar, G.A.(2010). Characterization of microalgal carotenoids by mass spectrometry

and their bioavailability and antioxidant properties elucidated in rat model. J. Agric. Food Chem., 58: 8553–8559.

Rather A H. and Singh, S. (2018). Preliminary evaluation of impact of monochromatic light on the biosynthesis of astaxanthin in green alga *H. pluvialis.* World News of Nat. Sci. 19:45-50.

Rather, A. H.; Singh, S. and Sameer, C. (2021). Antibacterial Activity of *Haematococcus pluvialis* Crude Astaxanthin Extract, Journal of Drug Delivery & Therapeutics. 11(2-s): 28-30

Santoyo, S.; Rodríguez-Meizoso, I.; Cifuentes, A.; Jaime, L.; García, G.; Reina, B.; Señorans, F.J. and Ibáñez, E. (2009). Green processes based on the extraction with pressurized fluids to obtain potent antimicrobials from *Haematococcus pluvialis* microalgae. Food Sci Tech, 42: 1213-1218.

Sarada, R.; Tripathi, U. and Ravishankar, G.A. (2002). Influence of stress on astaxanthin production in *Haematococcus pluialis* grown under different culture conditions, Process Biochemistry 37: 623–627

Seukep, A.J.; Kuete, V.; Nahar, L.; Sarker, S.D.and Guo, M. (2020). Plant-derived secondary metabolites as the main source of efflux pump inhibitors and methods for identification. J. Pharm. Anal. 10: 277–290

Shanmugapriya, K.; Kim, H.;Saravana, P.S.; Chun, B.S.and Kang, H.W.(2018). Astaxanthin-Alpha Tocopherol Nanoemulsion Formulation by Emulsification Methods: Investigation on Anticancer, Wound Healing, and Antibacterial Effects. Colloids Surf. B Biointerfaces 172: 170–179.

Singh, S.V.; Yallappa, M. S. and Deshpande, A.(2021). Astaxanthin – King of antioxidants as immune modulator and anti-inflammatoryfor enhancing productive performance and health of animals Indian J Dairy Sci 74(1): 1-7

Sowmya , R. and Sachindra, M.N.(2012). Evaluation of antioxidant activity of carotenoid extract from shrimp processing byproducts by in vitro assays and in membrane model system. Food Chemistry Volume 134, 1:308-314

Speranza, L.; Pesce, M.; Patruno, A.; Franceschelli, S.; Lutiis, M.A.D.; Grilli, A, and Felaco, M. (2012). Astaxanthin treatment reduced oxidative induced pro-inflammatory cytokines secretion in U937: SHP-1 as a novel biological target. Mar Drugs 10: 890-899

Stahl, W. and Sies, H. (2005) Bioactivity and protective effects of natural carotenoids. Biochim Biophys Acta Mol Basis Dis 1740: 101-107

Stein, J.R. (1973). ed. – Handbook of phycological methods. Culture Methods and growth measurements, pp. 448, Cambridge at the University Press, London, New York,

Suzuki, Y.; Ohgami, K.; Shiratori, K.; Jin, X.H.; Llieva, I.; Koyama, Y.; Yazawa, K.; Yoshidia, K.; Kase, S.and Ohno, S. (2006). Suppressive effects of astaxanthin against rat endotoxin induced uveitis by inhibiting the NF-kB signaling pathway. Exp. Eye Res., 82, 275–281.

Tanaka, T.; Makita, H.; Ohnishi, M.; Mori, H.; Satoh, K.and Hara, A. (1995). Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophyll's, astaxanthin and canthaxanthin. Cancer Res. 55: 4059–4064.

Tjahjono, A.E.; Hayama ,Y.; Kakizono, T.; Tereda, Y.; Nishio, N. and Nagai, S. (1994). Hyper accumulation of astaxanthin in a green alga *Haematococcus pluialis* at elevated temperatures. Biotech Lett;16:133–184.

Wayama, M.; Ota, S.; Matsuura, H.; Nango, N.; Hirata, A. and Kawano, S. (2013). Three dimension alultrastructura lstudy of oil and astaxanthin accumulation during encyst mentinthe green alga *Haematococcus pluvialis*. Polson 8:e53618 .doi: 10.1371 / journal. pone.0053618

Yen, G.C. and Duh, P.D. (1994). Scavenging effect of methanolic extracts of peanut hulls on free radical and active oxygen species, J Agric Food Chem, 42: 629-632.