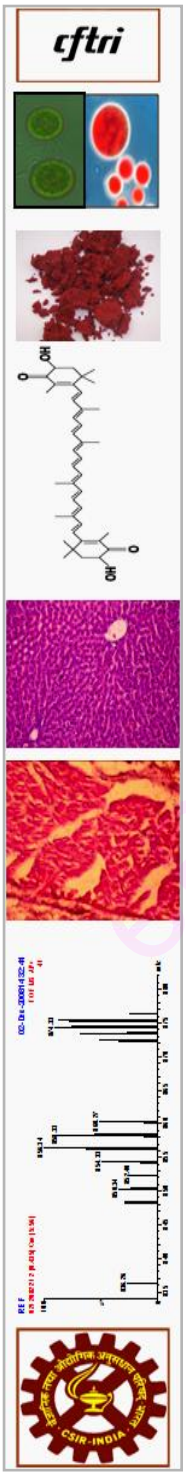
Production of Astaxanthin from Cultured Green Alga *Haematococcus pluvialis* and its Biological Activities



A Thesis submitted to the
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By

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January, 2011

DEDICATED TO,

MY DEAR FAMILY, TEACHERS &

BELOVED WIFE

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CERTIFICATE

I **Mr. Ranga Rao, A.,** certify that this thesis is the result of research work done by me under the supervision of **Dr. G. A. Ravishankar**, **Scientist-G & Head** at Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore**.** I am submitting this thesis of possible award of Doctor of Philosophy degree (Ph.D.) in **Biotechnology** of the **University of Mysore.**

I further certify that this thesis not been submitted by me for award of any other degree/diploma of this or any other University.

Signature of Doctoral candidate
Signed by me on date:

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CERTIFICATE

This is to certify that the thesis entitled "**Production of Astaxanthin from Cultured Green Alga *Haematococcus pluvialis* and Its Biological Activities"** submitted by **Mr. Ranga Rao, A.,** to the **University of Mysore** for the award of the degree of ***Doctor of Philosophy*** in **Biotechnology,** is the result of work carried out by him in **Plant Cell Biotechnology Department**, Central Food Technological Research Institute, Mysore under my guidance during the period October 2007 to October 2010.

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ABSTRACT

Micro algal biotechnology has gained importance due to its potential to produce bioactive molecules. Among the micro algae, *Haematococcus pluvialis* is a green alga belonging to chlorophyceae, which produces astaxanthin & its esters in large quantities under stress conditions. Carotenoids are being intensively investigated regarding their potential to prevent diseases and vitamin A deficiency. The present study is focused on the production of astaxanthin from *Haematococcus pluvialis* and its biological activities. This thesis consists of effect of various stress conditions on the production of astaxanthin in *Haemtococcus pluvialis;* isolation and characterization of astaxanthin & its esters; evaluation of stability of astaxanthin in various edible oils; safety evaluation of biomass; evaluation of its biological activity; bioavailability and vitamin A conversion; anticancer properties in rat experimental models.

Culture of *Haematococcus* was done using appropriate media & incubation conditions in laboratory. In sodium nitrate (25 mM) treated culture, the maximum biomass yield was 3.3 g/l, total carotenoid content was 2.9% and astaxanthin content was 2.5%, whereas in a potassium chloride 16 mM supplemented culture the biomass yield was 2.5 g/l, total carotenoid content was 2% and astaxanthin was 1.87%. Among various carbon source studied, ammonium carbonate (3 mM) treated culture showed the biomass yield of 2.9 g/l, total carotenoid was 2.6% and astaxanthin was 2.2%. Among the various solvents used, ethylacetate, isopropyl alcohol: hexane (1:1) and acetone were found to be efficient for extraction of the carotenoids from the *Haematococcus* cells.

Carotenoids, astaxanthin & its esters were quantified and identified by following techniques such as thin layer chromatography, high performance liquid chromatography and liquid chromatography mass spectrum using APCI mode. The major carotenoids were quantified in *Haematococcus* biomass and identified as astaxanthin & its esters followed by neoxanthin, violaxanthin, astaxanthin, lutein, zeaxanthin, β-cryptoxanthin and β-carotene. These were confirmed by their retention times and the absorption spectra of the respective reference standards. Based on the mass spectral data obtained from an astaxanthin mono-di esters such as ME $C_{16:0}$, ME $C_{17:2}$, ME $C_{17:1}$, ME $C_{17:0}$, ME $C_{18:4}$, ME $C_{18:3}$, ME $C_{18:2}$, ME $C_{18:1,}$ DE $C_{16:0}/C_{16:0}$, DE $C_{16:0}/C_{18:2}$, DE $C_{18:1}/C_{18:3}$, DE $C_{18:1}/C_{18:2}$ and DE $C_{18:1}/C_{18:1}$ were identified in *H. pluvialis*. Edible oils were also used as a vehicle of carotenoid delivery. To achieve maximum extractability various oils were tried. The maximum extractability of carotenoids was found in palm oil and olive oil. Astaxanthin & its esters were further confirmed by recording unambiguous ^{13}C NMR, ^{1}H NMR, HSQC 2D, NOESY 2D and COSY 2D NMR. Further, COSY and NOSEY spectrum confirmed the existence of both *cis* and *trans* forms of astaxanthin & its esters in *H. pluvialis.*

The *Haematococcus* extracts exhibited 80% antioxidant activity in β-carotene linoleate model system (β-CLAMS), 1, 1-diphenyl -2-picrylhydrazyl (DPPH) method, and hydroxyl radical scavenging model systems. Antibacterial properties of *H.*

pluvialis extracts were evaluated against selected bacteria. Among the different solvent extracts of *H. pluvialis*, chloroform extract exhibited highest antibacterial effect followed by ethyl acetate extract. Astaxanthin & its esters also showed significant antioxidant activity and hepatoprotective ability in carbon tetrachloride induced albino rats. Among the groups of experimental rats, the one which was treated with astaxanthin esters at 250 µg/kg b.w. showed maximum hepatoprotective activity i.e protection when compared to control treated group. However, pretreatment of rats with 250 µg/kg b.w of astaxanthin esters preserved catalase, peroxidase and SOD activities, when compared with control values in untreated animals.

Feeding trials, for experimental rats were carried out to assess the safety and efficacy of *Haematococcus* biomass as a source of astaxanthin. The study revealed that *Haematococcus* biomass was safe in both single and repeated dose in experimental rats. *In vivo* experiments demonstrated that astaxanthin from *Haematococcus* was effective in retinol formation and its accumulation in serum and liver. Time course study of carotenoids in rats after administration of *Haemtococcus* biomass showed peak levels in plasma, liver and eyes at 2, 4 & 6 h respectively. In the repeated dose study, the astaxanthin levels in plasma, liver and eyes of rat over 15 days after intubation of *H. pluvialis* biomass was recorded. In liver, astaxanthin levels were 1.7 and 1.8 fold higher than in plasma and eyes. In plasma and liver, antioxidant enzymes catalase, SOD, peroxidase activity was higher in astaxanthin treated rats when compared to untreated control rats.

The anticancer properties of astaxanthin & its esters was also studied in human glioma cell lines (LN-229, HNGC2) and liver hepatocellular carcinoma cell line (HepG2). The apoptosis cells were observed under phase-contrast and confocal microscope. Anticancer properties of astaxanthin & its esters were evaluated against skin carcinogenesis, which is reported for the first time. Tyrosinase enzyme activity was inhibited by using astaxanthin & its esters using *in vitro* models. Pre feeding of rats with astaxanthin esters 250 µg/kg b.w. prior to treatment with carcinogen showed 3-4 fold reduction in tumor index. Hematological and histopathological studies were examined which substantiate the protective role of astaxanthin esters. The plasma astaxanthin and retinol content were estimated in serum and liver homogenate. Biochemical changes like lipid peroxidation, catalase, superoxide dismutase, glutathione reductase activities were estimated in control, cancer induced animal groups. Astaxanthin metabolites isolated from plasma, liver from *in vivo* studies were characterized by HPLC and LC-MS (APCI) techniques to elucidate their structure. Epoxy carotenoids were tentatively identified in liver and plasma. This study emphasizes the influence of astaxanthin and its beneficial effects on the metabolism in experimental animals. Based on animal experiments and bioavailability studies, it was evident that astaxanthin & its esters could possibly be safely used as nutritional, antioxidant, anticancer agent.

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(Ranga Rao, A.,)

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LIST OF ABBREVIATIONS

Symbol	Abbreviation
µ	Micro
^{o}C	Degree centigrade
Klux	Kilolux
L	Litre
mL	Millilitre
min	Minute(s)
h	Hours
rpm	Revolution per minutes
mg	Milligram
µg	Microgram
M	Molar
mM	Millimolar
µM	Micromolar
w/v	Weight per volume
v/v	Volume per volume
w/w	Weight per weight
BBM	Bold basal medium
DW	Dry weight
OD	Optical density
b.w	Body weight
Rf	resolution front
APCI	Atmospheric pressure chemical ionization
BHA	Butylated hydroxyl anisole
CAT	Catalase
CLSM	Confocal laser scanning microscopy
DASX	Diester of astaxanthin
DL	Deciliter
DMBA	7, 12-dimethylbenz (a) anthracene
DMEM	Dulbecco's modified Eagle's medium
DPPH	1, 1-diphenyl-2-picryl hydrazyl
EDTA	Ethylene diamino tetra acetic acid
FAME	Fatty acid methyl esters
FBS	Fetal bovine serum
FCS	Fetal calf serum
FID	Flame ionization detector

GC	Gas liquid chromatography
H_2O_2	Hydrogen peroxide
HLDL	Human low density lipoproteins
HPLC	High pressure liquid chromatography
LC-MS	Liquid chromatography mass spectrum
MASX	Monoester of astaxanthin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MIC	Minimum inhibitory concentration
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
NBT	Nitroblue tetrazolium
PBS	Phosphate buffer saline
PDA	Photodiode array detector
RBC	Red blood cells
ROS	Reactive oxygen Species
SALP	Serum alkaline phophatase
SASX	Standard astaxanthin
SD	Standard deviation
SGOT	Serum glutamate oxaloacetae transaminase
SGPT	Serum glutamate pyruvate transaminase
SOD	Superoxidase dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TC	Total carotenoid
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
UV	Ultraviolet
WBC	White blood cells

INTRODUCTION & REVIEW OF LITERATURE

1. General introduction of algae

History of algae is as old as that of plants. The first reference on algae was found in early Chinese literature and mentioned as `Tsa`. It also appeared in Greek and Roman literature as 'phycos' and 'Fucus' respectively, while in the Latin language seaweed were designated by the term algae. Algae were used as food for long period, which was known by name `Limu'. Algae are relatively simple aquatic organism that capture light energy through photosynthesis, use it to convert inorganic substances into organic matter. Algae have been regarded as simple plants, but they actually span more than one domain, including both Eukaryota, belonging to Chlorophyceae, Rhodophyceae etc., (*Chlorella, Dunaliella* etc) and Prokaryota, belonging to cyanophyceae group (Blue green algae eg, *Spirulina*) (Gupta, 1981). Algae are classified into seven groups or divisions shown in Table 1. Algae range from single celled organisms (micro algae) to multicellular organisms, some with fairly complex differentiated form. The complete forms are known as macro algae which includes that of marine forms such as seaweeds. All algae have photosynthetic machinery basically derived from the cyanobacteria (Fritsch, 1977), producing oxygen as a by product of photosynthesis.

1.1. Microalgae as source of food

Historical records suggest that people collected macro algae and seaweeds for food around 2,500 years ago in China (Tseng, 1981). Europeans have collected seaweeds for food for 500 years. Of the macro algae, the most widely consumed one throughout the world has been the membranaceous red alga Porphyra. This alga commonly known as "Nori", "amanon" or "hoshinor" in Japan and "purple laver" in the West. The one genus of red algae represents that largest tonnage of aquaculture product in the world (McCoy, 1987) and was the first marine macro algae to be cultivated by man. Nori has been grown in Tokyo Bay for nearly 300 years (Lobban et al., 1985). It is directly eaten in soups or as a vegetable or used as a condiment. Presently China and Japan are the two major growers (Mumford, 1990). The Japanese grow over 500, 000 tons of Nori per year and consume over 100,000 tons directly per year. The Nori industry in Japan employs over 60,000 people and is estimated to support over 300,000 people (McCoy, 1987). The Chinese also have a very large Nori industry. Major commercial centers for Nori include marinan Islands, Saipan, and Guam. However, the world`s largest and most technically advance Nori farm facilities are present in the Philippines (McCoy, 1987). The blue green algae, another important

algae, was eaten by the Aztecs in Mexio, who called it "Tecuitlat" (Farrar, 1966). The same algae forms the part of food of the Kanembou tribe north of Lake chad in Central Africa, who make it into sauce called "dihe". Another blue green-green alga, *Phylloderma sacrum* is eaten is several region of java. In India, Burma, Thiland and Vietnam various species of *Oedogonium* and *Spirogyra* are eaten (Venkataraman and Becker, 1985). Most people in the United States of America ingest red or brown algae products everyday in chocolate, milk, candy, cosmetics, ice creams, salad dressing and many other household and industrial products. *Chlorella* is mainly sold in health food stores and as fish feed (Hills and Nakamura, 1978)

Table 1. Classification of microalgae

Division	Brief description of different classes	Examples
Cyanophyta	Cyanophyceae or blue green algae, fresh or marine forms	*Spirulina*
Rhodophyta	Rhodophyceae or Red micro algae, Seaweeds, thallus is highly differentiated , found in littoral zones, commercially important polysaccharides from this group	*Porphyridium sp* *Euchema,* *Gelidium* *Gracilaria*
Chlorphyta	Chlorophyceae, green algae, Large group of very differentiated forms, pigments resemble that of higher plants	*Chlorella, Dunaliella, Haematococcus Sp*
Euglenophyta	Euglenophyceae, green flagellates, mainly fresh water forms	*Astasia longa*
Chrysophyta	Xanthophyceae or golden brown algae, mainly fresh water.	*Fragillaria pinnata*
	Chrysophyceae or yellow brown algae, mainly fresh water forms.	*Prymensium parvam*
	Bacillariophyceae or diatoms, characterized by strong silicified cell membranes, fresh water and marine	
Pyrrophyta	Desmophyceae, mainly marine forms.	*Prorocentrum micans*
	Dinophycease free living marine unicellular organisms	*Dinophysis*
	Cryptophyceae, small poorly known group, marine or fresh water forms	
Phaeophyta	Phaeophyceae or brown algae, thallus with Ectocarpus, high differentiation, microscopic to Fucus, complicated filamentous bodies, majority laminaria found in littoral zones	*Ectocarpus,* *Focus,* *Laminaria*

(Adopted from Levring, 1979)

1.2 Microalgae as source of pharmaceuticals

Microalgae as source of natural products, novel compounds for pharmaceutical applications (Hoppe and Levring, 1979). Screening of marine algae for antitumor

activity began in the 1970s. Early discoveries included tubercidin, a heterocyclic nitrogen compound from the cyanobacterium *Tolypothrix byssoidea,* shown to have *in vitro* activity against P-388 lymphocytic leukemia. L-asparginase from the green microalga *Chlamydomonas,* inhibits growth of lymphosarcoma in mice. Marine blue green algae have been one target of a more recent Natural Cancer Institute screening program aimed at anticancer and antiviral (anti-HIV) activity that has identified a number of compounds for potential drug development, such as e.g. dibromoaplysiatoxin from *Lyngbya majuscula.* Microalgae, including cyanobacteria and colorless (apochlorotic) variants of diatoms, may also be potential sources of antiviral sulfolipids (eg: sulfoquinosovyl diglyceride) (Gustafson, 1989). Cyanobacterial extracts have shown activity against Herpes simples virus type-II and also against respiratory syncytial virus at higher concentrations (Lau et al., 1993). Cyanobacteria also produces a number of cytotoxic compounds namely, tubericidin and toyocamycin from *Streptomyces* (Patterson et al., 1991). *Spirulina* and *Dunaliella* extracts have shown anticancer activity against oral cancer cell lines as well as in case of tobacco induced buccal cancer in human volunteers (Shklar and Schwartz, 1988). This may be due to the bioactive compounds such as β-carotene and phycocyanin. The algal β-carotene (Sude et al., 1986) and phycocyanin (Gerwick et al., 1994) has shown the anticancer property in oral carcinogenesis. Caulerpenyne, is a sesquiterpene isolated from marine alga *Caulerpa taxifolia* has shown antiproliferative and apoptotic activity in human neuroblastoma cell lines (Caves et al., 2006). Red alga *Amphiroa zonata* has shown the presence of palmitic acid, which has shown antitumor activity in both *in vivo* and *ex vivo* (Laycock et al., 1989).

1.3. Microalgae as source of high value metabolites

Micro algal biomass and extracts from biomass have gained a firm position on the market. There is an increasing demand for sophisticated products from microalgae. The phylogenetically archaic cyanobacteria produce numerous substances which exhibit antioxidative effects, polyunsaturated fatty acids (PUFA), heat-induced proteins, or immunologically effective compounds. Some of these substances are even excreted by the algae (Cohen, 1999).

1.3.1. Pigments and carotenoids

Chlorophylls, carotenoids, algal tannins, fucoxanthin, phycocyanin, astaxanthin, phycoerythrin are some of the important pigments that can be extracted from algae for its use. Chlorophyll as the primary photosynthetic pigment, microalgae contain a

multitude of pigments which are associated with light incidence. The pigments improve the efficiency of light energy utilization (phycobiliproteins) of plants and protect them against solar radiation (carotenoids) and related effects. Algal pigments are in high demand for their unique utility & biological activities. β-Carotene from *Dunaliella* in health food as a vitamin A precursor; Astaxanthin from *Haematococcus* in aquaculture for coloring muscles in fish; Lutein, zeaxanthin and canthaxantin for chicken skin coloration; or for pharmaceutical purposes. The phycobiliproteins, phycocyanin and phycoerythrin, are unique to algae and some preparations are already being developed for food and cosmetics. This development will certainly go beyond applications in diagnostics and photodynamic therapy and extend to cosmetics, nutrition and pharmacy (Hirata et al., 2000). Carotenoids produced by microalgae are shown in **Table 2**

Table 2. Carotenoids from microalgae

Algal class	Common name	Carotenoid pigment
Cyanophyceae	Bluegreen algae	1,3,12, 13, 16, 19, 22, 55,26
Chlorophyceae	Green algae	1,7,10-14, 16, 17, 18, 19, 20-22, 24, 28, 29, 36, 43, 47, 48, 65-67
Charophyceae	Stoneworts	1, 6, 5
Euglenophyceae	Euglenoids	1, 13, 14, 16, 18, 19,23, 29, 31, 35, 36, 48, 70, 71
Phaeophyceae	Brown algae	1, 13, 14, 16, 19, 23, 29, 31, 35, 36, 48, 70, 71
Chrysophyceae	Golden and yellow green algae including diatoms	1, 4, 9, 14, 15, 20, 28, 29, 31, 33, 35, 36
Pyrrophyceae	Dinoflagellates	1,2,18,26,32,35,37-39,44,45,51,53,54,64
Rhodophyceae	Red algae	1,10,12,21,26,28-30, 40,42,46,65,68,69
Cryptophyceae	Yellow algae	1,2,4,21,42,46,65
Raphidophyceae	Yellow algae	1,4,12-14,20,26,28,29,36,51,63
Xanthophyceae		1,12,19,20,28,29,33
Eustigmatophyceae		1,4,12,20,26-29, 35,36
Prasinophyceae and Loxophyceae	Green flagellates	1,7,10-14,16,18-20,22,24,28,29,43,47,48, 65-67

(Adopted from Shahidi et al., 1998)

1.β,β-carotene, β,∈-carotene, 3. β, ψ-carotene, 4. ∈, ∈-carotene, 5. β,β-carotene-2-ol 6. β,β-carotene-2,2-diol, 7. β,∈-carotene-2-ol, 8. β-carotene epoxide, 9. β-carotene diapoxide, 10. Lutein, 11. Lutein epoxide, 12. Zeaxanthin, 13. Cryptoxanthin, 14. Cryptoxanthin epoxide, 15. Cryptoxanthin diapoxide, 16. Echinenone, 17. Lycopene, 18. Astaxanthin, 19. Canthaxanthin, 20. Antheraxanthin, 21. Alloxanthin, 22.3-hydroxycanthaxanthin, 23. 3-hydroxy-echinenone, 24. 4-Hydroxyechinenone, 25. 3'-hydroxyechinenone,26. Fucoxanthin, 27. Fucoxanthinol, 28.Violaxanthin, 29. Neoxanthin, 30.Taraxanthin, 31. Heteroxanthin, 32. Dinoxanthin, 33. Vaucheriaxanthin, 34. Vaucheriaxanthin ester. 35. Diatoxanthin, 36. Diadinoxanthin, 37. Diadinoxanthin epoxide, 38. Peridinin, 39. Peridinol, 40. Auroxanthin, 41. Aurochrome, 42. Crocoxanthin, 43. Loroxanthin, 44. Phytoene, 45.

Phytofluene, 46. Monadoxanthin, 47. Siphonaxanthin, 48. Siphonein, 49. Micronone, 50. Deepoxyneoxanthin, 51. 19'-hexanoyloxyfucoxanthin, 52.19-hexanoyloxyparacentrone-3-acetate, 53. Pyrrhoxanthin, 54. Pyrrhoxanthinol 55. Myxoxanthophyll, 56. Oscillaxanthin, 57. 4-ketomyxoxanthophyll, 58. Aphanizophyll, 59. O-methyl-methylpenthoside, 60.Mutachrome, 61.Caloxanthin, 62. Nostoxanthin, 63.19'-butanoyloxyfucocanthin, 64. Gyroxanthin, 65. α-carotene, 66. Prasinoxanthin, 67. Fritschiellaxanthin, 68. α-cryptoxanthin, 69. β-cryptoxanthin, 70. Eutreptiellanone, 71.Anhydrodiatoxanthin.

Most of the chlorophycean members contain multitude of carotenoids-neoxanthin, violaxanthin, lutein, zeaxanthin, and antheraxanthin (Jin et al., 2003b). Due to its carotenoid rich nature, *Chlorella vulgaris* is being used as natural colour ingredient in animal feed (Gouveia et al., 1996). Few green algae like *Haematococcus, Chlorococcum, Neochloris* produce carotenoid astaxanthin along with other carotenoids. Several carotenoids have been recognized as biotechnologically important which are presented in Table 3.

Table 3. Biotechnologically important carotenoid production in microalgae

Microalgae	Yield	References
	Lutein	
Chlorella zofingiensis	21 μg/ml	Del Campo et al., 2004
Chlorella protothecoides	225 μg/ml	Shi et al., 1999
Muriellopsis sp	35 μg/ml	Del Campo et al., 2001
	Zeaxanthin	
Dunaliella salina	6 mg/g	Jin et al., 2003a
Microcystis aeruginosa	Not reported	Chen et al ., 2005
Nannochloropsis	Not reported	Lee et al ., 2006
	Astaxanthin	
Haematococus pluvialis	30 mg/g	Lorenz and Cysewski, 2000 Brinda et al., 2004, Kamath et al., 2008
Chlorella zofingiensis	<1 mg/g	Ip and Chen, 2005
	β-carotene	
Dunaliella salina	100 mg/g	Garcia-Gonzalez et al., 2005 Chidambara Murthy, 2005
Dunaliella bardawil	>100 mg/g	Lers et al., 1990 Vanitha , 2007
	Canthaxanthin	
Chlorella emergonii	0.6 μg/ml	Arad et al., 1993

Bhosale and Bernstein, (2005).

1.3.2. Stable isotope biochemicals

Microalgae are suited as a source of stable isotopically labeled compounds. The ability to perform photosynthesis allows them to incorporate stable isotopes (^{13}C, ^{15}N and ^{2}H) from relatively inexpensive inorganic molecules ($^{13}CO_2$, $^{15}NO_3$ and $^{2}H_2O$) to more highly valued organic compounds (e.g., amino acids, carbohydrates, lipids and nucleic acids). Stable isotope biochemicals are used for two purposes (Apt and Behrens, 1999): incorporation into proteins, carbohydrates and nucleic acids to

facilitate their structural determination at the atomic level; and metabolic studies exploiting the increased mass of compounds labeled. Their market is reported to be higher than US$ 13 million/year (Apt and Behrens, 1999). Spectra stable isotopes (Columbia, MD, USA), a division of spectra gases (formerly Martek Stable Isotope) sells its marked amino acids at prices in the range from US$ 260/g to US$ 5900/g and its marked nucleic acids at about US$ 28/mg. It has developed a process for the autotrophic production of labeled PUFAs from microalgae using $^{13}CO_2$, in which $^{13}CO_2$ is directly sparged into the culture (Apt and Behrens, 1999).

1.3.3. Antioxidants

Microalgae, as phylogenetically the oldest plants, have adapted uniquely to extreme habitats over billions of years of evolution. Due to their phototrophic life, they are exposed to high oxygen and radical stresses. This has resulted in the development of numerous efficient protective systems against oxidative and radical stress. The protective mechanisms are able to prevent the accumulation of free radicals and reactive oxygen species and thus to counteract cell-damaging activities. In cultures of photosynthetically active microorganisms of high cell density, molecular oxygen is produced and an oxygen over-saturation is observed. The antioxidative potential of *Spirulina platensis* can increase 2.3-fold during oxygen stress. Because the antioxidative components originate from a natural source, their application in cosmetics for preserving and protecting purposes is developing rapidly. In combination with other antioxidative or bioactive substances from microalgae, especially sun-protecting cosmetics, they represent an area of high demand. For functional food/nutraceuticals, the radical-scavenging capacity of micro algal products is of growing interest, especially in the beverage market segment and in pharmaceutical applications for the therapy of oxidation associated diseases, like inflammations. The antioxidants may act by raising the levels of endogenous defence by up regulating the expression of genes encoding the enzymes such as superoxide dismutase, catalase, glutathione peroxidase etc. (Serafin, 2006). Most of the edible algal forms are rich sources of one or the other antioxidant form. They accumulate high amount of antioxidant principles eg., β-carotene from *Dunaliella*, astaxanthin from *Haematococcus* because, they have to survive in high stress conditions compared to higher plants. *Enteromorpha* and *Kappaphycus* are good sources of

ascorbic acid. Antioxidant activity of *Spirulina* and *Dunaliella* are well documented both *in vitro* and *in vivo* (Miranda et al., 1998; Chidambara Murthy et al., 2005)

1.3.4. Polyunsaturated fatty acids (PUFA)

Microalgae supply whole food chains with these vital components. Besides being a primary source of PUFA, these fatty acids from microalgae have further advantages over fish oils, such as the lack of unpleasant odor, reduced risk of chemical contamination and better purification potential (Cohen, 1999). Micro algal PUFA have a very promising biotechnological market both for food and feed, e.g., health-promoting purified PUFA are added to infant milk formulas in Europe (Cohen, 1991). PUFA of high pharmaceutical and nutritional value are shown in Fig 1.

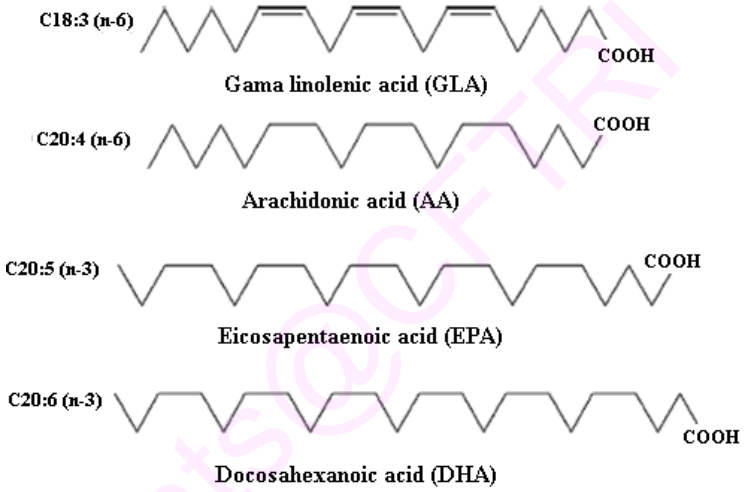


Fig. 1. PUFA of high pharmaceutical and nutrition value.

The importance of microalgae as a supplier of γ- linolenic acid was slightly weakened by the use of evening-primrose oil (Pulz and Gross, 2004). However, the preparation of EPA and DHA from marine organisms with phototrophic capability, like the dinoflagellate *Crypthecodinium*, for baby food or the health food market is an innovative approach (Apt and Behrens 1999; Radmer 1996). The application of this product line to foods was permitted on the basis of another organism *Ulkenia sp*. by EC regulatory boards in 2003. Products from *Odontella aurita* biomass is expected to have industrial potential. First the Martek company (USA) and then Nutrinova (a German company) announced the production of DHA products from microalgal biotechnology for human and other applications. Lipid-based cosmetics, like creams or lotions are gaining commercial importance because of their provision of both nourishing and protecting effects to the skin. For future developments in skin care,

other lipid classes from microalgae, like glyco and phospholipids have great potential (Muller-Feuga et al., 2003).

1.3.5. Lipids, oils, sterols and fatty acids

The total oil and fat content of micro algae ranges from 1% -70% of the dry weight and tends to be inversely proportional to the rate of growth with greater accumulations during stationary phase (Borowitzka, 1988). The percent of total lipid as neutral lipid, glycolipid, and phospholipid also varies widely within groups of microalgae (Borowitzka, 1988; Kates, 1987) and some species produce hydrocarbons. *Botryococcus braunii* is a chlorophyte capable of accumulating up to 90% of its colonial dry weight as a mixture of ten hydrocarbon compounds in globules occluded among cells embedded in a colonial matrix. Hydrocarbons up to C_{37} in size from different strains of *B. braunii* include straight chain alkadienes, branched triterpenoids (*botryococcenes*), and the tetraterpenoid lycopadiene (Okada et al., 1995). A wide range of common and rare sterols are also synthesized by microalgae, including, for example, cholesterols (cyanobacteria, rhodophytes), chondrillasterol (chlorophytes, euglenoids), clinoasterol (xanthophytes),dinosterol (dinoflagellates), ergosterol (chlorophytes,rhodophytes, euglenoids), epibrassicasterol (diatoms), poriferasterol (chlorophytes, chrysophytes), and sitosterol(cyanobacteria, chlorophytes, xanthophytes) (Goodwin, 1974). Micro algal lipids are mostly esters of glycerol and fatty acids with a chain length of C_4 to C_{22} and may be saturated or unsaturated. Cyanobacteria tend to have large amounts of polyunsaturated fatty acids while eukaryotic microalgae contain, in addition, a wide range of saturated and mono saturated fatty acids with fatty acid profiles widely variable among taxa. Specific fatty acid profiles vary widely among algal groups, with age and growth stage, and with environmental conditions. Linolenic acid ($C_{18:3}$) is common in green algae, whereas diatoms contain palmitic ($C_{16:0}$), hexadecenoic and C_{20} polyenoic acids. Red microalgae have high contents of arachidonic acid ($C_{20:4}$) as well as palmitic, oleic and linoleic acids. Chrysophytes contain significant quantities of highly unsaturated $C_{18:4}$ and $C_{20:6}$ acids in addition to unsaturated $C_{16:0}$ and $C_{20:0}$ compounds which are produced by a variety of microalgae. Eicosapentaenoic ($C_{20:5}$) and docosahexaenoic ($C_{22:6}$) acids are produced by some species of green and red microalgae, cryptophytes, dinoflagellates, prymnesiophytes and diatoms. Certain species have also been shown to synthesize unusual fattyacids such as, for example myristic in the diatom *Fragilaria,* lignoceric ($C_{24:0}$) in the premnesiophyte *lsochrysis,* gamma linolenic

($C_{18:3}$) in the golden-brown alga *Ochromonas,* and arachidonic ($C_{20:4}$) in the red microalgae *Porphyridium* (Chen et al., 1990).

1.3.6. Protein & amino acids

Most of the algal forms are known as rich sources of protein. They are considered as single cell proteins. The advantages of these proteins compared to plant and animal proteins are that these are simple and easily digestible ones (Becker, 2007). Nutritional quality of algal protein is very high compared to conventional plants that we use in our regular diet (Becker, 2007). Protein content in the algal forms range form 15-65% w/w. *Spirulina* contains 50-66% (Darcas, 2004, Becker and Venkatraman, 1982), *Chlorella sp* containing (54-63% (Laguna et al., 1993), *Dunaliella* containing upto 16-25% (Liu et al., 2000), *Nostoc* contains 40% (Knubel et al., 1990), *Scenedesmus* containing 45-65% (Fabric, 1970) *Enteromorpha* containing 29-35%, (Siva kumar, 2000), *Kappaphycus* containing 14-21% (Flurence, 1999), *Porphyra* 28-39% by dry weight (Becker, 2007), and *Ulva* can also yield 26% protein (Burtin, 2003). The micro algae can serve as a source of amino acids (Becker, 2007). This is mainly because they produce all the amino acids required for biological system.

1.3.7. Minerals and vitamins

Microalgae as source of minerals and vitamins, contain some of the biologically important minerals like calcium, zinc, selenium, cobalt, iodine, iron and potassium (Kikunaga et al., 1999). *Porphyra* species are especially rich in vitamin D (Aaronson, 2000). Algae are also known to contain both water and fat soluble vitamins. *Spirulina* and *Nori* are the two important sources of *cyanocobalamine* (vitamin B_{12}) (Berg et al., 1991). *Dunaliella* and *Spirulina* contains β-carotene, which is precursor of vitamin A (Borowitzka, 1988). Vitamin B_{12}, B_1 and biotin (Nakamura and Gowns, 1964), folic acid (B_9) and pantathonic acid (B_5) (Aaronson et al., 1980) by fresh water *Chlamydomonas* cells has been reported.

1.3.8. Nutraceuticals

Many species of micro algae such as *Spirulina, Chlorella, Scenedesmus* have been used as food for years and is still being used in several countries like China, Fiji, Mongolea (Prasad and Gupta, 2007). Carbohydrates in microalgae are in the form of starch, glucose or other polysaccharides have high digestibility (Becker, 2004). Some micro algae are rich source of ω-3 and ω-6 fatty acids (Tonon et al, 2002). The blue-

green microalga *Spirulina* had a long history in human nutrition. In 1975, *Spirulina, Chlorella* tablets made from dry powder were sold in the markets (Liang et al., 2004). Spray-dried biomass is generally utilized for health foods, food additives and feed supplements. (Venkataraman et al., 1995; Yamaguchi, 1997).

Spirulina is a rich natural source of protein, carotenoids, ω-3 and ω-6 polyunsaturated fatty acids, provitamins and other nutrients such as vitamin A, vitamin E, and selenium (Wu et al., 2005; Venkataraman et al., 1995). *Spirulina* has high protein efficiency ratio (PER) than those of cereals, vegetable and soya protein (Venkataraman, 1993). *Spirulina, Chlorella* are also utilized in the processing of common foods such as noodles, bread, green tea, health drink, candy (Liang et al., 2004). *Chlorella* health foods in the form of tablets, granules and drinks entered the market in 1964. More than 70 companies have their *Chlorella* health foods registered at Japan Health Food Association and their annual sales are estimated to be above 40 billion yen (Yamaguchi, 1997). Beneficial health effects of *Chlorella*, like preventive action against atherosclerosis, hypercholesterolemia, hypoglycemia in animal models have been reported (Jong-Yuh and Mei-Fen, 2005). β- carotene rich dried biomass of *Dunaliella* and its capsules and tablets are placed on the market as a health food (Metting, 1996). Microalgal oils have been commercially produced for incorporation into infant milk formulations, as dietary supplements and as food additives (Kyle and Gladue, 1996). β-carotene from the alga *Dunaliella salina* is the first high value algal product commercialized which is now being produced in Australia, USA and Israel (Spolaore et al, 2006). The US Company Mertek has 240 tonnes annual production of DHA oil from microalga *Crypthecodinium cohnii*. Similarly OmegaTech, also owned by Martek produce DHA oil (Spolaore et al., 2006). The list of companies producing microalgae as a source of nutraceuticals is given in Table 4.

1.4. Bioactive compounds from microalgae

The wide range of bioactive compounds produced by micro algae and their biological activity has been summarized in Table 5. They have been used as cheap and effective biocatalysts to obtain high added-value compounds including fine chemicals, vitamins, carotenoids, or polysaccharides (Holland, 1999; Harrigan and Goetz, 2002; Pulz and Gross, 2004). Microalgae such as *Phaeodactylum tricornutum, Isochrysis galbana, Crypthecodinium* sps., *Nannochloropsis* sps. are rich sources of polyunsaturated fatty acids (PUFA) - mainly Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA), (Apt and Behrens, 1999). DHA is important for proper

brain and eye development in infants and has been shown to support cardiovascular health in adults (Kroes et al, 2003).

Table 4. Nutraceutical products from microalgae.

Alga	Product	Activity	Company	Country
Crythecodinium	Docosahexaenoic acid (DHA)	Brain development	Martek/Omegatec	USA
Ulkenia	Docosahexaenoic Acid (DHA)	Treats brain, heart, mental disorder	Nutrinova/Celanese	Germany
Haematococcus	Astaxanthin	Treating carpal Tunnel syndrome	Cyanotec Corporation, USA	USA
Haematococcus	Astaxanthin	Anti-inflammatory, treats muscle soreness	Mera Pharmaceuticals Inc., USA	USA
Chlorella	Carbohydrate	Immune system	Ocean Nutrition	Canada
Odontella	Eicosapentaenoic acid (EPA)	Anti inflammatory	InnovalG	France
Spirulina	Vitamin B_{12}	Helps immune system	Panmol/Madaus	Austria
Rhodophyta (mix)	Biomass	Treats irritable bowel candidiasis	Rhodophyta (mix)	UK
Spirulina	Phycocyanin	Pigments	Parry Nutraceuticals Ltd., Chennai	India
Marine crustaceans	Astaxanthin	Eye health, Salmon fish	Parry Nutracetuicals Ltd., Chennai	India
Spirulina	Phycocyanin	Pigments	Hash Biotech Labs Pvt. Ltd.,Punjab.	India
Marine crustaceans	Astaxanthin	Eye health, Salmon fish	Hash Biotech Labs Pvt. Ltd.,Punjab.	India
Cyanobacteria	Pycocyanin	Color	Hash Biotech Labs Pvt. Ltd.,Punjab.	India
Spirulina	Allophycocyanin	Food color, Pharmacological	Hash Biotech Labs Pvt. Ltd.,Punjab.	India

(Adopted from Pulz and Gross, 2004)

Table 5. Bioactive compounds from microalgae and its biological activity.

Bioactive compound	Organism	Activity	Reference
Acetylated Sulfoglycolipids	*Oscillatoria raoi*	Antiviral	Reshef et al., 1997
Anabaenopeptin B	*Oscillatoria agardhii*	Protease inhibitor	Murakami et al., 1997c
Glycolipids	*Oscillatoria limnetica*	Antiviral (HIV-I)	Reshef et al., 1997
Glycolipids	*Oscillatoria trichoides*	Antiviral (HIV-I)	Loya et al., 1998
Oscillapeptin G	*Oscillatoria agardhii*	Tryrosinase inhibitor	Sano and kaya 1996
Aeruginosin 102 A Aeruginosein 102 B	*Microcystis virides*	Thrombin inhibitor	Matsuda et al., 1996
Aqueous extract	*Microcystis aeruginosa*	Antiviral (influenza A)	Nowotny et al., 1997
Kawaguchipeptin B	*Microcystis aeruginosa*	bactericide	Ishida et al., 1997c
		Algicide	Ikawa et al., 1996
Lipid	*Microcystis aeruginosa*	Leucin aminopeptidase	Ishida et al., 1997b
Microginin 299-A	*Microcystis aeruginosa*	inhibitor	
Microginin 299-B		Chymotrypsin	Murakami et al.,
Micropeptin 103	*Microcystis viridis*	inhibitor	1997a
Micropeptin 478-A	*Microcystis aeruginosa*	Plasmin inhibitor	Ishida et al., 1997a
Micropeptin 478-B	*Nostoc sps.*	Trypsin and thrombin	Pluotno and
Banyaside A and B		inhibitor	Cameli, 2005
Botophycin	*Nostoc linckia* *Nostoc spongeiae forme*	Cytotoxic	Singh et al., 2005
Cyanovirin N	*Nostoc ellipsosporum*	Antiviral (HIV-1)	Boyd et al., 1997
Cryptophycin	*Nostoc sp.* ATTCC 53789	Fungicide Cytotoxic	Singh et al., 2005
Nostopeptin A Nostopeptin B	*Nostoc minutum*	Elastase ihibitor	Okino et al., 1997
Microviridin	Nostoc minutum	Elastase ihibitor	Murakami et al., 1997b
Tenuecyclamides A-D	*Nostoc spongiaeforme*	Growth inhibitor	Banker and Carmeli, 1998
Hydrophilic extract Lipophilic extact	*Nostoc*	Antibacterial cytotoxic	Piccardi, 2000

Continuied

Nostocine A	*Nostoc spongiaeforme*	Cytotoxic Antiviral	Hirata et al., 2003
Calcium spirulan	*Spirulina platensis*	Anti-inflammatory	Hayashi et al.,
Phycocyanin	*Spirulina platensis*	Antioxidant	1996 Romay , 1999 Bhat and Madyastha, 2000
Aqueous extract	*Spirulina*	Hepato protective Antioxidant Antiproliferative	Vadiraja et al., 1998 Wu et al., 2005
Circinamide	*Anabaena criminalize*	Papain inhibitor	Shin et al., 1997
Dehydroradiosumi	*Anabaena cylindrica*	Trypsin inhibitor	Kodani et al.,
n Dendroamides	*Stigonema dendroideum*	Reversing multidrug resistance	1998 Ogino et al., 1988
Fisherellin-A	*Fisherella muscicola*	fungicide	Hagmann and Juttner, 1996
Lyngbyastatin 1	*Lyngbya majuscula*	Cytotoxic	Harrigan et al., 1998b
Nodulapeptin A Nodulapeptin B	*Nodularia spumigena*	Protracted toxic	Fujii et al., 1997
Phytoalexin	*Scytonema ocellatum*	Fungicide	Patterson and Bolis, 1997
Scyptolin	*Scytonema hofmanni*	Elastase inhibitor	Antonopoulou et al., 2005
Sulfolipids	*Phormidium tenue*	Antiviral (HIV-1)	Falch et al., 1995
Symplostatin 1	*Symploca hydnoides*	Cytostatic Antiviral	Harrigan et al.,
Polysaccharide	*Porphyridium*		1998a, Huheihel et al., 2002
Polyunsaturated	*Odontella aurita*	DHA-Postnatal brain	Makrides et al .,
fatty acids (PUFA)	Isochrysis galbana	Development	1995
Polyunsaturated	*Phaeodactylum*	EPA-prevention heart	Lebeau and
fatty acids (PUFA)	*tricornutum* *Crythecodinium*	disease Hypertriglyceridemia	Robert, 2003
		blood platelet aggregation	Singh et al., 2005
Curacin A	*Lyngbya majuscule*	Inhibitor of cell growth and mitosis	Burja et al., 2002
Polysaccharides	*Chlorella, Pyrenoidosa*	Immunomodulatory	Yang et al., 2006
Aqueous extract	*Chlorella vulgaris*	Antiviral, antibacterial	Hasegawa et al., 1995

1.5. Pigments

Coloration of food, with natural or synthetic color additives, should indicate good quality, assist marketing, and satisfy consumers (Clydesdale, 1993; Goyle and Gupta, 1998; Francis, 1985). Colorants are added to food matrices in specific technological steps in order to obtain and maintain the appropriate desired colors of food products. Colorants are also used to restore natural food colors lost by exposure to air, light temperature, moisture or improper storage conditions (Francis, 2000). Food colorants can also provide appropriate color to colorless foods; protect flavors and vitamins during storage or enchance the general appeal and nutritional value of foods. Color additives are classified as dyes (pigments) or lakes. Both types can be used as primary colors (pure colors without dilution) or secondary colors (blend primary colorants diluted with solvents or other additives). Dyes are commercial water soluble pigments, used as powders, granules, or liquids. The permission to use food colorants is bound to their safety and is strictly regulated by specific laws controlled at national and international levels. Individual country laws differ according to specific protocols, doses, and interpretations. The European Union (EU) has authorized 43 colorants as food additives and the United States (US) has authorized about 30.

1.5.1. Natural colors

Pigment as colorant in foods had always been of interest. This has influenced development of recipies & food processing. Added colours /pigments have been a major attractive proposition in processed food. The following aspects are of interest to food manufactures. 1. Food quality should be controlled by optical inspection, 2. Food processing steps may change food color, 3. colorants may be added to food as preservatives or simply to attract consumers. Food colorants can be conserved more or less during food processing. The pigments that color the original living biological material often possess essential functional properties like antioxidative effects, radical scavengers or are transmitters of signals or energy. In this way, intrinsic food colorants are involved in synergistic effects that they perform as components of molecular complexes. These supra molecular structures may, at least partly, be disturbed during food processing. Naturally occurring colourants not only impart attractive colouration to food but also have nutraceutical benefits. One group of natural colourants which has wide occurrence in nature is carotenoids. The role of carotenoids in human and animal health is widely recognized.

Among the sources of carotenoid, microalgal forms are being explored as rich source of carotenoids as shown in Fig. 2. Microalgal biotechnology has made significant progress for the production of biomass, mainly as a source of protein. It has been commercially produced for carotenoids like β-carotene, astaxanthin, lutein etc. The scientific knowledge of the beneficial role of carotenoids for prevention of specific diseases is rapidly gathering. Astaxanthin has gained importance in pharmaceutical, nutraceutical and pigmentation applications. Synthetic astaxanthin is the chief ingredient in the aquaculture feed which imparts the attractive red colour to salmon. *Haematococcus pluvialis* – a green alga is one of the natural sources known and its accumulate high amount of astaxanthin (2-3% w/w on dry weight basis) and its mono-diesters (70-75%). In view of this, *H. pluvialis* is selected as a suitable source for production of astaxanthin and its biological activities for the present investigation. The importance of *H. pluvialis* to produce astaxanthin as food colourant and nutritional supplements has not been fully exploited.

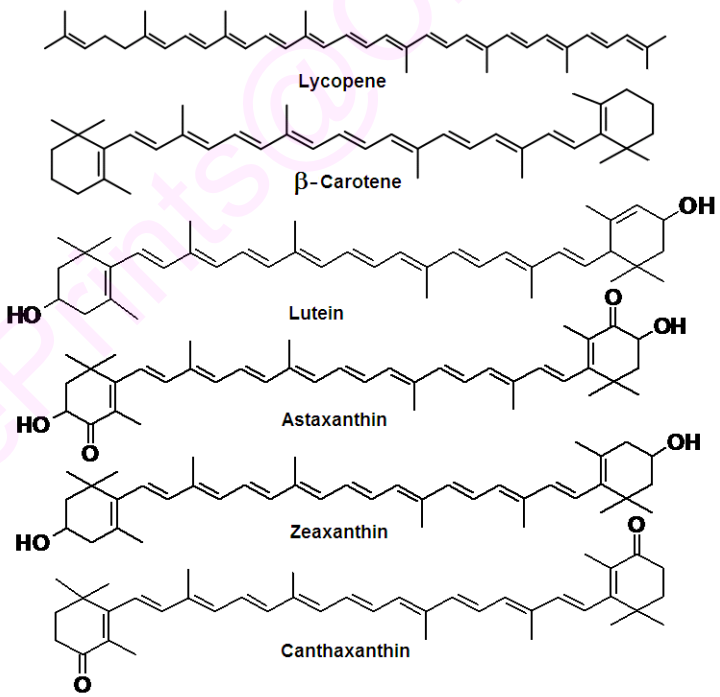


Fig. 2. Structure of major carotenoids in microalgae

1.5.2. Carotenoids

Carotenoids are natural pigments derived from five-carbon isoprene units that are polymerized enzymatically to form regular highly conjugated 40-carbon structures (with up to 15 conjugated double bonds). One or both ends of the carbon skeleton

may undergo cyclization to form ring β-ionone end groups, which additionally may be substituted by oxo, hydroxy or epoxy groups at different positions to form the different xanthophylls (Solomons and Bulux, 1994). At least 600 different carotenoids exercising important biological functions in bacteria, algae, plants and animals have been identified to date (Polivka and Sundström, 2004). Animals lack the ability to synthesize carotenoids and thus obtain these compounds via their diet. Carotenoids are essential constituents of the photosynthetic apparatus, primarily in the reaction centers of photosystems (or inserted in pigment–protein antenna complexes) where they act: (i) as accessory pigments for light-harvesting processes during photosynthesis, (ii) as structural stabilizers for protein assembly in photosystems, and (iii) as inhibitors of either photo- and free radical oxidation provoked by excess light exposure (Zhang et al., 1999). Several specific modifications of the basic structural moiety of carotenoids are found in natural algal carotenoids, including variations in the number of carbon atoms and the presence of unusual groups such as the allene groups and lactones found in peridinin (from marine dinoflagellates) and fucoxanthin (from coastal brown seaweeds, such as *Laminaria* sp.) (Pinto et al., 2003; Barros et al., 2001).

Carotenoids are organic pigments that are naturally occurring in the chloroplasts and in humans, four carotenoids (β-carotene, α-carotene, γ-carotene and β-cryptoxanthin) have vitamin A activity (meaning they can be converted to retinal), and these and other carotenoids can also act as antioxidants. In the eye, certain other carotenoids (lutein and zeaxanthin) apparently act directly to absorb damaging blue and near-ultraviolet light, in order to protect the macula lutea. Carotenoids are recognized worldwide for their unique biological characteristics. They are a group of molecules which can be found in most life forms and are responsible for diverse functions, ranging from their original evolutionary role as photosynthetic or lightquenching pigments to antioxidants, precursors of vitamin A, or pigments involved in the visual attraction of animals such as flower pollinators (Johnson and Schroeder, 1995). Carotenoids have been studied for many years because of their diverse roles in biological system. Britton (1995) has reported that carotenoids are not just "another group of natural pigments", they are substances with special and remarkable properties that form the basis of their many varied functions and actions in living organisms. The name 'carotene' was suggested by Wachenroder in 1831 for

the hydrocarbon pigment he had crystallized from carrot roots. Berzelius named the yellow pigments from autumn leaves as 'xanthophylls'. Many pigments of this class were separated by Tswett, who called the whole group 'carotenoids' (Olson and Krinsky, 1995). β-Carotene, α-carotene, lycopene, lutein and β-cryptoxanthin are the five most prominent carotenoids found in the human body (During and Harrison, 2004). In the human diet, plant food sources are the major contributors of carotenoids: carrots, squash, and dark-green leafy vegetables for β-carotene, carrots for α-carotene, tomatoes and watermelon for lycopene, kale, peas, spinach, and broccoli for lutein, and sweet red peppers, oranges and papaya for β-cryptoxanthin.

1.5.3. Carotenoids chemistry

Carotenoids are hydrocarbons containing C_{40} polyene chain backbones. The linear chain is cyclized at terminals, the molecules with hydrocarbon terminal are known as carotene and that of oxygenated terminals are known as xanthophylls (Goodwin, 1980). Those carotenes with at least half of the β-carotene molecules i.e. an unsaturated ionone ring having 11 carbon polyene side chain are classified as metabolic precursors of vitamin A. Most of the carotenoids can be described by the general formula $C_{40}H_{56}O_n$ where n is hydrocarbons (n=0) are termed as carotenes. The chemical feature of the carotenoid is a linear polyisoprenoid structure, a long conjugated chain of double bond and a near bilateral symmetry around the central double bond (Britton, 1995). Different carotenoids are derived essentially by modifications in the base structure by cyclization (i.e. formation of β- or ε-ionone rings) of the end groups and by introduction of oxygen groups giving them their characteristic colors and antioxidant properties (Rao and Rao, 2007). Majority are C_{40}-carotenoids and few bacterial carotenoids are with 30, 45 or 50 carbon atoms. In bacterial carotenoids, hydroxy groups at the ionone ring may be glycosylated or carry a glycoside fatty acid ester moiety. Furthermore, carotenoids with aromatic rings or acyclic structures with different polyene chains and typically 1-methoxy groups can be found. Typical fungal carotenoids possess 4-keto groups, may be monocyclic, or possess 13 conjugated double bonds. 3-hydroxy α- and β- as well as 5, 6- epoxy β-carotene derivatives are abundant in chloroplast of some algal groups and green plants.

Some reports also mention that the carotenoids, which possess hydroxy and carbonyl substitution on one or both of the molecule's end-groups, as xanthophylls,

e.g. astaxanthin, canthaxanthin, lutein, and zeaxanthin. The polyene chain and the other structural features influence the chemical properties (e.g., redox properties) of the carotenoids as well as their location and orientation within lipid bilayers in biological environments (El-Agamey et al., 2004). Carotenoids are known to exist in different geometric forms; *cis* and *trans* isomers. These isomers may be interconverted by light, thermal energy or chemical reaction; for example cooking of vegetable promotes isomerization of carotenoids from the *trans* to the *cis* form. β-carotene, with nine double bonds in its polyene chain that are free to assume *cis/trans* configurations, can theoretically form 272 isomers whereas its asymmetric isomer, α -carotene, can form 512. According to Olson and Krinsky (1995) synthetic β-carotene is almost entirely in the *trans*-isomeric form. The total possible number of compounds in the class, including all possible isomers, easily exceeds 200,000. Isomer specific biological functions clearly exist for carotenoids (Rock, 1997). The most popular industrial methods are given below. Badische Aniline and Soda-Fabric (BASF) synthesis. Here β-retinylriphenly phosphonium chloride and retinal were made to reach using KOH and ethanol form β-carotene (Fig. 3)

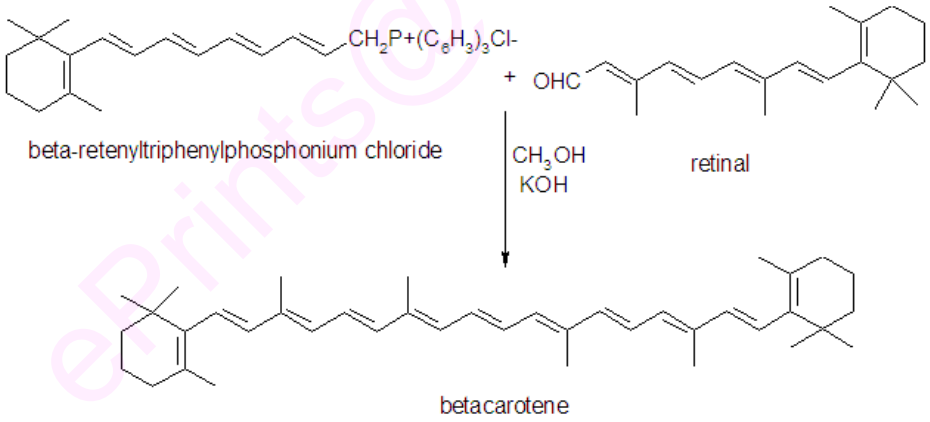


Fig. 3. Badische Aniline and Soda-Fabric (BASF) synthesis of β-carotene

Roche synthesis: This is based on Grignard reaction. In this method two molecules of β-C_{19} aldehyde are made to react using acetylene dimagnesium bromide to form C_{40} diol, which further converts to 15-15` didehydro-β-carotene, they will be converting to 15` β-carotene and all-trans β-carotene which takes very few seconds (Fig. 4).

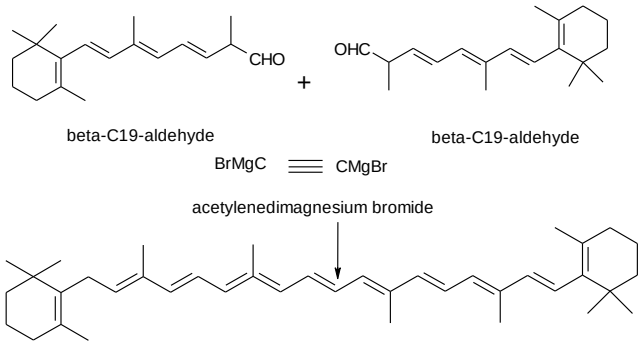


Fig. 4. Roche synthesis of β-carotene by Grignard reaction

1.5.4. Carotenoids as natural food colourants

The carotenoids are the most widely distributed group of pigments, occur naturally in large quantities, and are known for their structural diversity and various functions (Rodriguez et al., 2004). The carotenoids constitute a widespread class of natural pigments that occur in all three domains of life in the eubacteria, the archea, and the eucarya (Britton, 2006). Carotenoid are ubiquitous organic molecules, but they are not produced by the human body. They have been found to be essential to human health based on the nutritional understanding of vitamin A (retinol) and β-carotene (Otley, 2006). Carotenoids may also lend additional health benefits that may possibly reduce the risk of certain types of chronic diseases such as cancer and heart disease (Chaudhry, 2003). Carotenoids are also important natural source of orange, yellow, and red food coloring for the food and beverage industries (Otles and Atl, 1997). Carotenoids are lipid soluble pigments responsible for many of the brilliant red, orange, and yellow colors in edible fruits, vegetables, fungi, flowers and also in birds, insects, crustaceans, and trout (Linden and Lorient, 1999; Goodwin, 1980, 1982, 1992).

Synthetic food dyes are produced as fine powders or granules. Powders present the advantages of easy dissolution or incorporation in dry mixes but raise problems of dusting or clumping during manipulation. Genotoxicity and carcinogenicity of synthetic food colours, mainly azo dyes, has been documented by Combes and Haveland-Smith (1982). In animal model, the DNA damage induced by azo dyes has been reported by Tsuda et al., (2001). Because of the adverse effect of synthetic food colours, the current research is being focused on the natural food colours. Carotenoids are responsible for many of the brilliant red, orange and yellow

colour of edible fruits and vegetables. Carrot extract and red palm oil – rich in carotenoids have been widely used as colouring agents mainly to colour fats and margarine. Water-soluble forms of carotenoids are suitable for colouring of sugar confectioneries like candies, toppings, icings, fruit gums, fruit drops etc. An aqueous dispersion of carotenoids in large amounts of dextrin or sugars can be applied to colour breakfast cereals and dried infant food preparations (Pattnaik et al., 1997). The possible role of carotenoids and their metabolites in disease prevention is far from fully understood, because the bioavailability of carotenoids are complicated by multiple factors that affect their absorption, breakdown, transport, and storage (Yeum and Russel, 2002).

1.5.5. Carotenoids Pharmacology

The efficiency of absorption of carotenoids is highly variable. The carotenoids are in absorbed from in the intestine with the aid of dietary fat and incorporated into chylomicrons for transport in the serum (Rapola et al., 1998). The efficiency of absorption of β-carotene from β-carotene containing nutritional supplements can be as high as 70% or more. In foods, β-carotene exists either as a solution in oil or as part of a matrix within the vegetable or fruit. For example, in carrots, β-carotene exists in a complex matrix, comprised of indigestible polysaccharides, digestible polysaccharides and protein.

1.5.6. Carotenoids absorption

More than 600 carotenoids have been isolated from natural sources, but only about 60 have been detected in the human diet about 20 in human blood and tissues. β-carotene, α-carotene, lycopene, lutein and β-cryptoxanthin are the five most prominent carotenoids present in the human body. The internal absorption of carotenoids involves several crucial steps (1) the release of carotenoids from the food matrix, (2) the solubilization of carotenoids into mixed lipid micelles in the lumen, (3) the cellular uptake of carotenoids by intestinal mucosal cells, (4) the incorporation of carotenoids into chylomicrons, and (5) the secretion of carotenoids and their metabolites associated with chylomicrons into the lymphocytes. Absorption and metabolism vary to great extent in animal species. In case of humans, carotenoids can be absorbed intact by mucosal cells and subsequently appear unchanged in circulation and peripheral tissues (Bowen et al., 1993). In some of the rodent and animals β-carotene and other provitamin-A carotenoids are metabolized into vitamin A in

intestinal mucosal cells. If not it will not be absorbed in the body hence plasma concentration is very low and not comparable with that of human beings. Different proportions of carotenoid present in food and supplements are taken up by intestinal cells and are metabolized to vitamin A in the process of absorption in humans. Absorption of carotenoids takes place in the intestinal mucosa and uptake of these compounds by duodenal mucosal cells appears to be by passive diffusion, with a concentration difference between mixed micelle and that of cell membrane and this decides the rate of diffusion (Parker, 1996). After passive diffusion into enterocytes, unmetabolized carotenoids are incorporated into chylomicron and secreted by the lymph, followed by uptake by liver and release into circulation in association with very low density lipoproteins (VLDL) and ultimately in association with low density lipoptroteins (LDL) (Erdman et al., 1993). Generally two peaks are observed in plasma analysis of astaxanthin first one for the chylomicron associated astaxanthin and later for the astaxanthin associated with LDL. It is hypothesized that carotenoids may bind to cytosolic protein and this may play a role in the intracellular transport of these compounds in intestine or liver (Rock, 1997). The absorption of carotenoids in general are as shown in Fig. 5.

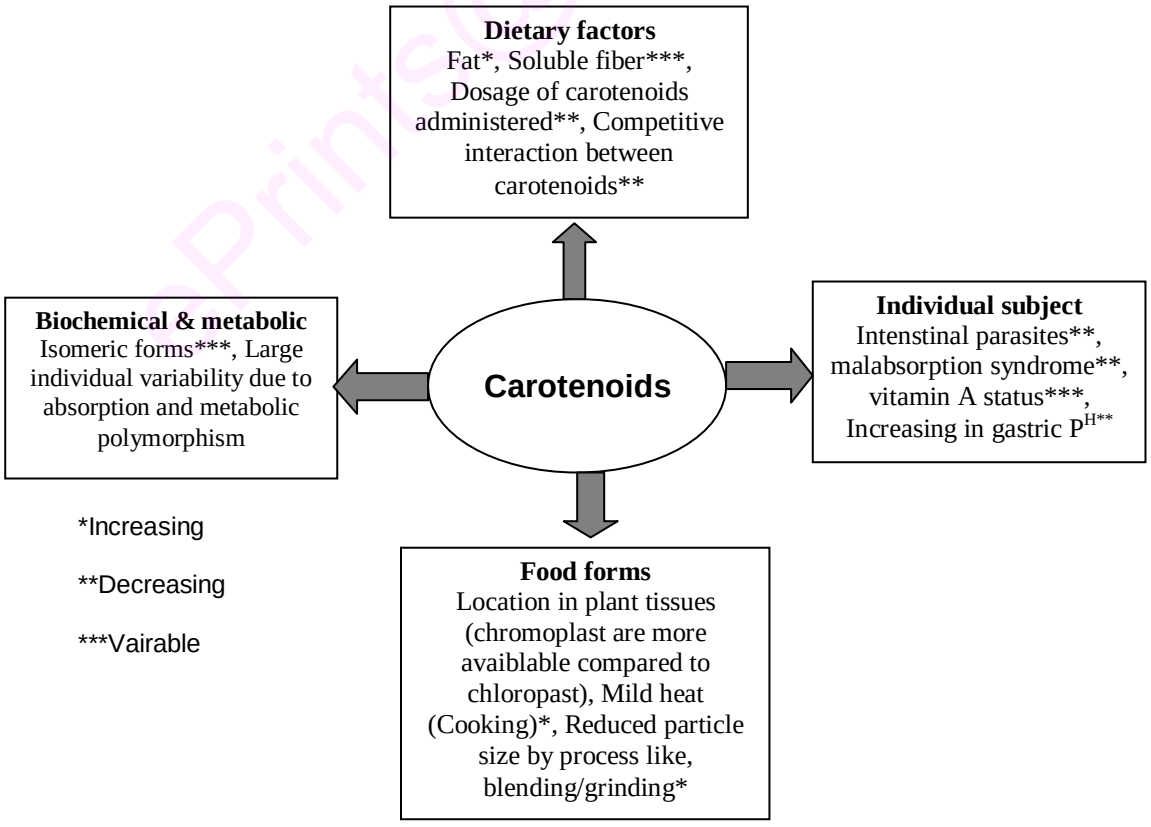


Fig. 5. Factors determing the absorption of carotenoids in humans

1.5.7. Carotenoids for health benefits

For human nutritional purposes, some carotenoids offer provitamin A activity (Mayne, 1996). Provitamin A carotenoids are generally converted to retinal via catalysis by the intestinal enzyme β-carotene 15, 15′-monooxygenase (Lindqvist and Andersson, 2002). Vitamin A deficiency is a problem that has prevailed in developing countries during the last decades. In the1990s, vitamin A deficiency has caused approximately 1.2 million deaths per year in children aged 1–4 years worldwide (Humphrey et al., 1992). Due to an assumed favorable correlation between a high intake of carotenoids and health benefits, the accepted pattern of a healthy meal includes the daily intake of at least five portions of fresh fruit and vegetables, providing about 6 mg of carotenoids. Sources of dietary carotenoids in humans include seafood, pink fleshed fishes (such as salmon and trout), fruits and vegetables, in particular watercress (16.6 μg carotenoids/g fresh weight and10.7 μg lutein/g fresh weight) and carrots (14.7 μg carotenoids/g fresh weight and 10.8 μg β-carotene/g fresh weight). Carotenoids directly provide photoprotection against UV light photooxidation in the skin (Sies and Stahl, 2004; Tapiero et al., 2004, Aust et al., 2005), while β-carotene was also shown to modulate UVA induced gene expression in human keratinocytes (Wertz et al., 2004, 2005). The ketocarotenoid astaxanthin is believed to play a key role in the amelioration/prevention of several human pathological processes, such as skin UV-mediated photooxidation, inflammation, prostate and mammary carcinogenesis, ulcers due to *Helicobacter pylori* infection and age-related diseases (Bennedsen et al., 1999; Guerin et al., 2002). Among the benefits of carotenoids to eye health, the occurrence of age-related macular degeneration (AMD) is strongly associated with lower levels of both zeaxanthin and lutein (xanthophylls) in the macula, while prospective epidemiological data showed a 19% lower risk of cataract in men taking high levels of both of these xanthophylls. In this context, zeaxanthin and lutein are the major carotenoids that accumulate in the macula of human retina and inhibit photo oxidative damage to the retina (Neelam et al., 2005). Many of the positive medical and nutritional trials have speculated that the antioxidant activity of carotenoids could be the key factor in reducing the incidence of many diseases, especially those suggestively mediated by light (Cantrell et al., 2003; Astley et al., 2004). Although there is a considerable epidemiological evidence linking high dietary intake of carotenoids to a decrease risk of certain cancers (such as lycopene against prostate cancer),controversy reigns in scientific discussions of the

health benefits provided by extra inputs of carotenoids, especially via supplementation schedules employing synthetic compounds.

1.5.8. Carotenoids metabolism

In intestinal cells, carotenoids can be incorporated into chylomicrons as intact molecules or metabolized into mainly retinol (vitamin A), but also in retinoic acid and apocarotenals. The polar metabolites are directly secreted into the blood stream *via* the portal vein. Within intestinal cells, retinol can be also esterified into retinylesters. Both intact carotenoids and their polar metabolites (retinyl esters) are secreted into the lymphatic system associated with chylomicrons. In the blood circulation, chylomicron particles undergo lipolysis, catalyzed by a lipoprotein lipase, resulting in the formation of chylomicron remnants that are quickly taken up by the liver. In the liver, the remnant associated carotenoid can be either (1) metabolized into vitamin A and other metabolites, (2) sortred, (3) secreted with the bile, or (4) repackaged and released with VLDL particles. In the bloodstream, VLDLs are transformed to LDLs, and then HDLs by delipidation and the carotenoids associated with the lipoprotein particles are finally distributed to extrahepatic tissues. Time course studies focusing on carotenoid appearances in different lipoprotein fractions after ingestion showed that chylomicron carotenoid levels peak early whereas LDL and HDL carotenoid levels reach peaks later. Representation of vitamin A absorption, digestion, transport to the liver and deliver to target tissues as shown in **Fig. 6.**

1.5.9. Carotenoids storage

Adipose tissue and liver are the major tissue storage depots for the carotenoids, although carotenoid traces have been found in lungs, kidney, cervix, prostate and other tissues (Schmitz et al., 1991). High concentration of carotenoids are found in tissues rich in LDL receptors, like corpus luteum, adrenal tissue, testis probably resulting from non specific in takeoff lipoproteins. Some of the carotenoids are specific in absorption, like macula of eye is rich in lutein and zeaxanthin, but not in other carotenoids (Handelman et al., 1998). Some of the concentration is found in pineal gland but not in case of brain stem (Olson, 1994). Difference in the distribution is also seen with respect to isomeric forms. In general, amount of *trans* exceeds that of *cis* in case of circulation in plasma, whereas increase in the proportion of *cis* β-carotene is observed in peripheral tissue (Stahl and Sies, 1993). In general carotenoids tissue content is directly proportional to dietary intake. Hence carotenoids are used as biomarkers for assessing intake of vegetables and fruits. The concentration

of carotenoids will also increase in peripheral tissue on high intake a part from plasma. Deposition of carotenoids in stratum corneum of epidermis occurs with repeated high dose of carotenoids leading to yellowing of skin and the condition is referred as carotenodermia. Average resident time of β-carotene is 51 days as revealed from human isotope tracer study (Nowotny et al., 1995).

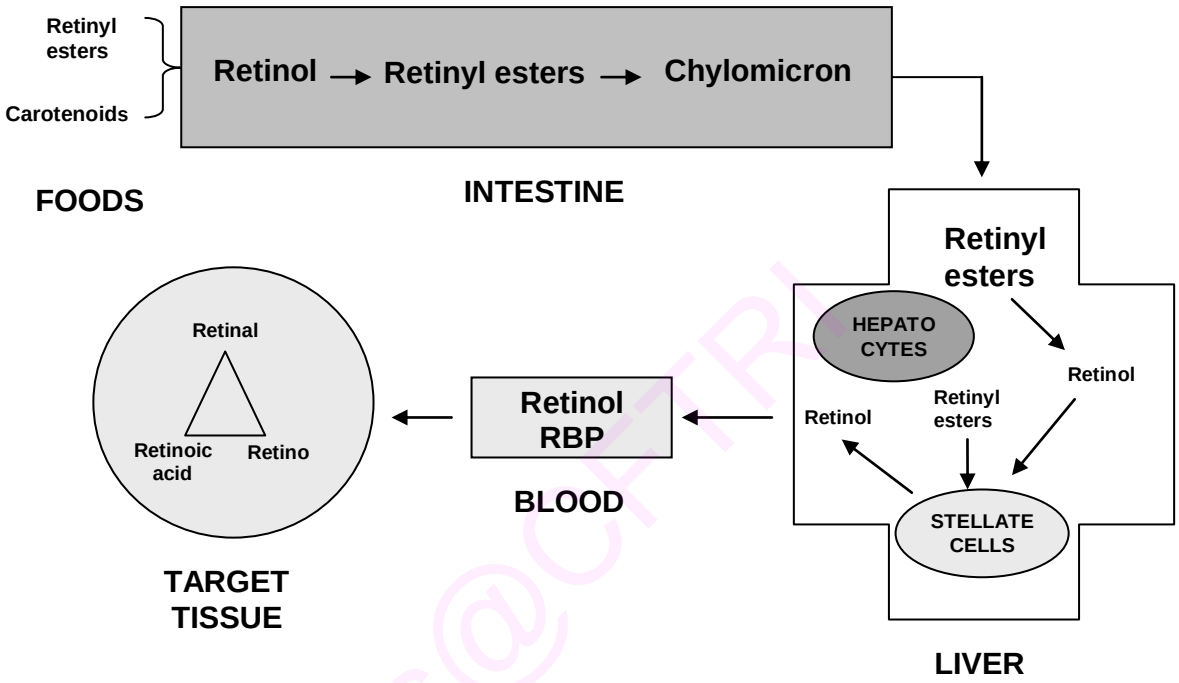


Fig. 6. Schematic representation of vitamin A absorption, digestion, transport to the liver and deliver to target tissues.

1.5.10. Transport and tissue distribution of carotenoids

In fasting human serum, the hydrocarbon carotenes (β-carotene and lycopene) are found primarily in LDL, while the xanthophylls lutein, zeaxanhin and β-cryptoxanthin are more evenly distributed between LDLs and HDLs (Krinsky et al., 2005; Clevidence and Bieri, 1993). The xanthophylls are primarily located at the surfaces of lipoprotein particles. Making them more likely to exchange between plasma lipoproteins. This hypothesis may explain their equal distribution between LDLs and HDLs. In humans, carotenoids were reported in liver, adrenals, tests, kidneys, lungs, skin, eyes, and adipose tissues. Adipose tissue seems to be the main storage site together with the liver accounting for at least 80% of carotenoid storage (Kaplan et al., 1990). It was suggested that the tissue distribution of carotenoids may correlate with the LDL uptake in tissues expressing LDL receptors at their faces (Parker, 1989),

but this does not explain why some tissues show marked enrichment in specific carotenoids, i.e. the human macula accumulates specifically the two xanthophylls, lutein and zeaxanthin.

1.5.11. Carotenoids biological activity

Carotenoids are known for their biological activity due to their capacity to transfer energy in photosynthesis and photoprotection (Krinsky 1994b). Carotenoids play vital role in photosynthesis in association with chlorophyll. The major functions of carotenoids include accessory pigments in light harvesting system and as photoprotective agents against oxidative damages. Based on epidemiological studies, a positive link is suggested between higher dietary intake and tissue concentrations of carotenoids and lower risk of chronic diseases (Johnson, 2002; Rao and Agarwal, 2000). β-carotene and lycopene have been shown to be inversely related to the risk of cardiovascular diseases and certain cancers whereas lutein and zeaxanthin to the disorders related to the eye (Ribaya- Mercado and Blumberg, 2004). The antioxidant properties of carotenoids have been suggested as being the main mechanism by which they afford their beneficial effects. Recent studies are also showing that carotenoids may mediate their effects via other mechanisms such as gap junction communication, cell growth regulation, modulating gene expression, immune response and as modulators of phase I and II drug metabolizing enzymes (Paiva and Russell, 1999; Astrog, 1997; Jewell and brien, 1999; Bertram, 1999). However, carotenoids such as α, β-carotene and β-cryptoxanthin have the added advantage of being able to be converted to Vitamin A and its related role in the development and disease prevention. The role of carotenoids in the prevention of diseases and their biological actions are summarized in Fig. 7.

1.5.11.1. Carotenoids bioavailability

Bioavailability is defined as the fraction of the ingested pigment that is absorbed and is available in the blood stream for its utilization in normal physiological function or for storage (Jackson, 1997). The term bioavailability thus covers several *in vivo* processes. 1. Release of the pigment from the food matrix and its solubilization in the gut bioaccessibility, 2. Uptake of the carotenoids by the intestinal cell followed by its secretion into the blood circulation (absorption), 3. Circulation of the pigment in the blood stream and its deliver to target tissues where it is stored and utilized for its biological activities. It was noted that at steady state plasma carotenoids amount to

approximately 1% of the total body content of carotenoids, whereas the highest concentration of β-carotene was found in the liver (Schmitz et al., 1991).

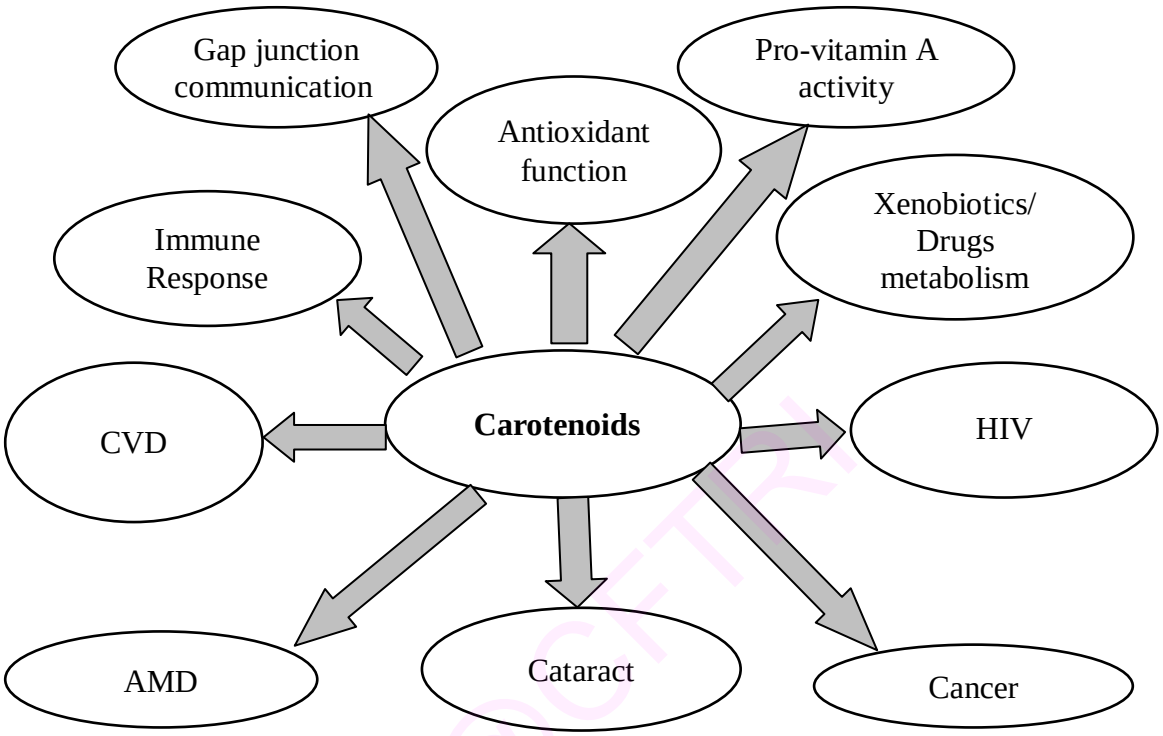


Fig. 7. Biological actions of carotenoids

1.5.11.2. Methods to determine bioavailability of carotenoids

Bioavailability of carotenoids are reviewed by Yeum and Russell (2002). Some of the methods include serum/plasma response after carotenoids ingestion (Rock and Swendseid, 1992). Chylomicron response after carotenoids ingestion (Johnson and Russell, 1992; Van Vliet et al, 1995), Oral Fecal balance Technique (Yeum and Russell, 2002), stable isotope applications (Blomstrand and Werner, 1967; Goodman et al., 1969) and macular pigment Density measurement (Yeum and Russell, 2002).

1.5.11.3. Plasma/serum response after carotenoid ingestion

The bioavailability of carotenoids is determined by measuring its plasma/serum concentration at different times after single or long term ingestion of the compound form supplements or food sources. A plasma concentration *vs* time plot is generated, from which is determined the area under the curve (AUC) value used as an indicator of the absorption of the compound. This method provides an estimate of relative bioavailability using simple procedure. Carotenoids are ingested and changes in serum concentration of carotenoids are measured at various time intervals following

ingestion (Parvin et al., 2000). Serum response curves are drawn using either single or repeated doses.

1.5.11.4. Safety and toxicity

United State Food and Drug Administration has declared β-carotene as generally recognized as safe for the consumption in 1979, as food colorant and dietary supplement (Diplock, 1995). This was supported by various studies like, mutagencity, embryotoxicity, teratogenecity of carotenoids (Bendich, 1988). Except in case of α, β-carotene and CARET (Carotene and retinol efficiency trail) where they found that, β-carotene is associated with increased risk of lung cancer in heavy smokers, it was considered to be completely safe in humans. Exact mechanism of increasing that risk of carcinogensis associated with smoking and β-carotene ingestion is not known (Erdman et al., 1996). Excess consumption by β -carotene or other provitamin-A carotenoids will not result in hypervitaminosis A as it is converted on demand of the body. But yellowing of skin or hyper carotenemia occurs with high dose of β-carotene for long period of time, and this will spontaneously reduce upon decrease in the carotenoids dose. Based on these reports no side effect has been observed in human subjects.

1.6. *Haematococcus species*

There are 15 *Haematococcus* species such as *H. pluvialis, H. allmani, H. buetschlii, H. capensis, H. carocellus, H. droebakensis var, H. droebakensis, H. grevillei, H. insignis, H. lacustris, H. murorum, H. salinus, H. sanguineus, H thermalis, H. zimbabwiensis* reported in the database. However seven species *H. pluvialis, H. buetschlii, H. capensis, H. carocellus, H. droebakensis, H thermalis, H. zimbabwiensis* have been currently accepted taxonomically. The five species, *H. pluvialis*, *H. buetschlii*, *H. capensis*, *H. droebakensis*, and *H. zimbabwiensis* are found in fresh water habitats. The green alga *Haematococcus pluvialis,* among the biological sources, has a high concentration of ketocarotenoid astaxanthin, up to 2.0-3.0% w/w on dry weight basis (Lorenz and Cysewski, 2000; Yuan and Chen, 1998).

1.6.1. Taxonomic classification

Phylum: Chlorophyta, Class: Chlorophyceae, Order: Volvocales,

Family: Haematococcaceae, Genus: *Haematococcus*, Species: *pluvialis*

1.6.2. Introduction

Haematococcus pluvialis is unicellular green alga that are commonly found in freshwater bodies. When the green cells of *Haematococcus pluvialis* experience environmental stress, such as high light intensity or phosphate deprivation, they differentiate from a vegetative stage to form aplanospores in a resting stage. At this point, the cell volume increases, producing a hard cellular wall that accumulates the red coloured xanthophyll, astaxanthin and its fatty ester derivatives. (Boussiba et al., 1992; Cohen et al., 2000, 2002). *Haematococcus* is reported to biosynthesize astaxanthin to protect itself against UV induced cellular damage (Kobayashi et al., 1992). Astaxanthin has recently been shown to have potential in reducing skin DNA damage and also to reduce time dependant sun burn in humans (Cyanotech, 2002). It has also been shown to be a powerful anti oxidant (Naguib, 2000). *Haematococcus* has been approved as a food supplement for use in fish farmed salmon, and the presence of this alga in the food chain, via salmon, is therefore clearly established. Consequently, since the mid 1990's, many companies have begun to market, worldwide the use of naturally derived astaxanthin as a dietary supplement for human use. Such companies include Mera Pharmaceuticals, Soft Gel Technologies, Inc., La Haye Laboratories and Cyanotech in the USA, Itano in Japan and Britannia Health in the UK. Since *Haematococcus pluvialis* is known to contain high amounts of astaxanthin, many of these companies are selling the dried form, or an extract of the algae to consumers who are interested in ingesting this natural product for their potential long term health benefits, rather than obtaining astaxanthin by consuming salmon meals on a daily basis. Astaxanthin biosynthetic pathway and life cylcle of *H. pluvilais* as shown in Fig. 8 & 9.

1.6.3. Origin and occurrence of astaxanthin

Astaxanthin (3, 3'-dihydroxy- β, β'-carotene-4, 4'-dione) is a naturally occurring carotenoid that is found in various organisms such as salmon, trout, lobsters and shrimps reported by Maher (2000) as shown in Table 6. Astaxanthin is responsible for the pink coloration in the flesh of these species (Turujman et al., 1997) and is not synthesized *de novo* in salmonoids, but is entirely obtained from their diet (Foss *et al.*, 1984). Astaxanthin is also found in the yeast (*Phaffia rhodozyma*) and the micro algae *Haematococcus pluvialis* and salmonoids obtain astaxanthin principally from ingestion of krill and micro algae (Grung, 1992).

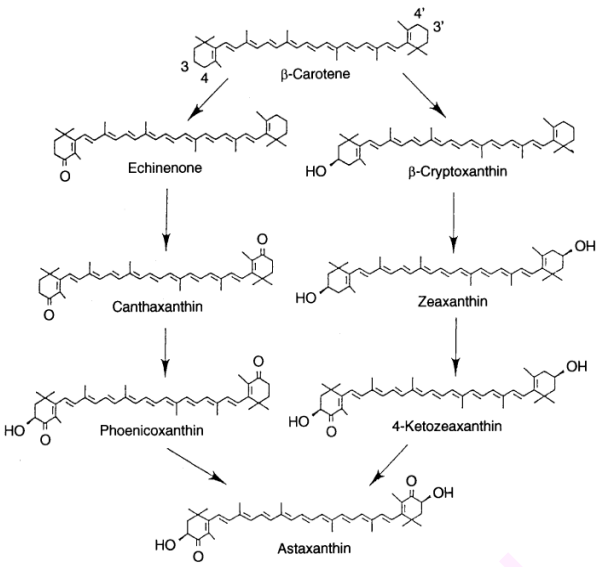


Fig. 8. Astaxanthin from *Haematococcus pluvialis* biosynthesis (adapted from Fraser et al., 1997).

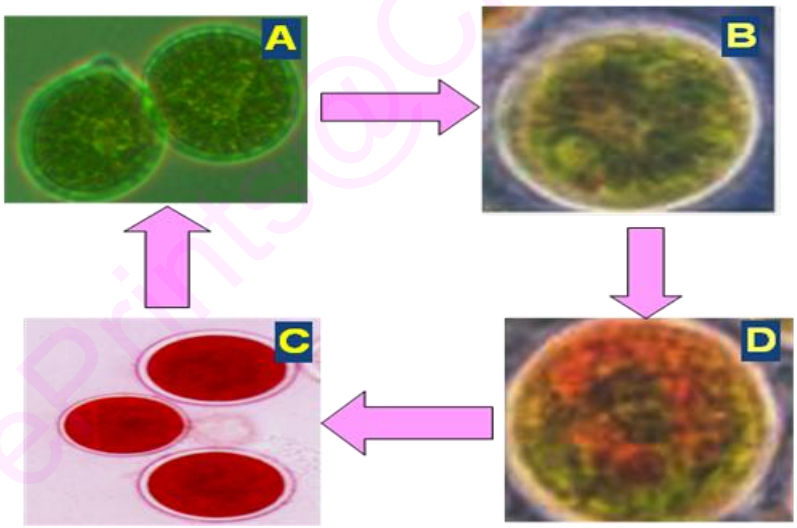


Fig. 9. Life cycle of *H. pluvialis*. A. Green motile vegetative cells, B. Intermediate non-motile cells, C. Intermediate carotenoid accumulating cells, D. Carotenoid rich encysted cells

1.6.4. Chemistry of astaxanthin

Astaxanthin was first chemically identified by Kuhn and Sorenson (1983). It is a complex molecule and the synthesis, being difficult, results in an expensive product costing approximately US $2,000 (Olaizola, 2003). Astaxanthin is derived from lycopene and it contains two terminal ring systems joined by a chain of conjugated double bonds or polyene system. This molecule has two asymmetric carbons located

at the 3, 3' positions of the benzenoid rings with hydroxyl group (-OH) and at 4, 4′ positions with keto (=O) group on the either end of the molecule. Astaxanthin can exist in three configurational isomers two enantiomers (3S, 3′S and 3R, 3′R) and a meso form (3R, 3′S) (Fig. 10) (Higuera-Ciapara et al., 2006). From all these isomers, the 3S, 3'S is the most abundant in nature. Astaxanthin producing organisms, including *Haematococcus,* synthesize the (3S,3′S)-isomer, yeast *Xanthophyllomyces dendrorhous* produces the opposite isomer having the (3R,3′R)-configuration (Visser et al., 2003). Synthetic astaxanthin consists of a racemic mixture of the two enantiomers and the meso form - 1:2:1 of isomers of (3S, 3′S) (3R, 3′S) and (3R, 3′R) respectively. Depending on their origin, astaxanthin can be found in association with other compounds such as fatty acid. Thus the mono or diesters of astaxanthin with fatty acids such as palmitic, oleic, linoleic etc. in one or both hydroxyl groups may be found. It may also be found free with the hydroxyl groups without being esterified. Synthetic astaxanthin is in free form, microalga is mixture of mono-diesters and free form as shown in Fig. 11.

Table 6. Astaxanthin from different sources

Oganism	Content	Reference
Green algae		
Haematococcus pluvialis	2-3%	Lorenz and Cysewski, 2000
Neochloris wimmeri	0.6	Orosa et al., 2000
Chlorococcum	<0.2	Zhang et al., 1997
Namochloropsis gaditana	<0.3	Lubian et al., 2000
Scenedesmus vacuolatus	0.01	Orosa et al., 2000
Chlorella zofingiensis	<0.01	Ip and Chen, 2005
Chlamydomonas nivalis	0.04	Bidigare et al., 1993
Fungi		
Xanthophyllomyces dendrorhous	0.4	Jacobson et al., 2000
Yeast-*Candida utilis*	0.04	Miura et al., 1998
Bacteria		
Mycobacterium lacticola	0.003	Simpson et al., 1981
Agrobacterium aurantiacum	0.01	Yokoyama et al., 1995
Paracoccus carotinifaciens	Not reported	Tsubokura et al., 1999
Brevibacterium sp	0.003	Neils and Leenheer, 1991
Animals		
Shrimp-*Pandalus clarkii*	0.015	Meyers and Bligh, 1981
Shrimp-*Pandalus borealis*	0.014	Shahidi and Synowiecki, 1991
Backs snow crab *Chinoecetes opilio*	0.011	Shahidi and Synowiecki, 1991

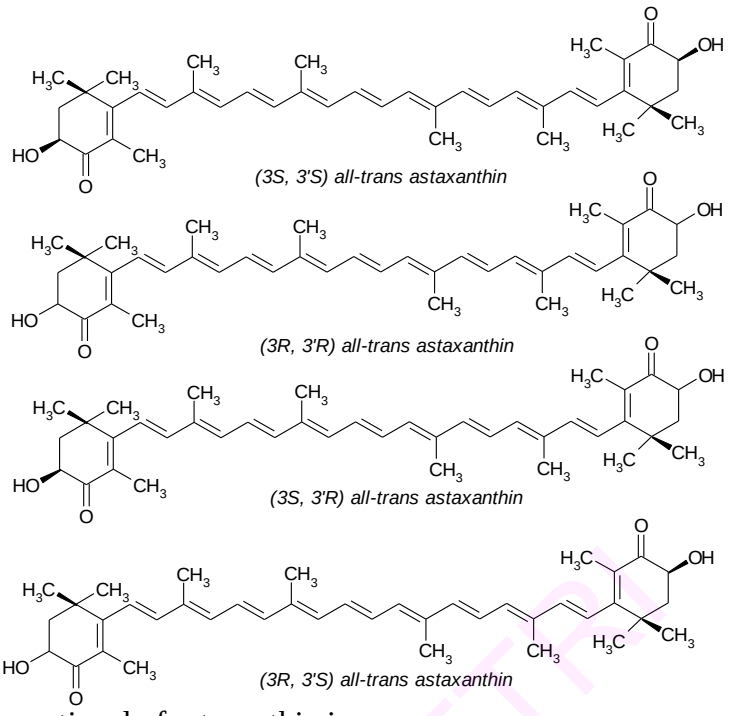


Fig. 10. Configurational of astaxanthin isomers.

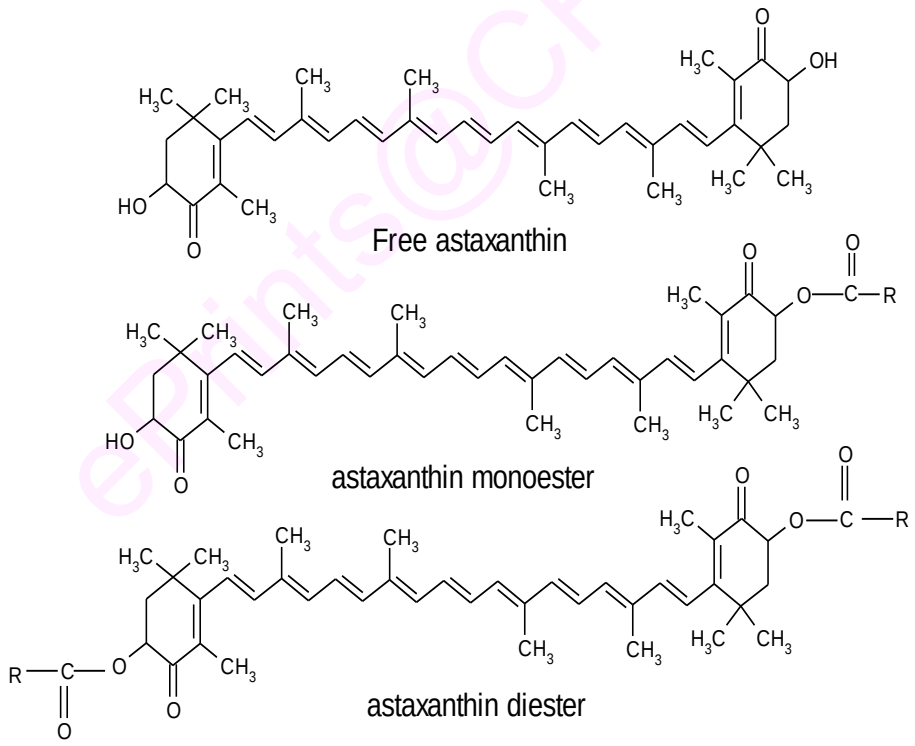


Fig. 11. Free astaxanthin, mono-diester forms of astaxanthin, R= saturated or unsaturated alkylchains), Adopted from Miao et al., (2006).

1.6.5. Production of astaxanthin

The commercial production of cultured *Haematococcus pluvialis* is undertaken by several companies round the world**.** Cyanotech and Mera pharmaceuticals in North America cultivate the algae using an open pond system, the Japanese firm Fuji Chemical Industries Ltd. Japan has an indoor facility in Sweden and dome shaped bioreactors in Hawaii, while the Israeli company, ALGA technologies uses solar powered photobioreactors in a closed, strictly controlled system (Wiener et al., 2003). All these companies harvest the algae at maximal astaxanthin concentration, the so called red stage of cultivation, before crushing (to increase bioavailability) and drying the material for production or extraction. Astaxanthin accumulation in *Haematococcus* is induced under stressful growth conditions. Thus, producers that use large-scale, outdoor, systems have adopted a two stage strategy whereby the first stage consists in growing *Haematococcus* biomass under conditions conductive to fast growth in enclosed photobioreactors followed by a second stage in which carotenogenesis is induced by changing the cells' environment to stress promoting conditions. *Haematococcus* astaxanthin can be produced indoors mixotrophically. The astaxanthin-rich cells are easily harvested by settling and centrifugation. Then, the cell biomass is cracked (to increase astaxanthin bioavailability) and dried. Finally, the dried product can be directly encapsulated or the astaxanthin extracted can be included in nutraceutical formulations (Olaizola and Huntley, 2003) Originally, *Haematococcus* astaxanthin producers attempted to enter the fish (especially salmon) feed market. However, price competition from synthetic astaxanthin (US $2000/kg) relegated *Haematococcus* astaxanthin producers to supply small, special markets. It is believed that present commercial producers cannot compete against synthetic astaxanthin on price alone. However, as production technology is optimized and production is transferred to lower cost locales, *Haematococcus* astaxanthin might compete against synthetic astaxanthin on price. Furthermore, and as the public becomes educated and demands natural pigmented salmon (and others) or regulations require the use of natural feed ingredients, *Haematococcus* astaxanthin could demand a premium price over synthetic astaxanthin, as has been the case in the vitamin E and β-carotene markets (Bahner, 1993a, 1993b)

1.7. Applications of astaxanthin

Astaxanthin is used for various applications such as nutraceutical, antioxidant, health, salmon and trout feeds and for human helath as shownin Fig. 12.

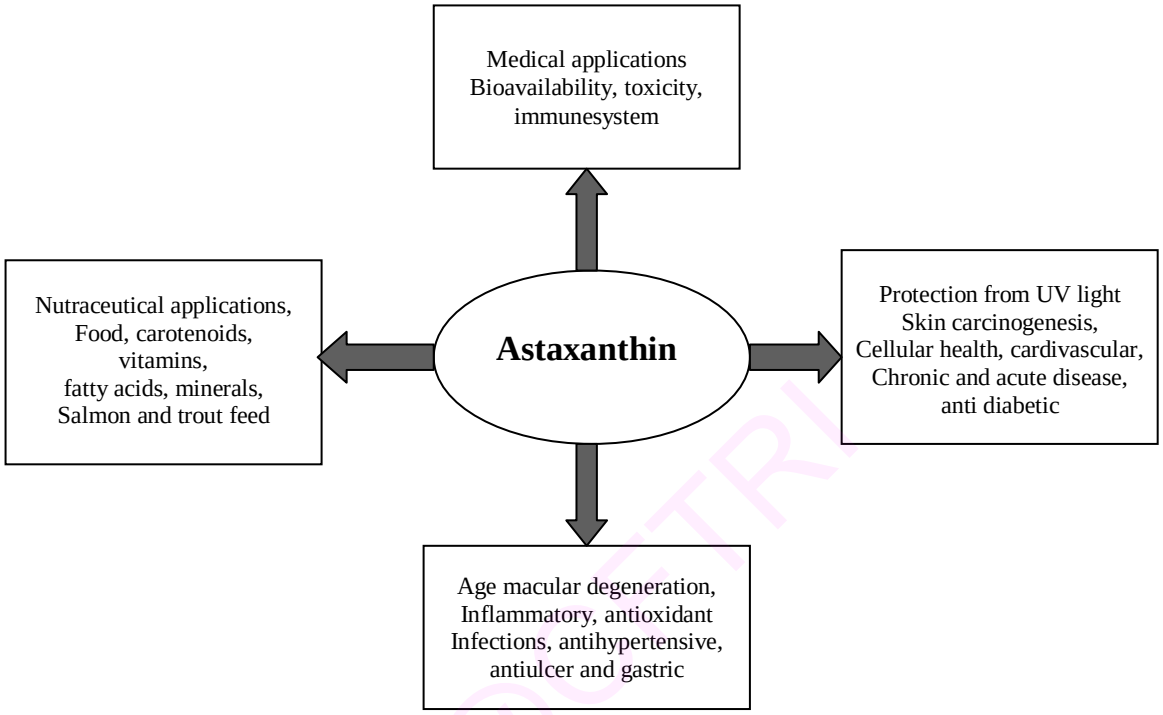


Fig. 12. Applications of astaxanthin

1.7.1. Toxicity and human clinical trials of astaxanthin

The safety and toxicity studies were conducted on *Haematococcus* derived astaxanthin. Although some of this data has been published in peer reviewed journals, many have also been derived from company sponsored research provided by astaxanthin supplement manufacturers. Toxicity studies have done on *Haematococcus pluvialis* biomass. No toxic effects were reported for *Haematococcus* from the dosage levels studied. When this data is extrapolated to an equivalent dosage form in humans, it strongly suggests that up to 5g of astaxanthin (present in the form of *Haematococcus* algae), could be potentially safely ingested by humans. These studies further suggest that an intake of 5mg of *Haematococcus* derived astaxanthin is unlikely to produce any toxic effects in human trials.

1.7.2. Bioavailability of astaxanthin

The bioavailability studies have been conducted on astaxanthin, *Haematococcus* meal as shown in (Table 8). These studies suggest that astaxanthin is rapidly metabolized over a 24 hour period and that a daily intake of up to 5mg astaxanthin in a capsular

form would not result in any residual build up of this material in the body. The various steps of digestion, absorption and plasma transport of dietary carotenoids in mammals have been reviewed (Furr, and Clark, 1997). In the plasma, non-polar carotenoids such as β-carotene, α-carotene or lycopene, are mostly transported by very low density lipoproteins (VLDLs) and low density lipoproteins (LDLs) and polar carotenoids, such as zeaxanthin or lutein, are more likely to be transported by LDLs and high density lipoproteins (HDLs). The only study on humans to date confirmed the bioavailability of astaxanthin supplied in a single high dosage of 100 mg and its transport in the plasma by lipoproteins (Osterlie et al., 2000).

1.7.3. Human clinical studies of astaxanthin

Human clinical trials have been undertaken on astaxanthin of *Haematococcus pluvialis* meal. Two studies have been reported specifically on the carbon dioxide extract of *Haematococcus* algae. The study conducted by Chew at Washington State University, specifically used US Nutra's carbon dioxide extracted oleoresin. Chews study of US Nutra's oleoresin found no adverse effects on examination of the subjects who participated in the trial of US Nutra's oleoresin (Chew et al., 2003) confirmed that laboratory immune function tests showed no deleterious effects with astaxanthin supplementation in human subjects as shown in (Table 7 & 8).

Table 7. Bioavailability of astaxanthin.

Source of astaxanthin	Tested material	Astaxanthin content	Toxicology study	Reference
Not stated	100 mg		Transport in the plasma by lipoproteins in a similar way to other carotenoids max levels (1.3+/- 0.1mg/L) were reached after 6.7 hours after administration elimination half life was 21 +/- 11hr.	Osterlie et al., 1999a, Guerin et al., 2003.
Carophyll Pink (Hoffman – LaRoche) containing all E-, 92 and 132 – astaxanthin (3R, 3'R: 3R, 3'S:35, 3'Sratio 1:2:1)	100 mg	100 mg	3 human volunteers were given a single meal containing 100mg of astaxanthin max plasma concentrations of astaxanthin (1.24 mg/ml) were observed after 6 hours.	Osterlie, 1999b.
Algal meal from *Haematococcus* (Astacarotene)	100 mg astaxanthin per kg feed in the form of an algal meal	100 mg	24 rats were divided into 2 groups. 12 were given feed without algal meal, the other group received the feed containing algal meal. Astaxanthin was found in particular in thigh and heart muscle, with no adverse effects reported but endurance tests were increased in those rats taking the algal meal.	Lignell, 2001 US Patent 6, 245, 818

Table 8. Human clinical trials on astaxanthin from *Haematococcus pluvialis*

Source of astaxanthin	Tested material	Astaxanthin content	Equivalent to CO_2 extracted oleoresin	Reference
Mera pharmaceuticals *H. pluvialis* algal meal (contains up to 2% total astaxanthin) (around 5mg per 250mg of algal meal	228mg of algal meal	3.85mg	33 human volunteer's daily ingestion for 29 days. Medical examination (urine & blood analysis) did not result in any safety concerns.	astafactor technical report 1.
Mera pharmaceuticals *H. pluvialis* algal meal (contains up to 2% total astaxanthin) (around 5mg per 250 mg of algal meal)	1.14g of algal meal	19.25mg of astaxanthin	33 human volunteer's daily ingestion for 29 days. Medical examination (urine & blood analysis) did not result in any safety concerns.	astafactor technical report 1.
		14.4mg	13 healthy patients were divided into 3 groups and given 3 levels of astaxanthin daily for 2 weeks. Maximum dose being 14.4mg/day. No ill effects were reported.	Miki, 1998 cited in astafactor technical report 1.
Astacarotene *Haematococcus* algal meal	100mg astaxanthin per kg feed in the form of an algal meal	4mg	20 healthy volunteers were given a capsule containing 4mg of astaxanthin, against 20 healthy volunteers receiving a placebo. No adverse effects were reported from the study but improvements in endurance test were increased in the astaxanthin group.	Lignell, 2001 US Patent 6, 245, 818
CO_2 extract of *Haematococcus* Algae	109 g astaxanthin/kg of oleoresin concentrate	2 or 8mgs	An 8 week double blind placebo control trial to investigate the immune boosting effects of astaxanthin was carried out, with subjects taking 0, 2 or 8mg astaxanthin capsules, once a day. No adverse effects were reported from the study.	Chew et al., 2003
CO_2 extract of *Haematococcus* Algae		2mg of astaxanthin capsule	35 adults, randomized, double blind, placebo controlled trial of 8 weeks duration. Subjects ingested 3, 2mg capsule per day. No adverse effects were reported.	Spiller et al., 2003

1.7.4. Astaxanthin as anti chronic and acute agent

Commercial *Haematococcus* astaxanthin had several benefits from astaxanthin supplementation. Astaxanthin works against chronic and acute diseases. Users indicated all conditions from which they suffered, from a list of acute and chronic health conditions. They had observed improvements as a result of *Haematococcus* astaxanthin supplementation. They have compared efficacy of *Haematococcus* astaxanthin with well known anti-inflammatory drugs. The result of *Haematococcus* astaxanthin supplementation was observed in 85% of the health conditions reported (Table 9). Of the 26 comparisons with popular brands of prescription drugs, *Haematococcu*s astaxanthin supplementation was reported to be as effective as or more effective than the anti-inflammatory drugs in 92% of the comparisons. Of the 62 comparative studies with over-the-counter (OTC) drugs including aspirin or ibuprofen, astaxanthin supplementation had reported an effective or more effective in 76% of the comparisons

Table 9. Astaxanthin supplementation on chronic and acute health

Health condition	Improves conditions		
	Number of reports	Number	%
Sore muscles and joints	146	128	88
Back pain	48	42	88
Cholesterol	37	29	78
Osteoarthritis	20	19	95
Prostate	15	11	73
Asthma	13	11	85
Menstrual cramps	8	6	75
Rheumatoid arthritis	7	6	86
Diabetes	5	1	20
Macular degeneration	5	3	60
Sunburn	5	5	100
Post-surgery inflammation	4	4	100
Fibromyalgia	3	3	100
Gastritis	3	3	100
Gingivitis	3	2	67
Peptic ulcers	2	2	100
Prostatitis	2	2	100
Ulcerative colitis	2	0	0
Total	328	277	85

1.7.5. Astaxanthin as source of medical and nutraceutical applications

Medical researchers had shown that astaxanthin may have significant pharmaceutical applications. *In vitro* experiments, *in vivo* pre-clinical studies and early-stage clinical trials have clearly indicated the possibility that astaxanthin itself, or in conjunction with other components, behaves like a prophylactic and curing agent against various diseases and health conditions presented in (Table 10 & 11). Lignell et al., (2001) had conducted a double blind study on 40 young, healthy male students. Capsules containing 4 mg of astaxanthin in the form of *Haematococcus* algal meal (manufactured by Astacarotene AB, Sweden), were given daily over a six month period. Another study was done by Chew, an academic based at Washington State University, USA, using 0, 2, or 8mg astaxanthin (109 g astaxanthin/kg as the oleoresin concentrate from *Haematococcus pulvialis*) in a double blind placebo control study with fourteen subjects (Chew, 2003). A twenty one patient trial investigating the effects of a super critical carbon dioxide extract of *Haematococcous* algae on preventing sunburn, took capsules containing 4mg of astaxanthin over a two week period (Cyanotech, 2002). A recent 35 patient double blind placebo controlled trial of 8 weeks duration, consumed an astaxanthin rich extract of *Haematococcus pluvialis*, at an intake of 6mgs per day (Spiller, 2003). No adverse effects have been

reported in these clinical trials or by companies currently marketing astaxanthin as a dietary supplement in the USA or Europe. These findings demonstrate that *Haematococcus* derived astaxanthin, including super critical CO_2 extracts of astaxanthin, have an extensive history of previous exposure in humans.

1.7.6. Astaxanthin conversion into retinol

The most important metabolic products of carotenoids are the retinoids, and the metabolic reactions of carotenoids in animals are essentially oxidative. However, pathways of reductive metabolism have recently been discovered, and this has opened up the possibility that xanthophylls could be precursors of retinoids (Goodwin, 1986). Only about 60 of the 600 known carotenoids (Pfander, 1987 **)** have been reported to be precursors of retinol, the main ones being α-carotene, γ-carotene, β-cryptoxanthin, echinenone, β-apo-l2'-carotenal and, of course, β-carotene. They have been demonstrated in goldfish *Carassius auratus* (Hata and Hata, 1972; Matsuno et al., 1991) and fancy red carp *Cyprinus carpio* (Matsuno et al., 1979) **.** Grangaud et al., (1962) first demonstrated that astaxanthin is converted into retinol in the liver. Gross and Budowaki, (1966) found that the conversion of astaxanthin, canthaxanthin and isozeaxanthin, via β-carotene, into retinol and 3-dehydroretinol in freshwater fish, guppies and platies. Barua and Goswami, (1981**)** reported that lutein is the precursor of 3-dehydroretinol in some freshwater fish. Schiedt et al., (1985) showed that retinol and 3-dehydroretinol are formed in retinol-depleted rainbow trout from any of a number of labelled carotenoids, particularly astaxanthin, zeaxanthin and canthaxanthin (Schiedt, 1985; Schiedt et al., 1986**).** All these investigations have been carried out with freshwater fish. The bioconversion of some xanthophylls such as astaxanthin, zeaxanthin, canthaxanthin, lutein and tunaxanthin into retinoids occurs in freshwater fishes, marine fish and mammals. Matsuno, (1991) reported that xanthophylls, astaxanthin, zeaxanthin, lutein and tunaxanthin, were probably directly bioconverted into 3-dehydroretinol without being first transformed into retinol. The bioconversion of astaxanthin into 3-dehydroretinol in mature rainbow trout has been reported by Guillou et al., (1989). Xanthophylls such as canthaxanthin, astaxanthin, zeaxanthin and lutein which are widely distributed in nature have been found to be precursors of retinoids not only in freshwater fish and marine fish (yellowtail) but also in a mammal (rat) as shown in (Fig 13.)

Table 10. Patents on astaxanthin health and nutrition applications.

Patent	Company name	Patent title
EP0786990	US Nutraceuticals	Use of astaxanthin for retarding and ameliorating central nervous system and eye damage
EP1217996	Astacarotene	Use of astaxanthin for treatment of autoimmune diseases, chronic viral and intracellular bacterial infections
US6475547	Astacarotene	Immunoglobulin-rich milk, production and use thereof
WO0023064	Astacarotene	Treatment of dyspepsia
US6410602	Astacarotene	Method of increasing the production and improving the quality of semen
US6335015	Astacarotene	Method of the prophylactic treatment of mastitis
US6262316	Astacarotene	Oral preparation for the prophylactic and therapeutic treatment of helicobacter sp. infection
US6245818	Astacarotene	Oedicament for improvement of duration of muscle function or treatmentof muscle disorders or diseases
US6054491	Astacarotene	Agent for increasing the production of/in breeding and production mammals
US5744502	Astacarotene	Method for increasing the production of/in breeding and productiona nimals in the poultry industry
US6433025	Cyanotech	Method for retarding and preventing sunburn by uv light
US6344214	Cyanotech	Method for retarding and ameliorating fever blisters and canker sores
US6258855	Cyanotech	Method of retarding and ameliorating carpal tunnel syndrome
EP1283038	Suntory Ltd	Compositions normalizing circadian rhythm
WO03013556	Itakura Hiroshige	Medicinal compositions having effects of ameliorating eye diseases and holding eye functions
WO03003848	Aanesen Berit Annie	The use of di-esters of astaxanthin for enhancing the growth of farmed fish
WO02094253	Fuji Chem Ind Co	Agents for relieving eye controlling function error
KR2000045197	Pacific Co Ltd	Healthy nutrition composition containing chitosan oligosaccharideand astaxanthin
WO02058683	Lycored	carotenoids as anti-hypertension agents
NZ299641	Suntory And Itano	Use of astaxanthin in pharmaceuticals for treating stress
US6277417	Triarco	Method of inhibiting 5alpha-reductase with astaxanthin
US2003/778304	Anderson And Petterson	Method of inhibiting the expression of inflammatory cytokines and chemokines
JP10276721	Suntory And Itano	Astaxanthin-containing food or drink

Table 11. Astaxanthin applications for human and mammalian health

Disease	Reference
Central nervous system and neurodegradative diseases	Tso and Lam, 1996
Eye health	Tso and Lam, 1996; Snodderly, 1995; Ohgami et al., 2003; Waagbo et al., 2003; Connor and Brien, 1998.
Joint health, muscle endurance	Malmsten, 1998; Lignell, 2001.
Inflammation and immune system	Bennedsen et al., 1999; Wang et al., 2000; Lignell, 1998; Lorenz et al., 2000; Hughes, 1999; Lignell, 2001; Lignell et al., 1998; Jyonouchi et al., 1991, 1993, 1994, 1995, 1996; Lee et al., 2003; Chew et al., 1999, 2003; Akyon, 2002; Okai and Higashi-Okai, 1996; Murillo, 1992.
Cardiovascular, heart, lipid peroxidation and blood	Murillo, 1992 ; Iwamoto et al., 2001; Zhang et al., 1991 ; Aoi et al., 2003 ; Kang et al., 2001, Rengel et al., 2000.
Cancer	Jyonouchi et al., 2000 ; Kurihara et al., 2002 ; Tanaka et al., 1994,1995a, 1995b; Kozuki et al., 2000 ; Gradelet et al., 1998 ; Black, 1998 ; Nishino et al., 2002 ; Li et al., 2002, Chew et al., 1999 ; Zhang et al., 1991.
Fertlity	Hansen et al, 2001; Lignell, 1998.
Sun burn and skin health, anti-aging and anti-wrinkling	Lyons et al., 2002 ; Savoure et al., 1995 ; Seki et al., 2001. Yamahita et al., 1995 ; Connor and Brien, 1998.
Benign prostatic hyperplasia (bph)	Anderson, 2001.

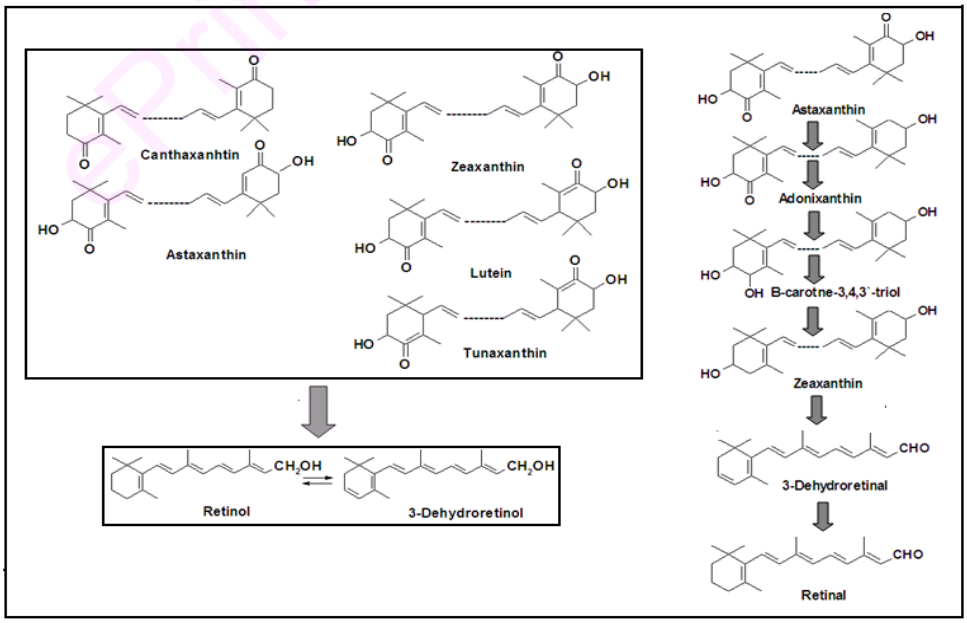


Fig. 13. Astaxanthin as retinoid precursor

1.7.7. Astaxanthin as a general biological antioxidant

Free radicals (e.g. hydroxyl and peroxyl radicals) and highly reactive forms of oxygen (e.g. singlet oxygen) are produced in the body during normal metabolic reactions and processes. Physiological stress includes air pollution, tobacco smoke, exposure to chemicals or exposure to ultraviolet (UV) light can enhance the production of free radicals. Phagocytes can also generate an excess of free radicals to aid in their defensive degradation of the invader. Free radicals can damage DNA, proteins and lipid membranes. Oxidative damage has been linked to aging, atherogenesis, ischemia-reperfusion injury, infant retinopathy, age related macular degeneration and carcinogenesis. Astaxanthin has been shown to be a powerful quencher of singlet oxygen by *in vitro* studies (Dimascio et al., 1990; Miki 1991), and is a strong scavenger of oxygen free radicals, at least ten times stronger than β-carotene (Miki, 1991). Experiments with red blood cells and mitochondria from rats have shown that astaxanthin is 100 times more effective at inhibiting lipid peroxidation than vitamin E (Miki, 1991). The results of these *in vitro* studies were confirmed *in vivo* with rats given dietary supplements of astaxanthin and subjected to oxidizing agents (Miki 1991). The antioxidative properties of astaxanthin have been demonstrated in a number of different biological membranes (Kurashige et al., 1990; Palozza and Krinsky, 1992; Nakagawa et al., 1997). Other tests have shown that astaxanthin is up to 1000 times more powerful than vitamin-E (Tso and Lam, 1996).

17.8. Astaxanthin for prevention of neurodegenerative diseases

Most of the microalgae products like *Spirulina* sp. and *Aphanizomenon flosaquae* are used as nutraceuticals. The medicinal benefits of nutraceuticals depend upon high quality manufacturing and effective control of production environments. Astaxanthin, a powerful bioactive antioxidant, has demonstrated efficacy in animal or human models against Alzheimer's and Parkinson's diseases, and macular degeneration (Snodderly, 1995). Astaxanthin also ameliorates the effects of LDL, protects against cancer, and repairs cell damage caused by lack of oxygen, hence a potent nutraceutical. The blood pressure was reduced by the introduction of astaxanthin to hypertensive rats. Blood pressure is a causative factor for many diseases including some associated with the eyes and brain. Astaxanthin works against stroke prone rats. The same study went on to demonstrate a neuroprotective effect (protection of brain function) in ischemic mice. Ischemia is the condition where there is a deficient supply of blood to the brain as a result of the obstruction of the arteries. In the case of these

mice, ischemia was induced by blocking the carotid artery. In humans, this condition can be caused by plaque buildup which can block the flow of blood through the carotid artery in the neck, the primary source of blood to the brain. Astaxanthin can attenuate the development of hypertension and may help to protect the brain from stroke and ischemic insult. In addition, Astaxanthin showed neuroprotective effects at relatively high doses by preventing the ischemia-induced impairment of spatial memory in mice. This effect is suggested to be due to the significant antioxidant property of astaxanthin on ischemia-induced free radicals and their consequent pathological cerebral and neural effects. Astaxanthin may have beneficial effects in improving memory in vascular dementia (Hussein et al., 2005). It appears that astaxanthin actually made the mice with restricted blood flow to their brains smarter by improving their memory. The implications of the studies are extremely exciting, as our aging population sees growing numbers of Alzheimer's patients, stroke sufferers and people afflicted by dementia caused by other factors. Further research in humans must be done to fully understand the potential benefit, but these pre-clinical experiments indicate that astaxanthin may help sufferers of many brain-related diseases and live better lives.

1.7.9. Astaxanthin protect from UV light

Exposure of lipids and tissues to light, especially UV-light, can lead to production of singlet oxygen and free radicals and photo-oxidative damage of these lipids and tissues. Carotenoids have an important role in nature in protecting tissues against UV-light mediated photo-oxidation and are often found in tissues directly exposed to sunlight. Astaxanthin can be significantly more effective than β-carotene and lutein at preventing UV-light photooxidation of lipids (Connor and Brien, 1998). Oxidative damage to the eye and skin by UV light has been widely documented and thus the unique UV protection properties of astaxanthin could be very important for eye and skin health.

1.7.10. Astaxanthin for prevention of cancer

Studies of the cancer-preventative properties of astaxanthin have been carried out on rats and mice by Takuji Tanaka and colleagues at the Gifu University School of Medicine. Dietary administration of astaxanthin proved to significantly inhibit carcinogenesis in the mouse urinary bladder (Tanaka et al., 1994), rat oral cavity (Tanaka et al., 1995a), and rat colon (Tanaka et al., 1995b). In addition, astaxanthin has been shown to induce xenobiotic-metabolizing enzymes in rat liver and also may

help prevent carcinogenesis (Gradelet et al., 1996). Several studies have demonstrated the anti-cancer activity of astaxanthin in mammals. Rats fed with a carcinogen but supplemented with astaxanthin had a significantly lower incidence of different types of cancerous growths in their mouths than rats fed only the carcinogen. The protective effect of astaxanthin was even more pronounced than that of β-carotene (Tanaka et al., 1995a). Dietary astaxanthin is also effective in fighting mammary cancer by reducing growth of induced mammary tumors by 50%, more so than β-carotene and canthaxanthin (Chew et al., 1999). Astaxanthin inhibits the enzyme 5-α-reductase responsible for prostate growth and astaxanthin supplementation was proposed as a method to fight benign prostate hyperplasia and prostate cancer (Anderson et al, 2001). Astaxanthin supplementation in rats was found to inhibit the stress-induced suppression of tumor-fighting natural killer cells (Kurihara et al., 2002). As noted earlier, Astaxanthin anti-cancer activity might be related to the carotenoids' role in cell communications at gap junctions, which might be involved with slowing cancer cell growth (Bertram, 1999), the induction of xenobiotic-metabolizing enzymes (Jewell and Brien, 1999) or by modulating immune responses against tumor cells (Jyonouchi et al., 2000). Chew and park, (2004) reported that astaxanthin, canthaxanthin and β- carotene inhibited tumor growth and showed the highest anti-tumor activity.

1.7.11. Astaxanthin support for the immune system

Astaxanthin has been shown to significantly influence immune function in a number of *in vitro* and *in vivo* assays using animal models. The majority of this work has been carried out by Harumi Jyonouchi and colleagues at the University of Minnesota. Astaxanthin enhances *in vitro* antibody production by mouse spleen cells stimulated with sheep red blood cells (Jyonouchi et al., 1991), at least in part by exerting actions on T-cells, especially T-helper cells (Jyonouchi et al., 1993). Astaxanthin can also partially restore decreased humoral immune responses in old mice (Jyonouchi et al., 1994). Furthermore, oxidants have been directly linked to the stimulation of inflammation genes in endothelial cells. Similarly, ROS have been attributed an aggravating role in the inflammation that accompanies asthma and exercise-induced muscle damage (Dekkers, et al. 1996). These immunomodulating properties are not related to provitamin-A activity, because astaxanthin, unlike β-carotene, does not have such activity (Jyonouchi et al., 1991). Studies on human blood cells *in vitro* have

demonstrated enhancement by astaxanthin of immunoglobulin production in response to T-dependent stimuli (Jyonouchi et al., 1995a). Other supporting data on astaxanthin and immune function, including studies on the mechanisms of action involved, may be found in Jyonouchi et al., (1995b), Jyonouchi et al., (1996), Okai & Higashi-Okai (1996), and Tomita et al., (1993). Both *in vivo* and *in vitro*, had shown that vitamin C and astaxanthin, a carotenoid, are not only free radical scavengers but also show antimicrobial activity against *H. pylori*. It has been shown that astaxanthin changes the immune response to *H. pylori* by shifting the Th1 response towards a Th2 T-cell response (Akyon, 2002). Because Astaxanthin can actually change the immune response, it is very effective at reducing *H. pylori*, which can help prevent certain types of gastric cancer and other stomach ailments.

1.7.12. Astaxanthin for treatment of infections

Astaxanthin may be effective as a prophylactic and/or therapeutic treatment of *Helicobacter* infections of the mammalian gastrointestinal tract, and an oral preparation has been developed for this purpose (Alejung and Wadstroem, 1998).

1.7.13. Astaxanthin for prevention of arteriosclerosis and related diseases

Astaxanthin has been shown in both *in vitro* experiments and in a study with human subjects to be effective for the prevention of the oxidation of low-density lipoprotein (Miki et al., 1998). This suggests that it could be used as a preventative for arteriosclerosis, coronary artery disease, and ischemic brain damage; a number of astaxanthin-containing health products are under development based on these findings (Miki et al., 1998). Astaxanthin has also been shown to enhance production of LDL and especially HDL cholesterol in the bloodstream of rats (Murillo, 1992).

1.7.14. Astaxanthin for prevention of inflammation

Astaxanthin diesters appear to exert a synergistic effect on anti-inflammatory agents, increasing the effectiveness of aspirin when the two are administered together (Yamashita, 1995). Astaxanthin is carried by VLDL, LDL and HDL in the human blood. An *in vitro* test and a study with human subjects ingesting daily dosages as low as 3.6 mg astaxanthin per day for two consecutive weeks demonstrated that astaxanthin protects LDL cholesterol against induced *in vitro* oxidation Miki et al., (1998). In an animal model study, astaxanthin supplementation led to an increase in blood levels of HDL (Murillo, 1992), the form of blood cholesterol inversely correlated with coronary heart disease. Thus, astaxanthin could benefit heart health by modifying blood levels of LDL and HDL cholesterol. Astaxanthin could also be

beneficial to heart health by reducing inflammation presumably associated with the development of coronary heart disease (Tracy, 1999).

1.7.15. Astaxanthin prevention cardiovascular disease

Astaxanthin has a variety of properties that can help people prevent heart disease and also help people with heart disease to minimize their risk of a heart attack or stroke. There is evidence that astaxanthin can help improve blood lipid profiles by decreasing low density lipoprotein and triglycerides, and by increasing high density lipoprotein. Murillo et al., (1992) demonstrated that astaxanthin raised high density lipoproteins in the rat model sytem. Astaxanthin and vitamin-E both supplements in rabbits that had high cholesterol, particularly astaxanthin, improved plaque stability in the arteries. All the rabbits that ingested astaxanthin were classified as "early plaques," as compared to the rabbits ingesting Vitamin E and also the control group (Li et al., 2004). Hussein et al., (2006) showed that astaxanthin increased high density lipoproteins, while decreasing both triglycerides and non-esterified fatty acids in the blood. A human clinical trial in Japan found a very promising effect on low density lipoproteins both *in vitro* test tubes and in human volunteers. The consumption of astaxanthin inhibits LDL oxidation and possibly to the prevention of atherosclerosis (Iwamoto, et al., 2001). Trimeks, (2003) reported that astaxanthin decreases the total cholesterol and of LDL of 17%, and an average decrease of triglycerides of 24%. Another potential benefit for cardiovascular health may be astaxanthin's ability to decrease blood pressure. Hussein et al, (2005a) indicated that astaxanthin can exert beneficial effects in protection against hypertension and stroke and in improving memory in vascular dementia. Astaxanthin may help with blood fluidity in hypertension, and that it may restore the vascular tone (Hussein et al., 2005b). There is one human study that is related to this anti-hypertensive animal research as well as the blood lipid research. This study centered on human volunteers supplementing with 6 mg of astaxanthin per day for only ten days. At the end of the ten day period, significant improvement in blood flow was found in the treatment group (Miyawaki, 2005). A very different type of animal study related to cardiac health was done by a different group of Japanese scientists at the Kyoto University of Medicine. The study found that mice that were fed astaxanthin and then run on a tread mill until exhaustion suffered less heart damage than mice that were similarly exercised without astaxanthin supplementation. They concluded that astaxanthin can decrease exercise induced damage in the heart as well as in the skeletal muscle (Aoi et al., 2003).

Astaxanthin was given to rats prior to heart attacks. It was found that astaxanthin significantly reduced the area of infarction and the damage caused to the heart by the heart attack (Gross and Lockwood, 2004). All are looking at making a unique, injectable delivery system for astaxanthin into a patented prescription drug for cardiovascular patients. The medical research to date clearly demonstrates that astaxanthin, already available as a low cost dietary supplement in most countries, has many diverse cardiovascular benefits as well.

1.7.16. Astaxanthin as anti skin carcinogenesis agent

Excessive exposure of unprotected skin to sunlight results in sunburn and can also lead to photo-induced oxidation, inflammation, immunosuppression, aging and even carcinogenesis of skin cells. Pre-clinical studies show that typical dietary antioxidants, such as α-tocopherol, ascorbic acid or β-carotene, could reduce such damage (Fuchs, 1998; Lee et al., 2000). Astaxanthin is believed to protect the skin and eggs of salmon against UV-light photo-oxidation (Meyers, 1993). Astaxanthin supplementation helped in protecting the retinal photoreceptors in the eyes of rats exposed to acute UV-light injury (Tso and Lam, 1996) and the *in vitro* protective effect of astaxanthin against UV-induced photooxidation (Connor and Brien, 1998) was stronger when compared with β-carotene and lutein. These findings suggest that astaxanthin has an excellent potential as an oral sun-protectant. Although diet supplementation with β-carotene or astaxanthin has demonstrated benefits in other types of cancer, the animal or clinical studies with these two compounds are inconclusive when it comes to skin cancer (Black, 1998; Savoure et al., 1995). More studies are needed to better understand the possible interactions between various antioxidants and their potential prooxidative role, to determine under which conditions supplementation with carotenoids such as astaxanthin can help reduce skin carcinogenesis.

1.7.17. Astaxanthin as anti age macular degeneration (AMD) agent

Astaxanthin protect form age related macular degeneration. Two of the leading causes of visual impairment and blindness are age-related macular degeneration (AMD) and age-related cataracts. Both diseases appear to be related to light-induced oxidative processes within the eye (Palozza and Krinsky, 1992; Naguib, 2000; Kurashige et al., 1990). It is therefore not surprising that factors related to oxidation have been shown in epidemiological studies to be related to an elevated risk for AMD. A high dietary intake of carotenoids, specifically lutein and zeaxanthin (from spinach, kale, and other leafy green vegetables) is associated with a reduced risk for both nuclear cataracts and

age related macular degeneration (Seddon et al., 1994; Lyle et al., 1999; Jacques, 1999). Lutein and zeaxanthin, two carotenoid pigments closely related to astaxanthin, are concentrated in the macula of the eye (Landrum et al., 1999). The structure of astaxanthin is very close to that of lutein and zeaxanthin but has a stronger antioxidant activity and UV-light protection effect (Connor and Brien, 1998). Astaxanthin has not been isolated in the human eye. However, an animal study (Tso and Lam, 1996) demonstrated that astaxanthin is capable of crossing the blood–brain barrier and, similar to lutein, will deposit in the retina of mammals. The retinal photoreceptors of rats fed with astaxanthin were less damaged by a UV-light injury and recovered faster than animals not fed with astaxanthin. Therefore, it can be inferred that deposition of astaxanthin in the eye could provide superior protection against UV light and oxidation of retinal tissues pointing to the potential of astaxanthin for eye health maintenance.

1.7.18. Astaxanthin in cellular health

In the mitochondria, multiple oxidative chain reactions generate the energy needed by the cell but produce large amounts of free radicals that need to be neutralized to maintain proper mitochondrial function. It is hypothesized that the cumulative oxidative damage to mitochondria is the main culprit for the senescence of cells, which in turn is responsible for aging. The efficacy of astaxanthin in preventing *in vitro* peroxidation of mitochondria of rat liver cells can be as high as 100 times that of vitaminE (Kurashige et al., 1990). This highlights the unique capacity of astaxanthin in helping to preserve mitochondrial functions and its unique potential in the fight against aging. Astaxanthin's superior role in protecting cellular membranes is believed to derive from its ability to protect both the inner part and external surface of membranes against oxidation. Antioxidants, carotenoids in particular, are not only essential to cellular health because they help protect cellular components against oxidative damage but also because they have a role in regulating gene expression and in inducing cell-to-cell communications and astaxanthin was reported to have a role in regulating CYP genes in rat hepatocytes, although it did not seem to have that effect in human hepatocytes, Also carotenoids are active inducers of communication between cells at the cell-gap junctions (Bertram, 1999; Kistler et al., 2002). Thus, it is hypothesized that carotenoids affect DNA regulating RNA responsible for gap-junction communications and that this role in cell-gap junctions communications might explain some of the anti-cancer activities of astaxanthin.

1.7.19. Astaxanthin prevention of ulcers, gastric injury, stomach cancer

Helicobacter pylori is responsable for chronic gastritis and stomach ulcers. Left untreated it can lead to more serious consequences including stomach cancer and lymphoma. It can be caused by eating a diet deficient in some very important nutrients such as carotenoids. A low dietary intake of antioxidants such as carotenoids and vitamin C may be an important factor for the acquisition of *H. pylori* by humans (Bennedsen et al., 1999). Astaxanthin was shown to be able to change the immune response to *H. pylori* (Akyon, 2002). In Denmark they have studied that astaxanthin-rich algae extract reduced the bacterial load and gastric inflammation in *H. pylori* infected mice (Bennedsen et al., 1999). In Sweden, both *in vitro* test tubes and in live mice astaxanthin in algae meal inhibited the growth of *H. pylori in vitro*. In the *ex vivo*, the mice that ate the *Haematococcus* algae meal showed lower bacteria levels and lower inflammation scores than untreated or control meal treated mice when tested one day after as well as ten days after the cessation of treatment (Wang et al., 2000). Astaxanthin's ability to prevent the gastric damage is due to naproxen and ethylalcohol. The results indicate that astaxanthin removes the lipid peroxides and free radicals induced by naproxen, and it may offer potential remedy of gastric ulceration (Kim et al., 2005a). Astaxanthin's effects on naproxen, its effects on ethyl alcohol showed significant protection against ulcers, and pretreatment increased the free radical scavenging activities of SOD, catalase and glutathione peroxidase. A histological examination clearly indicated that the acute gastric mucosal lesion induced by ethanol nearly disappeared after pretreatment with astaxanthin (Kim et al., 2005b). Recently, the anti-ulcer properties of astaxanthin fractions such as total carotenoid and astaxanthin esters from *Haematococcus pluvialis* were evaluated in our lab in ethanol induced gastric ulcers in rats (Kamath et al., 2008). This study is more vital evidence of the superiority of astaxanthin to other forms, as well as another piece of science demonstrating efficacy for astaxanthin in gastrointestinal health.

1.7.20. Can astaxanthin help in diabetics?

Since the astaxanthin can help reduce silent inflammation, that the use of astaxanthin should have some benefit in people with diabetes and/or in preventing diabetes. In Japan at the Kyoto University of Medicine and at the Institute of Natural Medicine, they examined a special type of mice that are diabetic and obese, the results demonstrated that astaxanthin significantly reduced the blood glucose level of these mice. The astaxanthin treated group maintained their ability to secret insulin and

concluded these results indicate that astaxanthin can exert beneficial effects in diabetes, with preservation of β-cell function (Uchiyama et al., 2002). Diabetes adversely affects many different organs of the body. In particular, diabetes can cause the kidneys to malfunction, causing a condition called "nephropathy". This second study used the same diabetic, obese mice to examine how astaxanthin could benefit the kidneys. The results after 12 weeks of treatment, the astaxanthin-treated group showed lower blood glucose compared with the non-treated group treatment with astaxanthin ameliorated the progression and acceleration of diabetic nephropathy in the rodent model of diabetes. The results suggested that the antioxidant activity of astaxanthin reduced the oxidative stress on the kidneys and prevented renal cell damage. Administration of astaxanthin might be a novel approach for the prevention of diabetic nephropathy (Naito et al., 2004). After treatment of 22 weeks, in rats, astaxanthin reduced blood pressure and improved cholesterol and triglyceride profiles, but it also showed a reduction in blood glucose levels. A significant reduction in fasting blood glucose levels as well as insulin resistance was noted, along with improvement in insulin sensitivity. A fascinating notation was made that astaxanthin actually decreased the size of fat cells. These results suggest that astaxanthin ameliorates insulin resistance by mechanisms involving the increase of glucose uptake, and by modulating the level of circulating lipid metabolites and adiponectin (Hussein et al., 2006). A recent study in diabetic mice showed that expression levels of genes extracted from the kidneys were decreased by astaxanthin. This research may lead to a better understanding of the genes and pathways involved in the anti-diabetic mechanism of astaxanthin (Naito et al., 2006).

1.7.21. Antihypertensive effects of astaxanthin

Astaxanthin is a biological antioxidant activity naturally found in a wide variety of aquatic living organisms. Hussein et al., (2005) investigated an antihypertensive effect of astaxanthin in spontaneously hypertensive rats (SHR), which have been widely used as a model to study the mechanism, pathophysiology, and management of hypertension. The administration of astaxanthin at the doses of 50 mg/kg for 5 weeks demonstrated a significant reduction in the systolic blood pressure (BP) (4%) and in the diastolic BP (10%), and also delayed the incidence of stroke in stroke-prone SHR. In the study using aromatic rings with intact and denuded endothelia, astaxanthin-induced vasodilation by both endothelium-dependent and endothelium- independent manners. They also investigated the effect of astaxanthin on nitric oxide (NO), which

plays a major role on regulation of vascular tone and arterial blood pressure-mediated vasorelaxation. Astaxanthin-mediated vasorelaxation is NO-dependent at the lower dose (30 μM), and is NO-independent at the higher dose (100 μM). Astaxanthin induced a significant reduction of the contractile responses of the aortic preparations to α-adrenergic receptor agonist, phenylephrine, suggesting that astaxanthin may decrease blood pressure by ameliorating the sympathetic pathway, especially via α-adrenergic receptor. Astaxanthin also demonstrated a significant reduction of the contractile responses to angiotensin -II, which has been reported to increase superoxide in cultured vascular smooth muscle cells (Griendling et al., 1994). Superoxide was increased in rats that became hypertensive by chronic infusion with angiotensin-II (Rajagopalan et al., 1996). Bonaventura et al., (2008) showed that endothelium potentiates the SNP-mediated vasorelaxation, suggesting a significant association between endothelium-mediated vasorelaxation and the sodium nitroprusside-mediated vasorelaxation. Hussein et al., (2006) investigated the effect of astaxanthin on plasma levels of Nitric oxide (NO) end products nitrite/nitrate (NO_2–/NO_3–, termed NOx) in spontaneously hypertensive rats (SHR). The synthesis of NO by vascular endothelium is responsible for vascular tone which plays an essential role in regulation of blood pressure (Rand et al., 1992). Because NOx are relatively stable in the blood, plasma NOx concentration has been reported to be an indicator of endogenous NO production (Rhodes et al., 1995). Oral administration of astaxanthin significantly reduced plasma NOx levels compared with control rats (Hussein et al., 2006). In a histopathological study, astaxanthin decreased coronary artery wall thickness compared with the control, and significantly reduced the elastic fiber in the aorta, suggesting the possibility that astaxanthin ameliorates hypertension-induced vascular remodeling (Hussein et al., 2006). The underlying mechanisms for development of hypertension in the metabolic syndrome, which is characterized by the simultaneous occurrence of metabolic abnormalities including obesity, glucose intolerance, dyslipidemia, are very complicated. Sympathetic over activity, oxidative stress, and activated renin-anigiotensin system have been suggested to be possible factors for developing hypertension in the metabolic syndrome (Yanai et al., 2008). Astaxanthin may be effective for the management of hypertension in the metabolic syndrome as well as essential hypertension.

1.7.22. Astaxanthin for salmon and trout feeds

The predominant source of carotenoids for salmonids has been synthetic astaxanthin, which has been used for pigmentation for the last 20 years, since FDA approval in 1996. Natural sources of astaxanthin for commercially raised salmonids have been utilized, which include processed crustacean waste from krill, shrimp, crab, and crawfish. However, crustacean waste products contain high amounts of moisture, ash, and chitin, which limits the use in salmonids feeds. Another natural source, *Phaffia rhodozyma*, requires a large amount of feed for sufficient pigmentation, leading to higher ash contents. The efficiency of dietary astaxanthin using microalgae for flesh pigmentation of Atlantic salmon and rainbow trout has been demonstrated by Torrisson et al., (1989) and Storebakken (1998). The algae were concluded to be a safe and effective source of pigment. Astaxanthin has been used to enhance the immune response of fish and shrimp for maximum survival and growth. Natural microalgal astaxanthin has shown superior bioefficacy over the synthetic form. Japan has been received for the use of astaxanthin as a pigment in feeds and foods; registration for approval is in progress for the United States, the European community, and Canada. An amount of 25–100 ppm of carotenoids in the final feed has been considered to give desired pigmentation in various salmonid species. In poultry astaxanthin has shown to reduce the mortality of chicks by 50%, and to reduce *Vibrio* infections in eggs, thereby improving the nutritional value of eggs, especially among European consumers. However, the livestock feed market for astaxanthin, which is presently small, may grow to a size comparable to the market for synthetic pigments, which is estimated at US$185 million. The largest market for astaxanthin, aquaculture, constituting 24% of total global fisheries production, is currently valued at US$35 billion per annum and is expected to grow to US$49 billion by 2010. Limited studies have been carried out on dietary astaxanthin intake by humans. In a study reported by Miki et al., (1998), astaxanthin was tested to protect low density lipoprotein from oxidation; 3.6–14.4 mg/day of an astaxanthin-containing drink was administered over a period of 2 weeks. Progressively slowed LDL oxidation with increasing doses of astaxanthin was observed and no ill effects were reported. Osterlie et al., (1999a, 1999b) reported that when 100 mg of synthetic astaxanthin in olive oil containing meal was given to male volunteers, maximum plasma concentration of 1.24 mg/L astaxanthin was observed in the first 6 hours postprandially. The relative concentration of total astaxanthin in HDL decreased compared to the other lipoprotein

fractions in the 72 hour study. Based on a study conducted with 40 healthy volunteers, Lignell, (1998) reported the effect of astaxanthin on mammalian muscle function. Volunteers received 1 capsule of 4 mg astaxanthin each morning in association with food. No significant difference was observed between the treatment and placebo group in any physical parameters measured. The effect of dietary astaxanthin on the health of humans as studied by Aquasearch, (2000) on 33 volunteers consuming daily 3.85 mg (low dose) and 19.25 mg (high dose) for a period of 29 days indicated no ill effects or toxicity due to consumption of astaxanthin as analyzed by medical and clinical parameters.

1.8. Growth and carotenogensis of microalgae

Research has been done on the physiology and growth conditions for producing the compounds of interest from micro algal form. Determining the exact and narrow range for each parameter without restriction on growth is difficult since the optimum conditions for a given algal strain varies considerably. Droop (1954) defined the culture condition for formation of astaxanthin in *Haematococcus* for the first time. Culture condition for indoor cultivation of *Haematococcus* and astaxanthin production has been reported by many authors (Sarada et al., 2002b; Orosa et al., 2000; Kobayashi et al., 2001; Fabregas et al., 2003). Many microalgae, including *Haematococcus*, are capable of autotrophic as well as heterotrophic growth (Sarada et al., 2002b; Kang et al., 2005). Heterotrophic cultivation has a potential for achieving high cell concentration and it has been demonstrated for production of *Chlamydomonas* biomass (Chen and Johns, 1996). Due to the problems of maintaining sterile conditions, heterotrophic system is not suitable for growth of most other microalgae. Few researchers have reported cultivation of microalgae in mixotrophic system where acetate is used as carbon source (Orosa et al., 2000; Martinez, 1997; Gong and Chen, 1997). Sequential heterotrophic-photoautotrophic cultivation of a green alga, *Haematococcus* was reported by Hata et al., (2001) where the algae was grown heterotrophically to high cell concentration, followed by illumination of the culture for astaxanthin accumulation. Astaxanthin production by *Haematococcus* in autotrophic, mixotrophic and heterotrophic medium was reported by Tripathi et al., (1999).

1.8.1. Current market status of astaxanthin

Haematococcus astaxanthin producers attempted to enter the fish (especially salmon) feed market. However, price competition from synthetic astaxanthin (US $2000/kg)

relegated *Haematococcus* astaxanthin producers to supply small, special markets. The present commercial producers cannot compete against synthetic astaxanthin on price alone. However, as production technology is optimized and production is transferred to lower cost locales, *Haematococcus* astaxanthin might compete against synthetic astaxanthin on price. Furthermore, the public becomes educated and demands natural pigmented salmon or regulations require the use of natural feed ingredients. Alternatively, as recent research has pointed to the possible functions of astaxanthin in the human body, a market for nutraceutical astaxanthin has started to develop. Although the size of this market is closely guarded by commercial producers it is expected that it could reach a size of several hundred million US$ within 5 to 10 years. Between the synthetic and natural astaxanthin prices varies, synthetic variety costs $2000 per kg where as the natural astaxanthin is still expensive at between $10,000 - 15,000 per kg (www.nutraingredients-usa.com). The range of astaxanthin products produced and marketed by various companies has been listed in Table 12.

Table 12. *Haematococcus* algae meal and astaxanthin products in world market

Product	Company	Particulars	Website
Asta Factor	Mera Pharmaceuticals Inc. USA.	Astaxanthin packaged as soft gel, dietary supplement derived from *Haematococcus*	www.astafactor.com
AstaPure™	Algatechnologies Ltd, Israel.	Dry algal biomass, astaxanthin beadlets and oleoresin	www.algatech.com
AstaXin® AstaCarox®	BioReal (Sweden) AB	Dietary supplement Containing *Haematococcus* - crushed and dried algae meal	www.bioreal.se
AstaEquus®	BioReal (Sweden) AB	Feed supplement for horses	www.bioreal.se
AstREAL	BioReal (Sweden)AB	Super critical fluid-oil extract derived from crushed algae	www.bioreal.se
BioAstin	BioAstin® Cyanotech Corporation, USA	*Haematococcus* extract – packaged in soft gel, beadlets; dietary supplement	www.cyanotech.com
Britaxan	Britannia Health Products Ltd. UK	Astaxanthin complex with other carotenoids packaged as capsule- dietary supplement	www.britanniahealth.co.uk
NaturAsta™	Jingzhou Natural Astaxanthin Inc,China	Dry algal biomass and astaxanthin soft gel	www.asta.cn
Naturose™	Cyanotech Corporation, USA	*Haematococcus* algae meal; pigmentation source for ornamental fish and animals	www.cyanotech.com
Novaasta®	BioReal (Sweden)AB	A feed supplement or animals	www.bioreal.se
Stazen	Stazen Inc., USA	Dietary supplement Containing *Haematococcus* - crushed and dried algae meal	www.stazen.com
Zanthin	Valensa International, USA	*Haematococcus* extract, softgel, beadlets	www.usnutra.com

1.8.2. Astaxanthin as nutraceutical

Antioxidant-rich foods in the diet are believed to contribute to the long-term health of all mammals. Carotenoids are important biological antioxidants that naturally occur in a wide variety of foods. These compounds are thought to support immune function and help to reduce cellular DNA damage associated with aging. The nutritional benefits of carotenoids extend beyond the role of some carotenoids as precursors of vitamin A. For example, the xanthophyll (oxygenated) carotenoids lutein and astaxanthin, and the carotene carotenoid lycopene, have all exhibited potential health benefits to animals and humans in scientific studies, yet none of them is a source for vitamin A (Rao and Agarawal, 2000; Shao, 2001; Guerin et al., 2003).

Astaxanthin, a xanthophyll (oxygenated) carotenoid, is known to be among the most potent antioxidant carotenoids *in vitro* and *in vivo,* yet unlike β-carotene it does not display any pro-oxidant behavior, even at high concentration and high oxygen tension (Dore, 2005). Astaxanthin is at least an occasional component in the human diet due to its presence in fish and crustaceans. Among human populations that consume large amounts of salmon, dietary astaxanthin may be more significant. *Haematococcus* algae have naturally high astaxanthin content. As a source of astaxanthin, the algal product is therefore practical for addition to dietary supplements than alternative sources such as salmon and shrimp meals, which may contain only a few ppm or less of astaxanthin. Lipid extracts of *Haematococcus,* with astaxanthin concentrations from 5-10%, are even more practical for this purpose. Natural astaxanthin is not a drug – it is not intended to prevent, cure, treat or mitigate any disease or specific condition. It is also not a vitamin; unlike β-carotene, astaxanthin has no provitamin A activity in humans. Astaxanthin has long been used as a pigmenting agent in diets for aquacultured salmon and trout. Although the purpose of added astaxanthin in feeds for salmonid fishes is to impart coloration to fish flesh, it has recently been suggested that carry-over of astaxanthin from farmed salmon into the human food chain should be beneficial to human health (Baker and Günther, 2004). In view of this it is based upon both the longstanding recognition of wild salmon as a healthful food and the growing body of scientific evidence revealing positive health effects of dietary astaxanthin on human beings and rodent models. These apparent effects include improvement of joint health, protection from sunburn, prevention of age-related macular degeneration, prevention of some types of cancer, enhancement of the immune system, and many others. The potential use of astaxanthin in human health management have been published (Maher, 2000; Naguib, 2001; Cronin, 2002; Guerin et al., 2003; Wiener et al., 2003; Dore, 2005).

Haematococcus algae have been used as a source of astaxanthin in human dietary supplements at least since 1995. The dietary supplements produced by these companies all contain a lipid extract of *Haematococcus pluvialis* algae. Recommended adult dosages range from 2-12 mg of astaxanthin per day. Human dietary supplements containing *Haematococcus* astaxanthin are marketed worldwide, which was approved by the United States Food and Drug Administration (USFDA) as a New Dietary Ingredient and launched in 1999, is now marketed for use in dietary supplements in at least 20 other non-EU countries. They are no reports of adverse reactions to any *H. pluvialis*-containing supplements after ten years of regular human consumption of such products till date.

Biotechnological approach for effective production of astaxanthin is through microalgae *Haematococcus pluvialis*, which can produce higher amount of astaxanthin and its esters along with other xanthophylls under manipulated condition. Details of the accumulation of astaxanthin and its esters during stress conditions and utility of astaxanthin is the aim of present research work. This study was undertaken with the following objectives. The aspects of safety and efficacy of the micro algal biomass and isolated astaxanthin and its esters is also borne in mind to add value to production and utilization of algal biomass in an eco friendly manner.

1.9. Objectives of the present study

- To study expression of astaxanthin production and enhancing the yield from cultured green alga *Haematococcus pluvialis.*
- To study the effect of expressed astaxanthin from *Haematococcus pluvialis* on biological activities, using *in vitro* and *in vivo* models.
- Evaluation of safety of *Haematococcus pluvialis* biomass in experimental animals.

CHAPTER-1

PRODUCTION OF ASTAXANTHIN FROM VARIOUS CULTURE CONDITIONS IN *H. PLUVIALIS*

2.0. Background

Microalgae are cultivated in autotrophic conditions to exploit their photosynthetic potential for production of value added products in an economical way. They are able to grow in autotrophic, mixotrophic and heterotrophic conditions. Their growth in autotrophic conditions is more advantageous because the organism can be grown with minimal contamination. Growth can be achieved in outdoor conditions and it is more economical than heterotrophic conditions. The adaptability of the organism to different environmental conditions varies among different microalgae. *Haematococcus pluvialis,* a green microalga is able to grow in both heterotrophic and autotrophic conditions. It has two distinct phases in its life cycle (i) motile vegetative growth phase and (ii) non-motile encysted carotenoid accumulation phase. Both the phases differ in their optimum culture conditions. *Haematococcus pluvialis* when experience environmental stress, such as high light intensity, phosphate deprivation, they differentiate from a vegetative stage to form aplanospores in a resting stage. At this point the cell volume increases, producing a hard cellular wall that accumulates the red coloured xanthophylls, astaxanthin and its fatty ester derivatives. *Haematococcus* can be grown in autotrophic, mixotrophic and heterotrophic conditions. It is grown autotrophically, with inorganic carbon, mixo-hetero-trophically using an organic carbon source, such as acetate. It is grown under nutrient-sufficient conditions and low average irradiance, as to achieve a high biomass yield.

Downstream processing represents a major issue in microalgal biotechnology. The biomass need to be separated from liquid media, which is usually done by centrifugation, although several alternative methods can be used, like flocculation, filtration, etc. (Molina-Grima et al., 2004). Harvesting of biomass is likely to remain as an active area of research, where experience has demonstrated that for every algal strain, it is possible to develop an appropriate harvesting system. The quality of the product is relevant to select the proper method. Further, the biomass needs to be quickly processed to avoid spoiling. Cell disruption can be performed by chemical methods (alkaline lysis, solvents) or mechanically (homogenizers, beads, ultrasound). Once more, the choice will depend on the microalgae and product obtained. Mendes-Pinto et al., (2001) have analyzed the recovery of astaxanthin from the hard-cell-

walled haematocysts of *Haematococcus* using different methods. The best result was reported in autoclaved and mechanically disrupted biomass, with the yield of astaxanthin being three fold higher than by other methods. In this context, it may be interesting to mention that the reddish, actively growing *Haematococcus* cells, prevalent in the biomass is generated by the one-step continuous photoautotrophic culture system (Del Río et al., 2005a), are rather more fragile than haematocysts and break more easily. Astaxanthin bioavailability and extraction is thus facilitated in this type of biomass (Del Río et al., 2005b). Ultrasonication of suspended microalgal cells can be used to distrupt small amount of biomass, but this cannot be applicable for large scale (Bermejo Roman et al., 2001). Hence it may not require such hard disruption process, however gentle shaking or vibration should be able to extract all the constituents. Solvent extraction of microalgal constituents is widely used to extract metabolites like, astaxanthin, lutein, β-carotene and essential fatty acids. Solvents like, hexane, ethanol, chloroform, diethylether are employed for extraction of fatty acids such as ecosapentanoic acid (Grima et al., 2003). In view of this background, the present study focused on influence of different nitrogen, salt and carbon sources on growth, total carotenoids, and astaxanthin production in *Haematococcus pluvialis* for maximizing the yields.

2.1. Materials and Methods

2.1.1. *H. pluvialis*

H. pluvialis (SAG 19-a) was obtained from Sammlungvon Algenkulturen, Pflanzen Physiologisches Institut, Universitat, Gottingen, Gottingen, Germany. Stock cultures were maintained in autotrophic bold basal medium (Usha et al., 1999). Cultures were incubated at 25 ± 1 °C under a continuous light intensity of 1.5 klux.

2.1.2. Glasswares

All the glasswares used for experiment viz., conical flasks, culture tubes, culture bottles, measuring cylinders, volumetric flasks and etc, were from Borosil or Vensil Ltd., Mumbai, India. Chemicals used for indoor cultivation are from Loba Chemicals Ltd (Mumbai, India).

2.1.3. Stock culture maintenance

Stock cultures of *H. pluvialis* were maintained on both semisolid and liquid autotrophic Bold's basal medium (BBM), (Kanz and Bold, 1969). The composition of the BBM is provided in the below **Table 13.**

2.1.4. Normal growth condition

The inoculated slant and liquid cultures were incubated in culture room under controlled temperature at 25 ± 1°C and light intensity of 1.5 klux. Light was provided by cool white fluorescent set of lamps (40W; Phillips India Ltd, Kolkata, India) and the light intensity was measured using lux meter (TES 1332, Taiwan).

Table 13. Composition of Bold basal medium

Components	**g/L**
$NaNO_3$	0.25
K_2HPO_4	0.075
KH_2PO_4	0.175
$MgSO_4.7H_2O$	0.073
$CaCl_2.2H_2O$	0.024
NaCl	0.025
$FeSO_47H_2O$	0.005
EDTA	0.05
KOH	0.031
Trace elements	1 ml
PH	7.0
***Trace elements** (for preparation of 1L stock solution)	
H_3BO_3	11.42
$ZnSO_4.7H_2O$	8.82
$MnCl_2.4H_2O$	1.44
MoO_3	0.71
$CuSO_4.5H_2O$	1.57
$Co(NO_3)_2$	0.49

The above medium was solidified with agar (15g/L) to prepare slants.

The media was distributed into 150 ml conical flasks, closed with cotton plugs and sterilized by autoclaving at 121°C for 20 min and allowed to cool at room temperature before inoculation. Inoculation was carried out under aseptic conditions in laminar air

flow hood. The *H. pluvialis* slants and the liquid cultures were sub cultured at every 4 week and 2 week intervals respectively.

2.1.5. Growth in carbon source enriched condition

The two-tier vessel consisting of two 250 ml narrow neck Erlenmeyer flasks (Husemann and Barz, 1977) was used for enriching CO_2 in the culture environment. Upper compartment of the flask contained 50 ml culture and the lower compartment of the flask contained 100 ml of 3M buffer mixture ($KHCO_3/K_2CO_3$) at specific ratio, which generated a partial pressure of CO_2 at 2% in the two-tier flask (Tripathi *et al.*, 2001b). The mouths of the upper and lower compartments were sealed tightly using cotton plug and parafilm.

2.1.6. Growth measurement

2.1.6.1. Dry weight

Known volume of culture was centrifuged; the algal biomass was washed with distilled water and dried in a hot air oven (Sanyo, Electrical Biomedical Co. Ltd., Japan) at 60°C till constant weight was attained. Biomass weight was expressed as dry biomass (g/L).

2.1.6.2. Chlorophyll content

Known quantity of *H. pluvialis* cells were taken into graduated tube and centrifuged at 5,000 rpm for 15 min. The lower part of the pellet was used for extraction of chlorophyll. This pellet was extracted in acetone by homogenizing using pestle and mortar. This was subjected to vortexing for few minutes followed by centrifugation under low light at 3,000 rpm for 10 minutes. The absorbance of supernatant was measured spectrophotometrically (Shimadzu 160A, Japan) at 645nm and 661.5nm against acetone blank.

Concentration of chlorophyll a, b total content were calculated by the equation of Lichtenthaler (1987) and expressed as (µg/ml) content

$$\text{Chlorophyll a} = 11.24 \times \text{O.D}_{661.5} - 2.04 \times \text{O.D}_{645}$$

$$\text{Chlorophyll b} = 20.13 \times \text{O.D}_{645} - 4.19 \times \text{O.D}_{661.5}$$

$$\text{Chlorophyll a +b} = 7.05 \times \text{O.D}_{661.5} + 18.09 \times \text{O.D}_{645}$$

2.1.6.3. Total carotenoid and astaxanthin content

The total carotenoids were extracted in acetone as mentioned above and analyzed spectrophotometrically by measuring the absorbance at 470 nm (Lichtenthaler, 1987).

Astaxanthin content was determined at 480 nm using an absorption coefficient, A_1% of 2500 by the method of Davies (1976).

Carotenoids content (mg/vol.) = (OD_{450} x volume of the sample taken x dilution of the sample x 10)/2500

Astaxanthin content (mg/vol) = (OD_{480} x volume of the sample taken x dilution of the sample x 10)/2500

2.2. Influence of different stress conditions (carbon, salt, nitrogen) on the production of astaxanthin.

2.2.1. Effect of different nitrogen concentration on growth and astaxanthin production

The experiment was carried out in Erlenmeyer flasks (250 ml) containing 80 ml of Bold basal medium with different nitrogen sources (10, 15, 20, 25, and 30 mM) such as sodium nitrate, potassium nitrate, ammonium nitrate, and calcium nitrate in terms of nitrogen equivalent to potassium nitrate, which is a nitrogen source in Bold basal medium to study their effect on *H. pluvialis* growth and astaxanthin production. One set of cultures with different nitrogen sources grown for two weeks were subjected to stress by adding 0.2% NaCl together with 4.4 mM sodium acetate and the cultures were further incubated for a period of two weeks at 25 ± 1 ^oC with 1.767 erg m^{-2} s^{-1} light intensity and 16:8 h light dark cycle. All the experiments were carried out in triplicates.

2.2.2. Effect of different carbon source on growth and astaxanthin production

The experiment was carried out in Erlenmeyer flasks (250 ml) containing 80 ml of Bold basal medium with different carbon sources (3, 5, and 7 mM) such as potassium hydrogen carbonate, ammonium carbonate, sodium carbonate, di potassium carbonate, and sodium bicarbonate in terms of carbon source equivalent to ammonium carbonate in Bold basal medium to study their effect on *H. pluvialis* growth and astaxanthin production. One set of cultures with different nitrogen sources grown for two weeks were subjected to stress by adding 0.2% sodium chloride together with 4.4 mM sodium acetate and the cultures were further incubated for a period of two weeks at 25 ± 1^oC with 1.767 erg m^{-2} s^{-1} light intensity and 16:8 h light dark cycle. All the experiments were carried out in triplicates.

2.2.3. Effect of different salt concentration on growth and astaxanthin production

The experiment was carried out in Erlenmeyer flasks (250 ml) containing 80 ml of Bold basal medium with different salinity concentrations (16 mM, 32 mM, and 64 mM) such as potassium chloride, sodium chloride, calcium chloride, magnesium chloride, manganese chloride, ammonium chloride and cobalt chloride equivalent to sodium ions, which is a salt source in Bold basal medium to study their effect on *H. pluvialis* growth and astaxanthin production. The culture flasks were incubated for 3 weeks at 25 ± 1^{o}C with 1.767 erg m^{-2} s^{-1} light intensity and 16:8 h light dark cycle. The studies involved three replications for analyses.

2.3. Downstream processing of carotenoids from *H. pluvialis*

2.3.1. *Haematococcus* biomass extraction in different solvents

Carotenoids were extracted from freeze dried samples of *Haematococcus* biomass using mixture of polar and non-polar solvents. Five volumes of solvent was taken with the sample (100 mg) in mortar along with glass powder and subjected for mechanical grinding for 2-3 minutes and the same was transferred into Borosil glass tubes and sonicated using MS-72 probe producing diameter 2.0 mm, with 70G horn of amplitude 200μm_{ss} (Bendalin, Sonoplus ultrasonic, Homogenizer, CE electronics, Berlin). This process was repeated 2-3 times or till the sample is colorless. Whole process was carried out under yellow light in order to minimize the loss due to photo degradation. This was centrifuged at 3,000 rpm for 5 minutes and the supernatant was collected and absorbance was measured at 480 nm spectrophotometrically. The solvents used were acetone, methanol, ethanol, hexane, petroleum ether and chloroform.

2.3.2. *Haematococcus* extraction in different edible oils

Extraction of carotenoids from oil was done as given in chapter-II. Freeze dried, *Haematococcus* biomass were mixed with edible oils such as coconut, gingilly, palm oil, olive, groundnut, rice bran, mustard and sunflower oils in the ratio of 1:4, vortexed for 3 min and kept under low light for 2-3 h followed with extraction by pulsed sonication for 4-5 min. Centrifuged at 5,000 rpm for 10 minutes and supernatant was decanted. Carotenoid content in supernatant was measured immediately using optical density at 480 nm spectophotometrically.

2.4. Results and Discussion

2.4.1. Maintenance of *H. pluvialis* stock culture

Haematococcus pluvialis stock cultures were maintained on agar slants of Bold's basal medium (BBM) as explained in materials and methods. Liquid cultures were maintained under normal culture condition (Fig. 14) and after 15 days of growth period, exposed to 3.5 Klux to induce carotenoid accumulation.

Fig. 14. Maintenance of *H. pluvialis* culture

2.5. Influence of different nutrient conditions (nitrogen, carbon, salt) on the production of biomass, total carotenoid and astaxanthin.

2.5.1. Influence of nitrogen source on growth of *H. pluvialis* culture

Nitrate is the major source of nitrogen for algae (Borowitzka and Borowitzka, 1998), essential for the formation of the protein, amino acids and other vital components of plants and algal cells. Effect of different concentrations 10 mM, 15 mM, 20 mM, 25 mM and 30 mM sources of nitrogen such as sodium nitrate, potassium nitrate, ammonium nitrate, and calcium nitrate in cultures grown for 3 weeks were studied (Fig. 15). Addition of NaCl 0.2% together with sodium acetate 4.4 mM showed differences in biomass yield, total carotenoid and total astaxanthin production.

Fig. 15. Effect of nitrogen sources on growth in *H. pluvialis*

2.5.2. Effect of different nitrogen sources on biomass yield, total carotenoid and astaxanthin production in *H. pluvialis*

Among the nitrogen sources, sodium nitrate at 25 mM concentration influenced high biomass yield 3.3 g/L, total carotenoid content of 2.9% and total astaxanthin content of 2.5% on 21st day (Fig. 16-18).

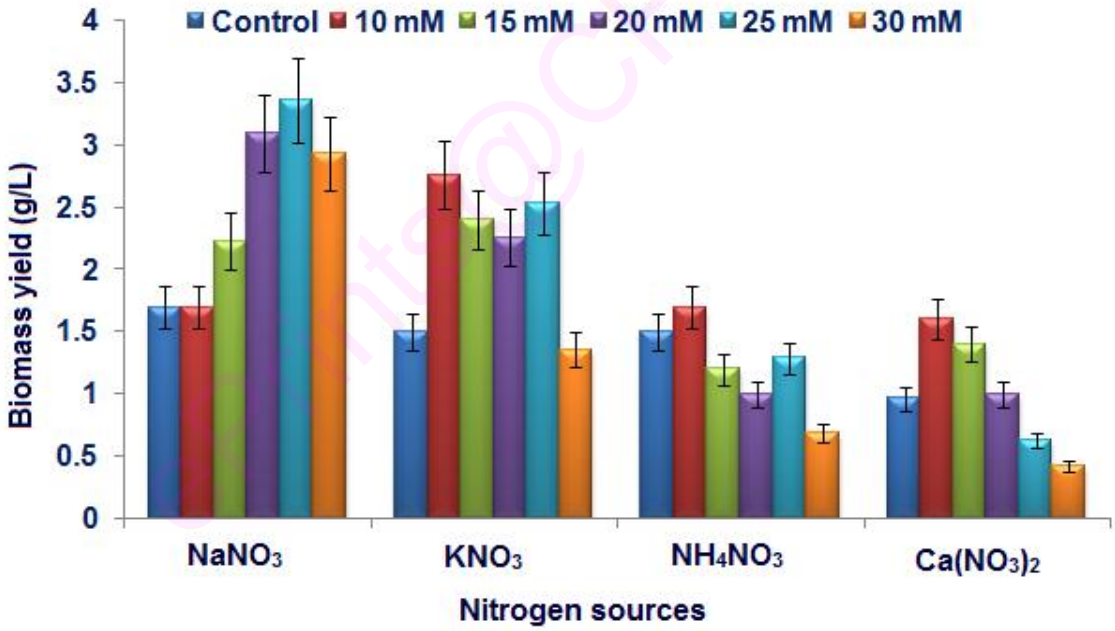


Fig. 16. Effect of different nitrogen sources on biomass yield in *H. pluvialis*

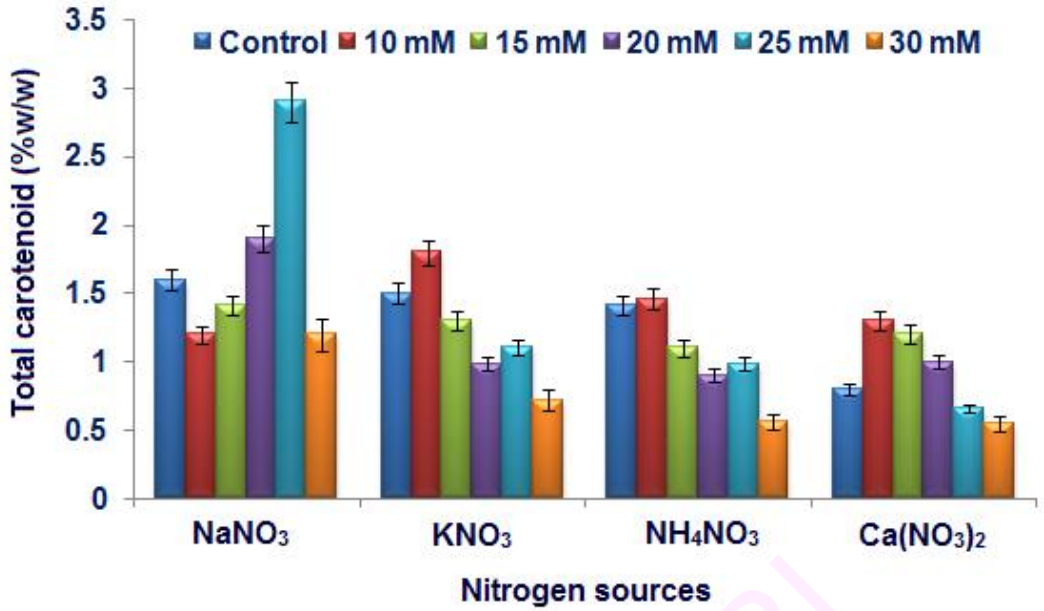


Fig. 17. Effect of different nitrogen sources on total carotenoid content in *H. pluvialis*

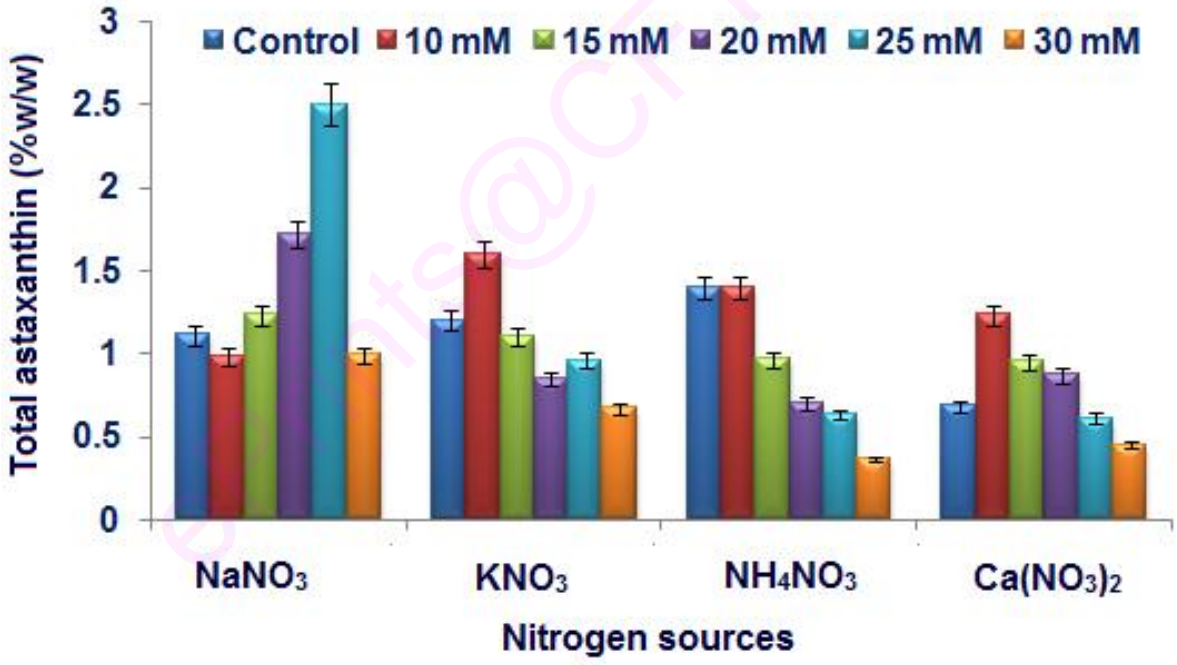


Fig. 18. Effect of different nitrogen sources on total astaxanthin production in *H. pluvialis*

2.5.3. Effect of nitrogen sources on carotenoid composition by HPLC

Influence of nitrogen source such as sodium nitrate, potassium nitrate, ammonium nitrate, and calcium nitrate at 10 mM, 15 mM, 20 mM, 25 mM and 30 mM concentration was studied on carotenoid composition and astaxanthin production in *H. pluvialis*. In the order of elution through a C_{18} column, the carotenoids,

xanthophylls and astaxanthin esters were separated within 30 min. The maximum carotenoid composition was found to contain neoxanthin 0.7%, violaxanthin 0.9%, free astaxanthin 3.2%, lutein 0.9%, zeaxanthin 4.2%, β-carotene 2.1% and astaxanthin ester 88% was observed in sodium nitrate treated culture at 25 mM concentration, which was generally higher that in control culture & other treatments.

2.6. Influence of carbon sources on growth of *H. pluvialis* culture

Influence of different concentrations (3 mM, 5 mM and 7 mM) of sodium bicarbonate, potassium hydrogen carbonate, sodium carbonate, dipotassium carbonate, ammonium carbonate and its effects on growth, biomass yield, total carotenoid and total astaxanthin content were studied for 3 weeks are shown in **Fig. 19.**

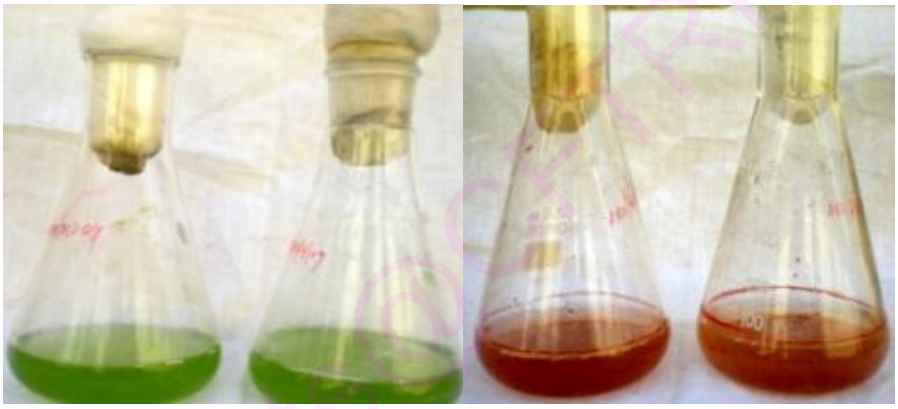


Fig. 19. Effect of carbon source on growth of *H. pluvialis* culture.

2.6.1. Effect of carbon source on biomass yield and carotenoid composition in *H. pluvialis*

After 3 weeks incubation of the culture the biomass yield, total carotenoid and total astaxanthin content were observed. Among the carbon sources, ammonium carbonate at 3 mM concentration influenced maximum biomass yield of 2.9 g/L, total carotenoid 2.6% (w/w) and total astaxanthin content 2.2% (w/w) (**Fig. 20-22**). The relative percentage of xanthophylls, carotenoids and esters such as neoxanthin 0.3%, violaxanthin 0.7%, free astaxanthin 2.6% lutein 1.3%, zeaxanthin 3.5%, α-carotene 0.6%, β-carotene 1% and astaxanthin esters 90% were recorded in ammonium carbonate and sodium carbonate treated culture at 3 mM concentration.

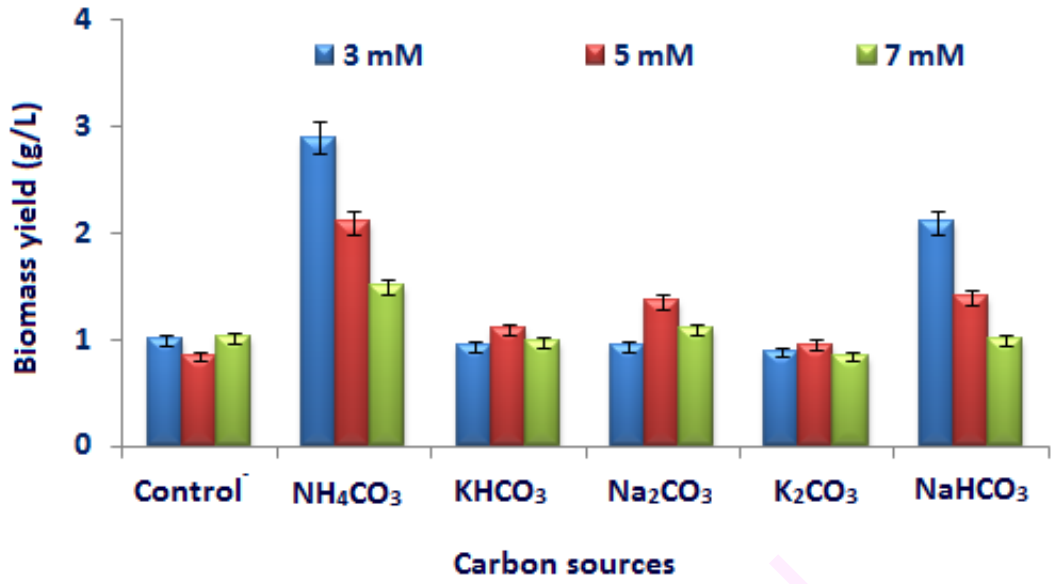


Fig. 20. Effect of carbon sources on biomass yield in *H. pluvialis* culture

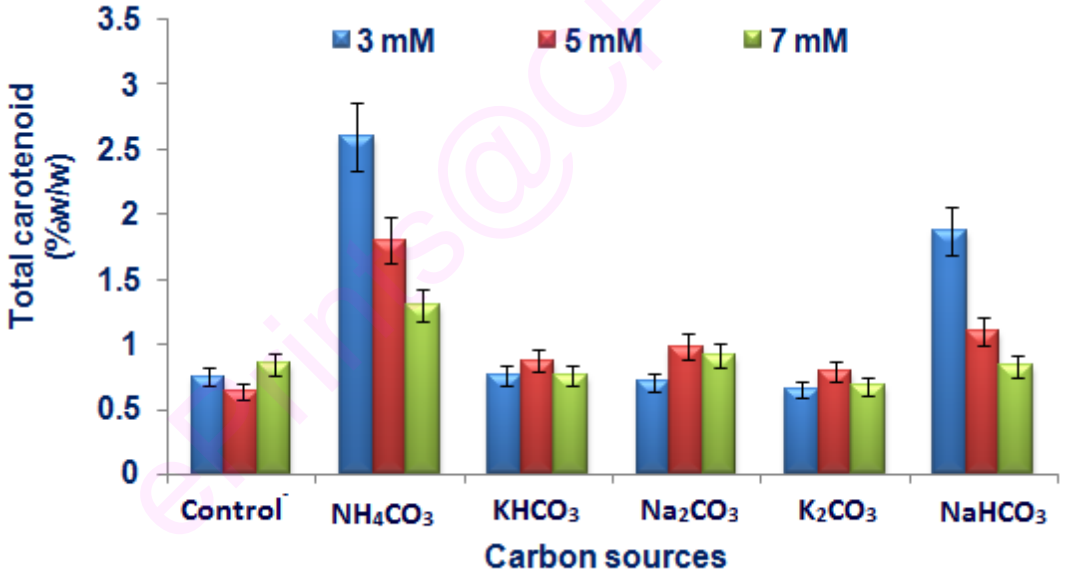


Fig. 21. Effect of carbon sources on total carotenoid content in *H. pluvialis*

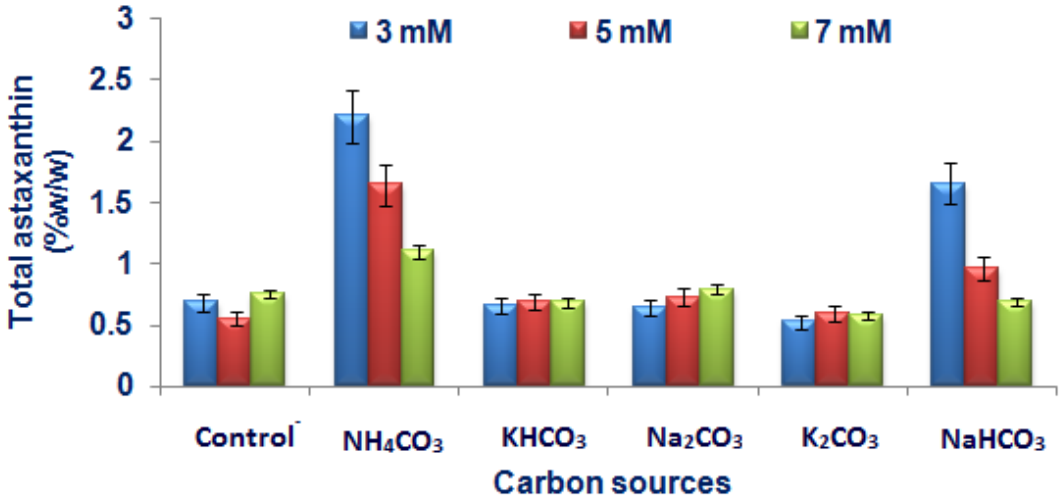


Fig. 22. Effect of carbon sources on total astaxanthin content in *H. pluvialis.*

2.7. Effect of salt stress on growth of *H. pluvialis* culture

It was necessary to determine the optimal levels of salt stress for culture of algae. The structure of the cell membrane is the one, which makes it more comfortable to adapt to range of salt content. Even though algae can survive in range of sodium chloride medium, it can multiply well in particular range of salt concentration. Influence of different salt concentrations (16 mM, 32 mM and 64 mM) of potassium chloride, sodium chloride, calcium chloride, magnesium chloride, manganese chloride, ammonium chloride and cobalt chloride and its effects on biomass yield, total carotenoid and total astaxanthin were studied in *H. pluvialis* culture (**Fig. 23).**

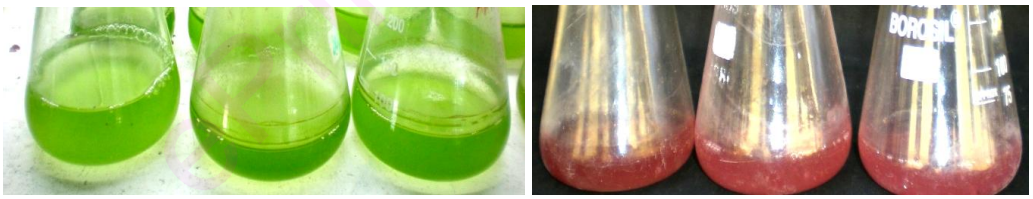


Fig. 23. Effect of salt stress on growth in *H. pluvialis* culture

2.7.1. Effect of different salt stress on biomass yield, total carotenoid and total astaxanthin content in *H. pluvialis*

Among the salt stress, potassium chloride at 16 mM influenced maximum biomass yield 2.5 g/L, total carotenoid 2% (w/w) and total astaxanthin levels, 1.97% on 21st day (Fig.s 24-26)

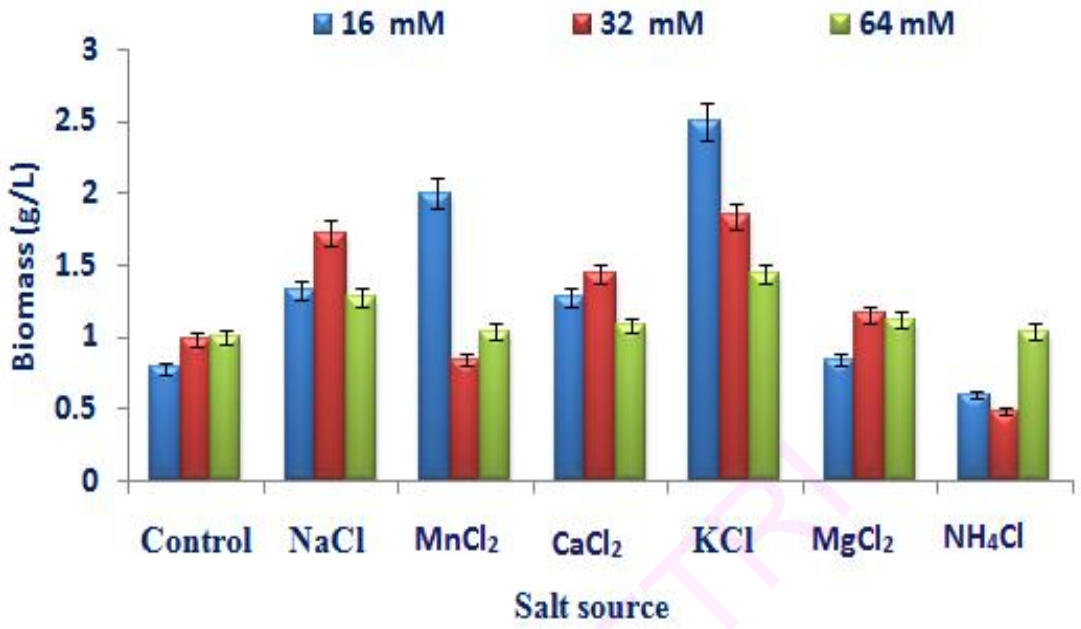


Fig. 24. Effect of different salt source on biomass yield in *H. pluvialis*

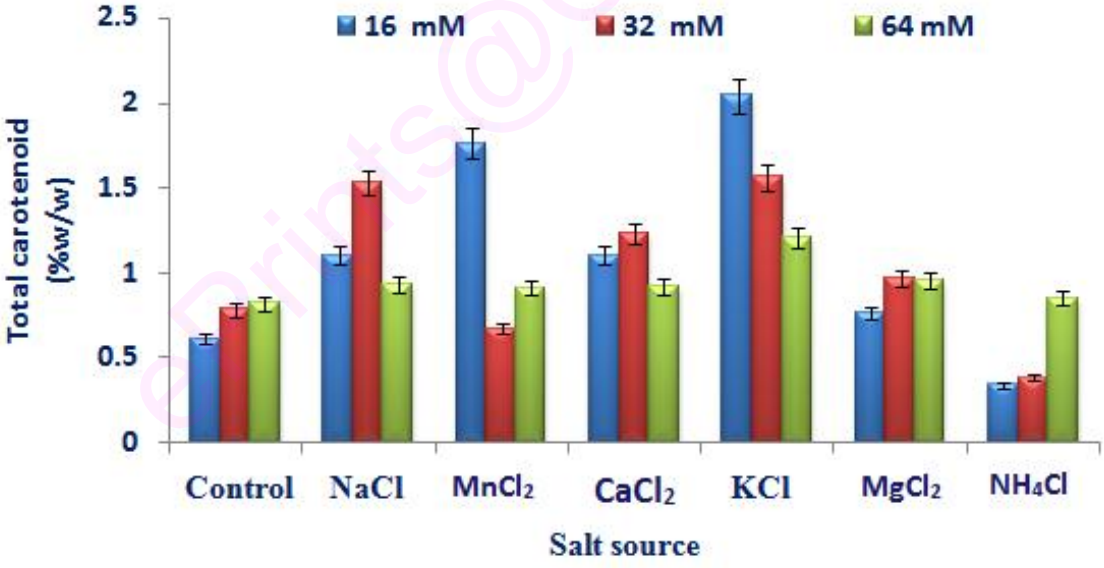


Fig. 25. Effect of different salt source on total carotenoid in *H. pluvialis*

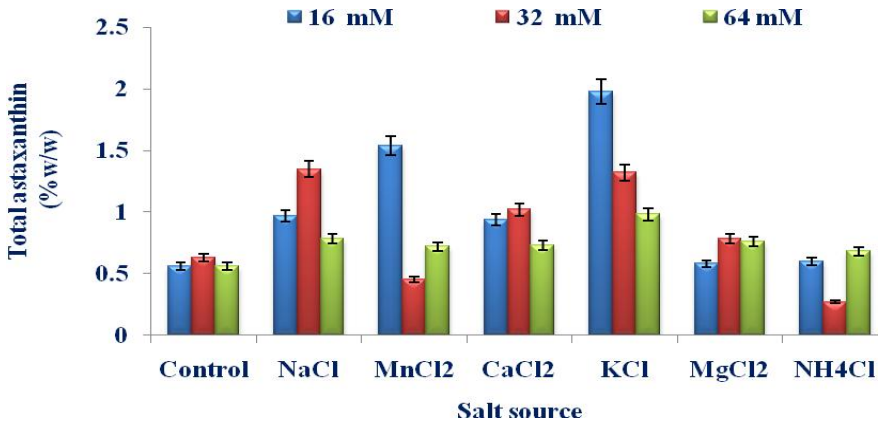


Fig. 26. Effect of different salt source on total astaxanthin in *H. pluvialis*

2.7.2. Effect of salt stress on carotenoid composition by HPLC

Influence of different salt stress at 16 mM, 32 mM and 64 mM concentration of potassium chloride, sodium chloride, calcium chloride, magnesium chloride, manganese chloride, ammonium chloride and cobalt chloride on carotenoid composition in *Haematococcus* was evaluated. The higher carotenoid composition was found to be neoxanthin 0.2%, violaxanthin 0.8%, free astaxanthin 3.6%, lutein 1.8%, zeaxanthin 6.2%, *β*-carotene 1.1%, and astaxanthin esters 85.9% in potassium chloride treated culture with 16 mM KCl concentration, when compared to control culture & other treatments

2.8. Extraction of carotenoids from *H. pluvialis* biomass using different organic solvent systems

Different solvents acetone, petroleum ether, ethylacetate, methanol, hexane, hexane: acetone (1:1), hexane: isopropyl alcohol (1:1), chloroform used for extraction of carotenoids from best medium of dry biomass of *H. pluvialis*. Among the different solvents, ethylacetate and acetone:hexane could extract more carotenoids, where as methanol extract had chlorophylls contained for extraction of carotenoids. Acetone, chloroform, ethylacetate, n-hexane, n-hexane:isopropyl alcholol (1:1) extracted carotenoids and xanthoplylls. Content of relative percentage of carotenes extracted is as shown in Table 14.

Table 14. Extraction of carotenoids using different solvents in *H. pluvialis* biomass.

Solvent	Relative % of extractability
Ethylacetate	98.54[a]
Isopropyl alchol:hexane (1:1)	90.34[b]
Methanol	51.23 [a]
Chloroform	45.67[e]
Acetone	78.34[c]
Petroleum ether	62.81[d]
n-Hexane	65.42[d]
Acetone:n-hexane (1:1)	45.47[e]

Values are mean ± SD (n=3). Values are not sharing a similar superscript within the same column are significantly different (P<0.05) as determined by ANOVA.

2.8.1. Extraction of carotenoids from *H. pluvialis* using edible oils

Extaction of carotenoids by dry biomass of *H. pluvialis* using different edible oils was tried in order to have oil based carotenoids formulations. Results revealed maximum extractability of carotenoids was in palm oil 51.29 µg/g followed by olive oil 47.26 µg/g. Amount of carotenoids extracted by various edible oils are shown in Table 15. The results showed that carotenoids were extractable in oil by repeated extraction process.

Table 15. Extraction of carotenoids in *H. pluvialis* biomass using edible oils

Edible oil	Amount of carotenoid extract (µg/gram)
Mustard oil	31.21[e]
Sunflower oil	39.80[c]
Olive oil	47.26[b]
Groundnut oil	35.53[d]
Ricebran oil	40.91[c]
Gingelly oil	36.28[d]
Coconut oil	30.15[e]
Palm oil	51.29[a]

Values are mean ± SD (n=3). Values are not sharing a similar superscript within the same column are significantly different (P<0.05) as determined by ANOVA.

2.9. Discussion

Microalgae, in general, have the ability to use different organic and inorganic nitrogen compounds as nitrogen, carbon and salt source. Commercial production of β-carotene by the green halotolerant microalga *Dunaliella* has been successful in open ponds and resembles the process of biomass production of *Spirulina* practiced in several countries (Borowitzka, 1992). However, little research has been performed on the scale up of astaxanthin production in *Haemtococcus* for several reasons viz. Slow growth, low growth temperature and light requirement (Kobayashi et al., 1991). *H. pluvialis* can grow as heterogeneous culture which utilizes some other organic carbon sources in small amount and acetate is commonly used as a complementary organic carbon source (Chen et al., 1997, Dong and Zhao, 2004). Astaxanthin production from *Haematococcus* is difficult to compare, due to differences in culture condition, strains, salt stress etc. Little information is available on effect of different nitrogen sources on growth, astaxanthin production in *Haematococcus.* In the present studies the effect of different nitrogen sources on growth, biomass yield, total carotenoid and astaxanthin production in *Haemtococcus* was evaluated.

Nitrate concentration plays very important role in the cell division rate and in the accumulation of secondary carotenoids of *Haematococcus* (Boussiba and vonshak, 1991). The synthesis of astaxanthin requires nitrogen, and most likely reflects the need for continuous synthesis of protein in order to support the massive accumulation of the pigment. Nitrogen is an effective way to enhance astaxanthin accumulation and other xanthophylls in *Haematococcus* (Fig. 15-18). The ratio of chlorophyll and carotenoids could be a good indicator of the physiological state of the culture as observed previously by Droop (1954) and Borowitzka et al., (1991). The present study showed the enhancement of the biomass yield by 1.9 fold, total carotenoid content 2.2 fold and total astaxanthin content 1.8 fold higher, when compared to control at 25 mM concentration of sodium nitrate. The carotenoid composition was evaluated in various levels of nitrogen treated culture showed differences. However, the effect of nitrogen was concentration dependent, higher concentrations inhibited growth and markedly increased astaxanthin content in *Haematococcus.* Gong and Chen (1998) reported the influence of several medium components such as nutrients, trace elements and nitrogen source which increased astaxanthin content in *Haematococcus*. Fabregas et al., (2003) reported that nitrogen deficiency played a

greater effect than high light intensity on astaxanthin synthesis in flagellated cells. Some studies showed that *Phaffia rhodozyma* can utilize many kinds of nitrogen sources including ammonium salts, nitrates and organic nitrogen sources (Johnson, 1991; Du et al., 2005).

In another set of experiment, influence of different carbon sources such as sodium bicarbonate, potassium hydrogen carbonate, sodium carbonate, di-potassium carbonate, and ammonium carbonate were studied in *H. pluvialis* and their effects on biomass yield, total carotenoid content and astaxanthin content was observed for a period of three weeks. Among the carbon sources, in ammonium and sodium bicarbonate increased the biomass yield by 2-3 fold and total carotenoid content by 2 fold in *H. pluvialis* at 3 mM concentration. Astaxanthin content increased in ammonium carbonate source provided when compared to control culture (Fig. 19-22). Light, temperature and inorganic carbon availability are the most important factors controlling the photosynthetic productivity of algal system. Carbon is the major element (40-50%) in algal biomass, which in turn meets the requirement of inorganic carbon (Weissman, 1988). Ding et al., (1994) reported that under steady state conditions astaxanthin content in *H. pluvialis* was 0.41%. Droop (1954) and Borowitzka et al., (1991) reported that the acetate appeared to be an important carbon source, enhancing both growth and carotenogenesis. However, the effect of acetate was concentration dependent, higher concentrations inhibiting growth but markedly increasing astaxanthin content (Kakizono et al., 1992). Cordero et al., (1996) reported astaxanthin production with different stress conditions where the alga *Haematococcus* was cultivated in a bioreactor employing two stage cultivation processes. Zych et al., (2009) reported that the assimilation of individual organic compounds (5 mM sugars and L-asparagine) under mixotrophic growth conditions in *Haematococcus* strains. The results obtained in the present study clearly indicated that stress is a cumulative factor of the physiological state of the culture and the culture conditions. Enhanced astaxanthin production under stress conditions precedes drastic chlorophyll degradation. Sodium nitrate, potassium chloride and ammonium carbonate were more effective for the formation of astaxanthin in *Haemtococcus* culture. Acetate supplementation along with salinity further enhanced astaxanthin production in *Haematococcus*

In potassium chloride treated culture the biomass yield increased by 3.5 fold, total carotenoid by 3.3 fold and astaxanthin content by 3.1 fold at 7 mM concentration, when compared to control culture (Fig.s 23-26). The accumulation of astaxanthin in cysts cells under stress conditions has been reported both in the dark (Kobayashi et al., 1997b) and in the light conditions (Spencer, 1989, Borowitzka et al., 1991, Harker et al., 1995). Harker et al., (1995) reported that the addition of sodium chloride to *H. pluvialis* culture, improved the astaxanthin yield. Sarada et al., (2002a) found that the age of culture was crucial to trigger astaxanthin production in salt stress induced cultures. The total astaxanthin and carotenoid contents were increased in *H. pluvialis* during stress conditions reported by Usha et al., (1999). The total carotenoid accumulation produced in the best condition in the present study, was 2.9% w/w, and astaxanthin accumulation of 2.5% w/w. Under stress conditions the chlorophyll content decreased drastically in culture. The results indicated that the response to stress varied with media pH-7.0 being optimum for astaxanthin production.

Extraction of astaxanthin from encysted cells is a critical step in downstream processing as the cells consisted of thick cell wall which hinders solvent extraction of astaxanthin from intact encysted cells of *Haemtococcus*. Downstream processing accounts for the main cost of the product. The methodology to be employed for the process also decides the choice of downstream technique. As explained earlier drying of the biomass will lead to loss of carotenoid content. For the therapeutic supplementation of astaxanthin at the rate of 10-25 mg astaxanthin/day, direct oil extraction method can be employed in oils which take up carotenoids upto 1200 ppm. Different methods have been tried to extract carotenoids from algal biomass, including direct extraction into edible oils, extraction in organic solvents and continuous extraction using different solvents with biphasic systems, without compromising the viability of cells (Hejazi *et al.*, 2001). There are several reports on the treatment of cells to enhance the extractability and bioavailability of astaxanthin. Kobayashi et al., (1997) evaluated different conditions for selective removal of chlorophyll and extraction of astaxanthin from *Haematococcus* cells. They recovered 70% astaxanthin when red cysts were treated with 40% acetone at 80 °C for 2 min followed by lyophilization or enzymatic treatment. Mendes-Pinto et al., (2001)

compared the effect of different cell disruption processes like acid, alkali, and enzyme treatment, autoclaving for 30 min, at 121 °C, spray drying inlet 180 °C, outlet 115 °C, and mechanical disruption methods for extraction of astaxanthin. They found that autoclaving and spray drying treatments facilitated cell disruption by ultra sonication, thereby resulting in better astaxanthin recovery (85%) which is otherwise resistant to disruption by ultra sonication alone. They also reported that enzymatic treatment of *Haematococcus* cells exposure to alkali or acid resulted in a significant loss (20-35%) of total carotenoids as a direct result of processing. Bubrick (1991) described grinding of dried *Haematococcus* biomass at cryogenic temperature (170 °C) in the presence of butylated hydroxytoluene. Boussiba et al., (1992) described treatment of cyst cells with 5% KOH in 30% methanol to destroy the chlorophyll, and extraction with DMSO with a few drops of glacial acetic acid by homogenization and heating at 70 °C for 10 min to recover astaxanthin. Although Mendes-Pinto et al., (2001) reported treatment with HCl for 15 and 30 min at room temperature, they could achieve efficient extraction of astaxanthin only after sonication. Sarada et al., (2006) reported that 90% extractability of astaxanthin resulted when treated with HCl at 70 ^{o}C alone without homogenization. An ideal extraction procedure should employ a solvent, which can extract more pigment while being sufficiently volatile so that it can be easily removed without causing significant degradation of the compounds. Therefore in order to make a concentrate of carotenoids it was found that the best method was to use a solvent for extraction and resuspend in oil. Present process utilizes ethylacetate which is capable of extracting maximum content of carotenoids and can be easily removed so as to suspend the extract in oil, which acts as a stable carrier matrix. Among the solvents ethylacetate and n-hexane: isopropyl alcohol (1:1) showed maximum extractability of carotenoid content data presented in (Table 15 & 16). *Haematococcus* was directly mixed with the vegetable oils and the astaxanthin from cyst cells was extracted with recovery yields of over 87.5% as reported by Kang and Sim (2008). Plam and olive oil showed maximum extractability, which may be due to the chemical nature of these oils, which implies that the chemical nature and natural antioxidant contents in theses oils, is ideal for stability. Further these carotenoids were used to check biological activities such as antioxidant, bioavailability, hepatoprotective properties, and also anticancer activities.

2.10. Salient features

The aim of present investigation was to study the method for culture of *Haematococcus* and factors responsible for growth as well as astaxanthin production. Effect of different carbon, nitrogen sources, salt stress on the growth, total carotenoid and total astaxanthin production were evaluated after culture of the algal cells for three weeks. In sodium nitrate treated culture, increase in biomass accumulation (1.9 fold, total carotenoid 2.2 fold and total astaxanthin 1.8 fold were observed over conventional potassium nitrate treatment. Potassium chloride 7 mM concentration treated culture also registered increase in biomass yield 3.2 fold, total carotenoid 3.5 fold and total astaxanthin 3.3 fold, when compared to control culture. Ammonium carbonate at 3 mM was effective as carbon source which enhanced biomass yield 2.9 fold, total carotenoid 3.2 fold and total astaxanthin 3 fold over the untreated control. Astaxanthin was extracted from *Haematococcus* biomass using various edible oils and solvents. Among the solvents, ethylacetate, isoprophyl alcohol:hexane (1:1) and acetone were found to be efficient. Among the edible oils, the maximum extractability of carotenoids was in palm oil 51.29 µg/g followed by olive oil 47.26 µg/g.

CHAPTER-II

ISOLATION AND CHARACTERIZATION OF ASTAXANTHIN & ITS ESTERS by HPLC, LC-MS AND STABILITY STUDIES

3.0. Background

The present study mainly focuses on isolation, characterization, identification and quantification of carotenoids from *Haematococcus pluvialis* and its stability. The algal biomass obtained from the methodology described in chapter-I, were subjected for quantification of carotenoids and other pigments. These pigments were separated by using preparative TLC and further confirmed by HPLC using standards. Many reported methods of HPLC protocols are able to resolve only few of the carotenoids present in algae on C_{18} silica column. Hence in order to get maximum separation of pigments an isocratic elution methodology was standardized. The pigments extract was subjected to column chromatography using different solvents on silica, alumina and magnesium oxide, and compounds thus separated were identified by absorption maxima in various solvents. Further the carotenoids including xanthophylls were confirmed by mass spectroscopy using positive ion mode as described below. As carotenoids are complex mixtures associated with fatty acids and having almost similar physicochemical properties, it is very difficult to separate, and identify. Hence a number of analytical techniques, like spectrometry, analytical and preparative TLC, column chromatography, preparative HPLC, ESI, and LC-MS positive mode were adopted to confirm them. Further astaxanthin and its esters were confirmed by separating and reliably elucidating the structures of the most commonly occurring isomers by ^{13}C NMR, ^{1}H NMR, HSQC 2D, NOESY 2D and COSY 2D NMR. Apart from that, this section deals with stability of astaxanthin in various edible oils under different temperatures. Stability of carotenoids is most crucial in the process of biological value for the compounds in the form of formulations. Throughout the world, there is growing interest in health foods containing natural bioactive nutrients such as carotenoids and antioxidant vitamins. The loss of carotenoids at room temperature in carrot, orange peel and sweet potato were found to be as high as 60–90% (Cinar, 2003). However, freeze drying of the sample reduced the loss to 50%. Arya et al., (1983) observed only a 32% colour loss in papaya at room temperature. These studies indicated that carotenoid behavior during storage is as important as its stability during processing. Therefore, astaxanthin, being a superior antioxidant, shows great promise but there is very little information on the stability of this compound. As astaxanthin is a lipophilic compound, studies on its stability in the same form would expand its application in food and pharmaceutical formulations. The present study is focused on isolation and characterization of astaxanthin & its esters as

well as stability of a carotenoid extract. Since astaxanthin constituted a major proportion of the total carotenoids the results are expressed in terms of astaxanthin.

3.1. Materials and methods

3.1.1. Chemicals used for experiments

All the media chemicals were of analytical grade obtained from either Qualigens Fine Chemicals, Mumbai or Loba Chemicals, Mumbai, or Himedia, Laboratories, Mumbai. or Ranbaxy Fine Chemicals, Mumbai, India. Chemicals such as ascorbic acid, ferrous sulphate, hydrochloric acid, hydrogen peroxide, α-tocopherol, potassium ferric cyanide, sodium carbonate, potassium iodide, potassium chloride, hydroxylamine hydrochloride, sodium acetate, and ferrous chloride were obtained from Loba Chemicals Ltd (Mumbai, India). Tween-40, folin-ciocaltaeu reagent, ethylene diamine tetra acetic acid and linoleic acid were obtained from Ranbaxy fine chemicals Ltd, (Mumbai, India). Nitroblue tetrazolium, trichloroacetic acid, thiobarbituric acid, butylated hydroxyl anisole , butylated hydroxyl toluene, 1,1-diphenyl-2-picrylhydrazyl, nash reagent, tris-Hcl buffer, egg lecithin standard astaxanthin, violaxanthin, lutein, zeaxanthin, and α, β-carotene were obtained from Sigma Chemicals Ltd., St. Louis, Missouri, USA. HPLC grade solvents acetonitrile, methanol and dichloromethane were obtained from Rankem Chemicals Ltd (Mumbai, India). Analytical grade solvents acetone, hexane, chloroform, methanol, and petroleum ether were purchased from Sisco Chemicals Laboratorary (Mumbai, India),

3.1.2. Extraction of carotenoid from *H. pluvialis*

Known quantity of *Haematococcus* biomass were homogenized and extracted repeatedly with acetone. The absorbance of extracts was read at 450 nm to estimate the content of carotenoid. Astaxanthin was determined at 480 nm using an extinction coefficient of 2500 at 1% level by the method of Davies (1976).

3.1.3. Identification and estimation of carotenoids and chlorophyll

Spectrophotometric determination was done by measuring optical density of astaxanthin at 480 nm and concentration was calculated using extinction coefficient as 2500. In order to know the levels of astaxanthin and chlorophyll, absorption spectrum was analysed using wavelength of 400-800 nm in acetone and also used for quantification.

3.1.4. Identification of carotenoids by TLC

The *H. pluvialis* extract were spotted on silica gel TLC readymade plates (Merck New Delhi, 20×20 cm, 0.5mm thickness) and separated using different solvent

systems as mobile phase including acetone, ethyl acetate, n-heptane and hexane in different proportions (Fiksdahl et al., 1978). Rf values for each spot were calculated and individual spots were scraped from the TLC plate and extracted with acetone or chloroform, absorbance spectra of the same was measured to confirm the compound. Solvent extract of *H. pluvialis* was subjected to preparative TLC using activated silica gel plates (0.5 mm thickness, manually prepared) with various solvent systems like acetone : hexane, acetone :n-heptane:ethylacetate in different proportion.

3.1.5. Seperation of astaxanthin & its esters by TLC

Total carotenoid (TC) from *Haematococcus* biomass was extracted as described previously (Sarada et al., 2002b). Total carotenoid was subjected to preparative thin layer chromatography (TLC) using the solvent system acetone: hexane (3:7 v/v) and, separated astaxanthin mono (MASX), diester (DASX) bands were scraped from TLC plates and resuspended in acetone (Kamath et al., 2008; Yuan and Chen, 1998).

3.1.6. Separation of carotenoids by column chromatography

The total carotenoids from the freezed-dried biomass were extracted using acetone, petroleum ether, hexane, and all the individual extracts obtained were pooled. Partitioning of carotenoids between xanthophylls and carotenes was carried out using different solvents and separated carotene and xanthophylls fractions were subjected to column chromatography (Jungalwala and Cama, 1962). The carotene fraction was resolved on neutral alumina column (45 x 1.5cm), when the column was eluted (gradient elution) with hexane containing increasing amounts of acetone. Three ml each of eluted fractions were collected and their UV and visible spectra were determined.

3.1.7. Identification of carotenoids by HPLC

Carotenoids were analyzed using HPLC (Shimadzu 10AS, Kyoto, Japan) reverse phase 25 cm × 4.6 mm, 5 μm, C_{18} column (Supelco) with an isocratic solvent system consisting of dichloromethane: acetonitrile: methanol (20:70:10, v/v/v) at a flow rate of 1.0 ml/min (Ranga Rao et al., 2009). All the carotenoids were monitored at 450 nm and 476nm with UV- visible detector (Shimadzu, Kyoto, Japan). The peak identification and λ_{max} values of these components were confirmed by their retention times and characteristic spectra of standard chromatograms recorded with a Shimadzu model LC-10AVP series equipped with (SPD–10AVP) photodiode array detector. They were quantified from their peak areas in relation to the respective reference standards.

3.1.8. Identification of carotenoids by MS-ESI positive mode

The whole cell extract in acetone were subjected to direct MS analysis (MS-Qtofultima, No.Qtof GAA 082, Waters Corporation, Manchesters, UK). It was done by positive electro spray ionization (ESI) using time of flight (TOF) mode. Capillary voltage was 7000 V, fragmentation voltage was 70 mV, using drying temperature of 210 ^{o}C. Gas flow was (N_2) was 6.5 ml/min, m/z scan was at the range of 100-1000 daltoms, Scan rate was 1.0 seconds/cycle. *Haematococcus* extracts (100 µl) was injected directly into the system.

3.1.9. Identification of carotenoids by LC-MS (APCI)

The carotenoids were identified in *H. pluvialis* biomass by using the Waters 2996 modular HPLC system (auto-sampler, gradient pump, thermo-regulator and DAD), coupled to a Q-Tof Ultima (UK) mass spectrometer. In brief, APCI source was heated at 130^{o}C and the probe was kept at 500^{o}C. The corona (5 kV), HV lens (0.5 kV) and cone (30 V) voltages were optimized. Nitrogen was used as sheath and drying gas at 100 and 300 l/h, respectively. The spectrometer was calibrated in the positive mode and $[M+H]^+$ ions were recorded. Mass spectra of carotenoids were acquired with an m/z 400-2000 scan range at 450 nm by a diode array detector and confirmed with respective standards. Standard astaxanthin was analyzed by both positive electro spray ionization as well as atmospheric pressure chemical ionization (APCI) in order to know the variation in mass pattern.

3.1.10. Characterization of astaxanthin, mono-diesters from *H. pluvialis* by Nuclear magnetic resonance (NMR)

The occurrence of the astaxanthin & its esters were confirmed by ^{13}C NMR, ^{1}H NMR, HSQC 2D, NOESY 2D and COSY 2D NMR spectra using NMR (Bruker Avance 500 MHz, Germany), UV and MS spectral data.

All the NMR experiments were recorded using Bruker Avance 500 MHz, Germany (500 MHz for ^{1}H and 125 MHz for ^{13}C) at 300K using 5 mg of samples in $CDCl_3$. The NMR spectrometer was controlled HP workstation XW4100, operating with MS Windows Professional V 2002. NMR software TOPSPIN 1.3 software (Bruker Biospin GmbH, Rheinstetten, Germany) in addition ACD NMR version 13.0 professional academic version (free software) was used for data analysis. The acquisition parameters were provided along with spectrum

3.1.10.1. ^{1}H NMR

Proton NMR was recorded by dissolving 5mg of the compound in 500 µl $CDCl_3$. The NMR spectrometer was controlled by an topspin 1.3 software (Bruker Biospin GmbH, Rheinstetten, Germany). ^{1}H NMR spectra were acquired with the pulse program ***zg30***. 32 scans were acquired with a spectral width of 10330.578 Hz, a relaxation delay of 2 s and 64 K time domain data points.

3.1.10.2.^{13}C NMR

The ^{13}C NMR was collected at 125MHz with ***zgdc*** pulse program, number of data points 16K, number of scans 2K, spectral width 26455.027 Hz, FID resolution 1.614687 Hz, acquisition time 0.3097265 sec, receiver gain 16384, dwell time18.900 µsec, pre scan delay 6.00 µsec, relaxation delay 2.00 sec,

3.1.10.3. HSQC

Hetero nuclear single quantum correlation experiment was carried out with *hsqcetgp* pulse program. The experimental parameters are given below. Number of data points 16K, size of fid f2 channel 2K, f1 channel transients 256, number of scans 16, spectral width 10330.578 Hz. FID resolution 5.044228 Hz, acquisition time 0.0992216 sec, relaxation delay 2.00 sec.

3.1.10.4. COSY

^{1}H COSY spectra were recorded with the pulse program *cosygpqf*. 256 transients with 2 K complex data points and a spectral width of 10330.58 Hz were accumulated in the F2 dimension, as well as 256 complex data points in the F1 dimension.

3.1.10.5. NOESY

NOESY spectra was recorded with pulse program *noesyph.* 256 transients with 2 K complex data points and a spectral width of 10330.58 Hz were accumulated in the F2 dimension, as well as 256 complex data points in the F_1 dimension.

3.2. Stability of astaxanthin and its esters from *H. pluvialis*

3.2.1 Standard tocopherol

Tocopherol concentrate (71.2%) was obtained through the courtesy of the Henkel Co. (Kankakee,IL,USA) consisted of α:12.7%, β:1.5%, γ:44.2%, δ:12.8%. Various authenticated edible oils used for experiments were purchased from local market such as ground nut oil (Safal, KOF, Kadugodi, Bangalore, Karnataka), gingelly (N.S.Karthikeyan & Co, Kangayam, Tamilnadu), Palm oil (Palm Sakthi, Mangalore), sunflower oil (Sunpure, M. K.Agrotech Pvt. Ltd, Mandya Dist, Karnataka), mustard (R.R.Omerbhay Pvt.Ltd, Thane, Mumbai, Maharashtra), rice bran oil (Saffola Gold

Ltd, Jalgaon, Maharashtra), coconut (Parachute, Maharashtra), olive oil (Johnson & Johnson, Mumbai, Maharashtra).

3.2.2. Preparation of carotenoid stock solution.

The stock solution of carotenoid was prepared after extraction of carotenoid with acetone from encysted cells of *Haematococcus* followed by complete evaporation of solvent in Rota vapor and dispersion into sunflower oil in concentrated form at 5% (w/w) carotenoid level. Since the carotenoid stock is very concentrated, the amount of sunflower oil (around 2%) added with carotenoid to each oil is insignificant.

3.2.3. Stability of astaxanthin in various edible oils

From the stock solution of the carotenoids, known quantity is dispersed in different edible oils viz coconut, rice bran, groundnut, mustard, gingelly, olive and sunflower at 0.1% (w/w) carotenoid level. At this concentration the oils looked bright red. One set of different oil vials with carotenoid were incubated at room temperature while the second set of vials were incubated in a water bath maintained at 70 ^{o}C and third set at 90 ^{o}C for a period of 8 h. At hourly intervals carotenoid content was estimated in the heat treated samples. After the heat treatment, the oils were stored at room temperature for a period of 3 months. Stability of carotenoid in oils was analyzed in terms of content, profile and colour value. Stability of astaxanthin in oils was studied at 120 and 150 ^{o}C in Sanyo MOV 212F convection oven for a period of 8 h. At regular intervals of 2 h, samples were taken for carotenoid estimation. All the treatments were carried out in triplicates and the average values are presented.

3.2.4. Extraction of astaxanthin from oils

The carotenoid from the oils was extracted after saponification and separation on silica column. The carotenoid fraction was eluted by isooctane: ethyl acetate (95:5) ratio (Koswig et al., 1990). Carotenoid profile was analyzed by TLC and HPLC and the profile was compared with the extract from *Haematococcus* cells as described earlier 3.1.3 & 3.1.7.

3.2.5. Estimation of peroxide value

Peroxide value in different oils both in presence and absence of carotenoid was estimated by titrimetric method and expressed in terms of milliequivalents of active oxygen per kg oil as per the procedure of AOCS (Cd -53 1997).

3.2.6. Extraction of tocopherols from oils

Extraction of tocopherols from oils was carried out as per the procedure of Joshi and Desai (1952). Known quantity of oil sample was taken into conical flask to which 4 ml of 0.5% ethanolic pyrogallol solution was added and was boiled for 1 min. To this 2 ml of 40% KOH was added which was boiled for another 3 min. This was cooled and 25 ml of water was added. The mixture was transferred into a separating funnel and tocopherol was extracted with hexane 2-3 times, washed with water and solvent was completely evaporated. This extract was used for HPLC analyses.

3.2.7. HPLC analyses of tocopherols

The tocopherol profiles of different oils were analyzed by the procedure of Tan and Brzuskiewicz (1989) by HPLC (Shimadzu 10AS) using a reverse phase (Supelco) 25 cm × 4.6 mm, 5μm, C_{18} column with an isocratic solvent system consisting of acetonitrile: methanol (60:40 v/v) at a flow rate of 1.0 ml/min and the absorbance was read at 295 nm. Compounds were identified and quantified by comparing their retention time and peak areas with respective authentic standards.

3.2.8. Estimation of total phenolics in oils

The concentration of total phenolic content in different oils was determined according to the method of Taga et al., (1984) and expressed as caffeic acid equivalents. Samples and standards were prepared in (60:40 v/v) acidified (3 g/L HCl) methanol/water. Test samples/standards of 100 µL were added to 2 ml of 2% Na_2CO_3. After 2 min, 100 μL of Folin–Ciocalteu reagent was added and the mixture was allowed to stand at room temperature for 30 min. The absorbance was measured at 750 nm. The blank consisted of all reagents and solvents without test samples or standards. The standard caffeic acid prepared at concentrations of 0.01–0.1mg/ml. The phenolic concentration was determined from the standard graph.

3.2.9. Measurement of hunter (colour) values

The colour of the oil samples with carotenoids are measured in terms of hunter colour values of ‘a’, ‘b’, ‘L’ and ‘DE’ using color measuring spectrophotometer (Minolta color measuring system CM-3500d) both before and after different treatments.

3.2.10. Statistical analysis

All the experimental analyses were done in triplicate. Result values were expressed as mean ± SD in case of *in vitro* experiments. In case of *in vivo* analysis values were expressed as mean ± SD and one way analyses of variance (ANOVA) was used and

the test was used for comparison of mean values. All tests were considered to be statistically significant and highly significant at $p < 0.05$ and 0.001 respectively.

3.3. Results

3.3.1. Different stage of *H. pluvialis*

Fig. 27 shows different stages of astaxanthin from *H. pluvialis*. Green motile vegetative cells (A), intermediate non-motile cells (B), intermediate carotenoid accumulating cell (C), carotenoid rich encysted cells (D), carotenoid extract from *H. pluvailis* cells (E), Separations of carotenoids from thin layer chromatography (F), astaxanthin from encysted cell (G) spectrum of astaxanthin (H), structure of astaxanthin and its esters (I-K).

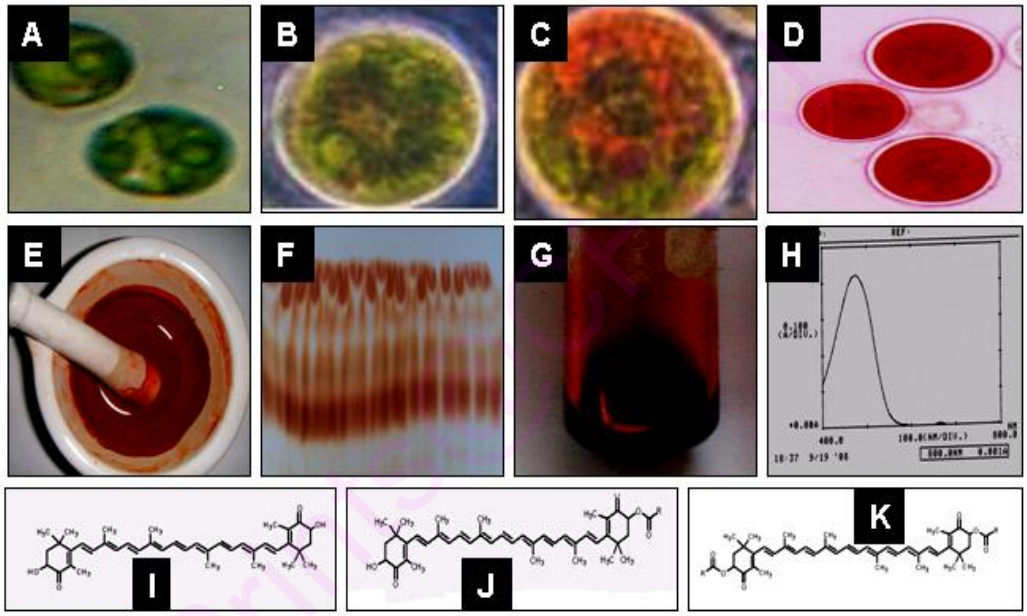


Fig. 27. Different stages of astaxanthin from *H. pluvialis*. Green motile vegetative cells (A), intermediate non-motile cells (B), intermediate carotenoid accumulating cell (C), carotenoid rich encysted cells (D), carotenoid extract from *H. pluvailis* cells (E), Separations of carotenoids from thin layer chromatography (F), astaxanthin from encysted cells (G) and spectrum of astaxanthin (H) and structure of astaxanthin & its esters (I-K).

3.3.2. Identification and estimation of total carotenoids and chlorophyll content in *H. pluvialis* extract by spectrophotometry

Acetone extract of *H. pluvialis* contained 2.2% (w/w) total carotenoid, 0.22% (w/w) chlorophyll and 0.18% phenolics. Astaxanthin content was found to be 1.94% of biomass on dry weight basis (i.e it constituted 88% of total carotenoid). The absorption spectra of the extract from *Haematococcus pluvialis* cells showed a major

absorption peak at 470-474 nm (carotenoid) and a minor peak at 661-663 nm (chlorophyll) (Fig. 28)

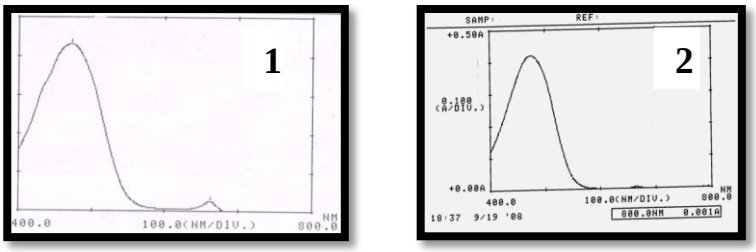


Fig. 28. Spectra showing the presence of astaxanthin and chlorophyll in solvent extract. 1. *Haematococcus* extract, 2. Standard astaxanthin

3.3.3. Carotenoid composition in *H. pluvialis* biomass by HPLC

The HPLC profile of carotenoids in the *H. pluvialis* is shown in Fig. 29. Algal biomass contained three classes of pigments such as xanthophylls, chlorophylls, hydrocarbon carotenoids. All these carotenoids in the order of elution through C_{18} column separated within 30 min. The detectable xanthophylls comprised of neoxanthin (peak 1), violaxanthin (peak 2), astaxanthin (peak 3), lutein (peak 4), and zeaxanthin (peak 5), followed by unidentified (peaks), β-cryptoxanthin (peak 7), and β-carotene (peak 8). These carotenoids were eluted under isocratic conditions and confirmed by their retention times and the absorption spectra of the respective reference standards (Fig. 30). Carotenoid composition of *H. pluvialis* is shown in Table 16. Among the carotenoids, *H. pluvialis* biomass contained astaxanthin esters (AE, 77.58%) as major, followed by neoxanthin (0.9%), violaxanthin (0.3%), astaxanthin (3.8%), lutein (1.4%), zeaxanthin (4.2%), β-cryptoxanthin (5.3%), β-carotene (1.7%) and unidentified peaks (2.8%).

3.3.4. Identification of carotenoids from *H. pluvialis* by LC-MS (APCI)

LC-MS (APCI) has been applied for analysis of different carotenoids of *H. pluvialis.* LC-MS (APCI) was used for determination of molecular mass of each peak. Because the ionization mode was positive, most of the m/z data are $[M+H]^+$ and mass data of compounds identified are given in Table 17. Based on the mass fragmentation interpretation, violaxanthin, neoxanthin, astaxanthin, lutein, zeaxanthin, α-carotene, β-carotene, echinenone, 7, 8, 7', 8'-tetradehydroastaxanthin, and antheraxanthin were identified in *H. pluvialis* extract. Carotenoids and its mass spectral fragmentation data has shown in Fig. 31 & 32.

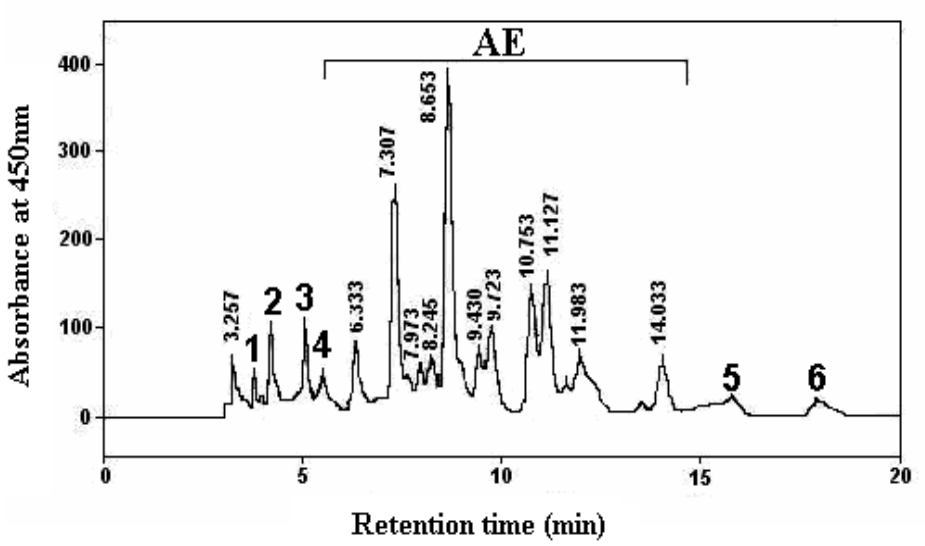


Fig. 29. HPLC profiles of carotenoids from *H. pluvialis* (1) violaxanthin, (2) astaxanthin, (3) lutein, (4) zeaxanthin, (5) α-carotene and (6) β-carotene and AE refers to astaxanthin esters

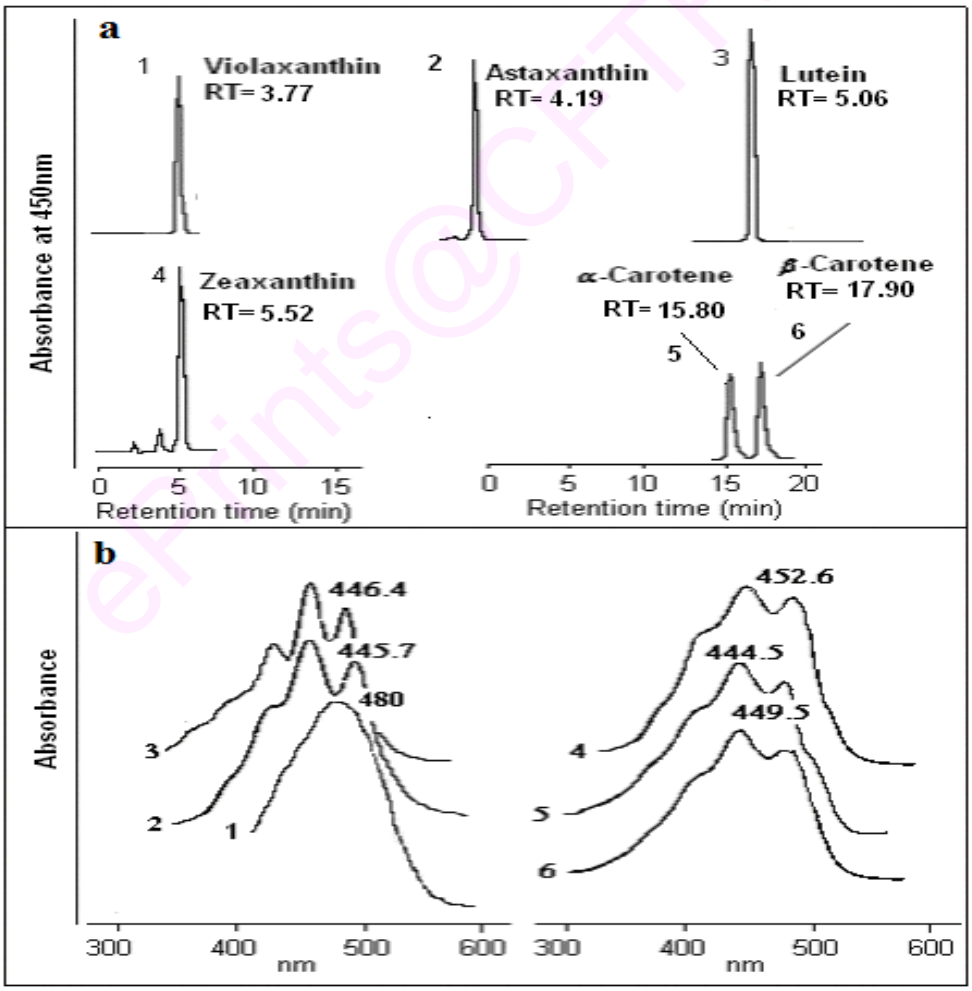


Fig. 30. HPLC profiles of standard carotenoids (a) and absorption spectra (b) of carotenoids eluted from *H. pluvalis* (1) astaxanthin, (2) violaxanthin, (3) lutein, (4) zeaxanthin, (5) α-carotene, and (6) β-carotene.

Table 16. Percentage of carotenoids in *H. pluvialis* biomass analyzed by HPLC.

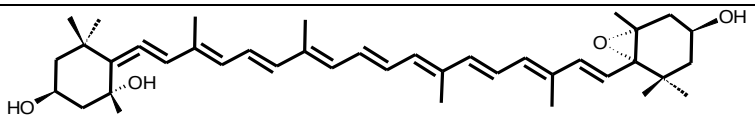
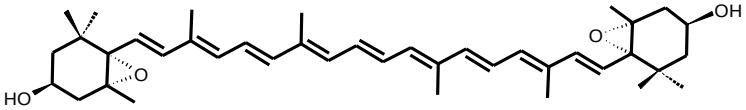
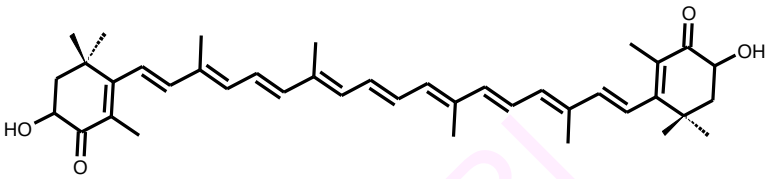
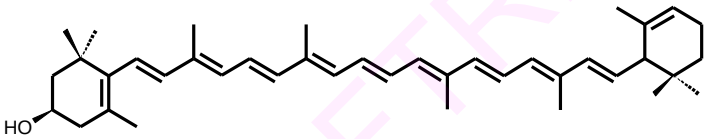
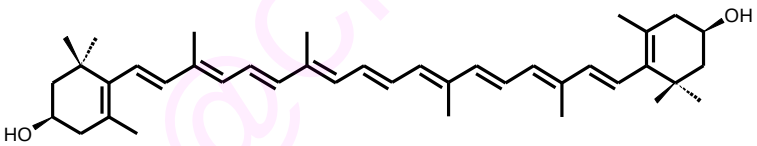
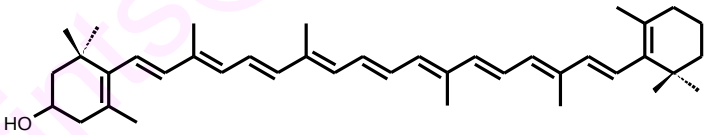
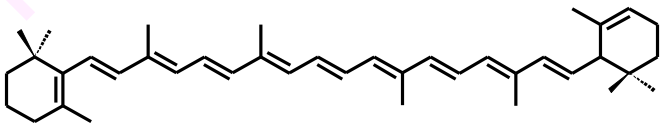
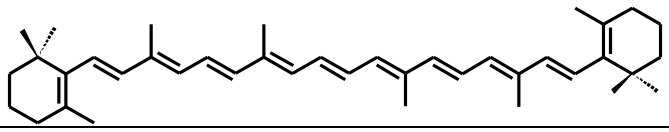
Peak no	Carotenoids	*H. pluvialis* (%)
1	Neoxanthin	0.9 ± 0.05
2	Violaxanthin	0.3 ± 0.12
3	Free astaxanthin	3.8 ± 0.25
4	Lutein	1.4 ± 0.20
5	Zeaxanthin	4.2 ± 0.38
6	Unidentified	2.8 ± 0.16
7	β-Cryptoxanthin	5.3 ± 0.25
8	β-Carotene	1.7 ± 0.98
AE	Astaxanthin esters (mono and di-esters)	77.58 ± 3.12

Values are mean ± SD (n=5), ND=not detected.

Table 17. Identification of carotenoids from *H. pluvialis.*

S.No	Identification of carotenoids	Molecular formula	Approximate molecular mass	Fragmentation mass	Identity
1a	Violaxanthin	$C_{40}H_{56}O_4$	600.41	601.60	$[M+H]^+$
1b	Neoxanthin	$C_{40}H_{56}O_4$	600.41	601.60	$[M+H]^+$
2	Astaxanthin	$C_{40}H_{52}O_4$	596.38	596.62	$[M+H]^+$
3	Lutein	$C_{40}H_{56}O_2$	568.42	569.54	$[M+H]^+$
4	Zeaxanthin	$C_{40}H_{56}O_2$	568.42	569.54	$[M+H]^+$
5	α-Carotene	$C_{40}H_{56}$	536.43	537.51	$[M+H]^+$
6	β-Carotene	$C_{40}H_{56}$	536.43	537.51	$[M+H]^+$
7	Echinenone	$C_{40}H_{54}O$	550.41	551.53	$[M+H]^+$
8	7,8,7',8'-Tetradehydroastaxanthin	$C_{40}H_{48}O_4$	592.35	593.53	$[M+H]^+$
9	Antheraxanthin	$C_{40}H_{56}O_3$	584.42	585.51	$[M+H]^+$

Fig. 31. Chemical structures of major carotenoids identified in *H. pluvialis* biomass by LC-MS (APCI).

Peak No	Carotenoid	Structure of compound	Formula
1	Neoxanthin		$C_{40}H_{56}0_4$
2	Violaxanthin		$C_{40}H_{56}0_4$
3	Astaxanthin		$C_{40}H_{52}0_4$
4	Lutein		$C_{40}H_{56}0_2$
5	Zeaxanthin		$C_{40}H_{56}0_2$
6	β-Cryptoxanthin		$C_{40}H_{56}0$
7	α-Carotene		$C_{40}H_{56}$
8	β-Carotene		$C_{40}H_{56}$

Carotenoids elucidated from algal biomass extracts by APCI ion mode showing their chemical structure, molecular mass and molecular formula.

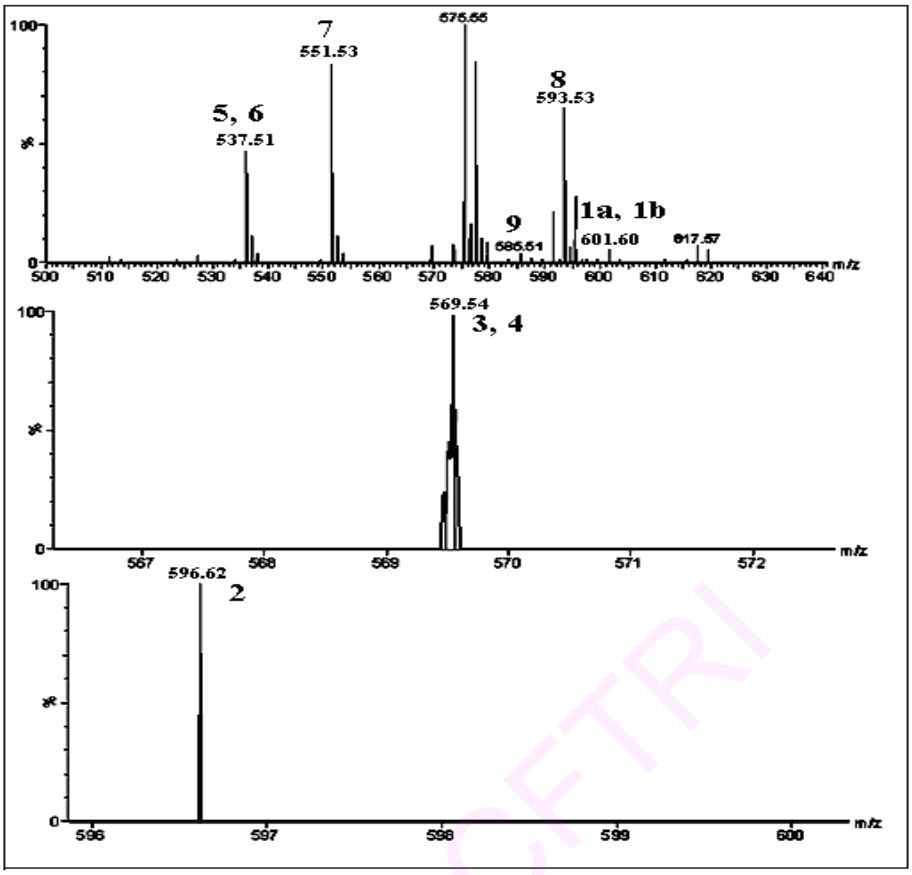


Fig. 32. LC-MS (APCI) profile of carotenoids from *H. pluvialis* extract, (1a) violaxanthin, (1b) neoxanthin, (2) astaxanthin, (3) lutein, (4) zeaxanthin, (5) α-carotene, (6) β-carotene, (7) echinenone, (8) 7, 8, 7', 8'-tetradehydroastaxanthin, and (9) antheraxanthin.

3.3.5. Identification of astaxanthin and its esters by TLC

Acetone extracts of *Haematococcus* dissolved mixing in acetone and n-hexane (3:7) a solvent system showed yellow, yellowish green, pale yellow, red bands. Major spot (R_f) was confirmed as astaxanthin by comparison with standard (Fig. 33, Table 18)

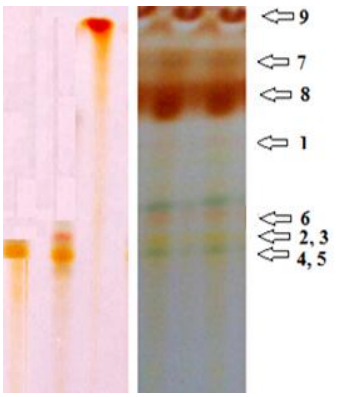


Fig. 33. Separation of astaxanthin & its esters by TLC. 1.astaxanthin, 2.lutein, 3.zeaxanthin, 4 & 5. Chlorophylls a & b, 6. β-cryptoxanthin, 7. Mono-ester of astaxanthin, 8. Diester of astaxanthin, 9. β-carotene**.**

Table 18. Rf values of the different carotenoid fractions from *H. pluvialis*

Sl. no	Possible compound	Rf
1	Astaxanthin	0.54
2	Lutein	0.58
3	Zeaxanthin	0.61
4	Chlorophyll a	0.64
5	Chlorophyll b	0.64
6	β-cryptoxanthin	0.69
7	Mono-ester of astaxanthin	0.77
8	Di-ester of astaxanthin	0.82
9	β-carotene	0.97

Solvent system used for TLC is acetone: hexane: ethylacetate = (3:7:0.5**)**

3.3.6. Preparative TLC for separation of carotenoids

By preparative TLC using acetone: hexane: ethylacetate (3:7:0.5) solvent system carotenoids were resolved into nine spots, which were scraped, re-extracted in acetone and further analyzed by HPLC using isocratic solvent system. Some of the spots identified were astaxanthin, zeaxanthin, lutein, chlorophylls, monoester of astaxanthin, diester of astaxanthin **(Fig. 34).**

Fig. 34. Separation of astaxanthin & its esters from *H. pluvialis*. Comparision with synthetic astaxanthin, diester, mono-ester, free astaxanthin and crude extract of *H. pluvialis*

3.3.7. Separation of carotenoids by column chromatography

Separation of carotenoids was done on a glass column (300 x 10 mm) using various adsorbants namely, silica gel G, magnesium oxide and diatomaceous earth, alumina and silica impregnated with 10% phosphoric acid. Among all the materials tried, silica impregnated with 10% phospahric acid (over night) provided better separation. The solvents used for elution were in the order of n-hexane (100%), acetone:n-hexane

(1:9, 2:8, and 1:1) and acetone (100%). Based on the UV absorption spectroscopy, the major carotenoids were identified (Fig. 35 & 36, Table 19).

Table 19. Different carotenoids identified in *H. pluvialis*

Sl.no.	Absorption maxima	Name
1	418, 438, 450	Neoxanthin
2	420, 443, 470	Violaxanthin
3	476,478, 480	Astaxanthin
4	420, 445, 475	Lutein
5	426, 452, 479	Zeaxanthin
6	422, 444, 474	α-carotene
7	425, 453, 479	β-carotene

Fig. 35. Separation of carotenoids from column chromatography

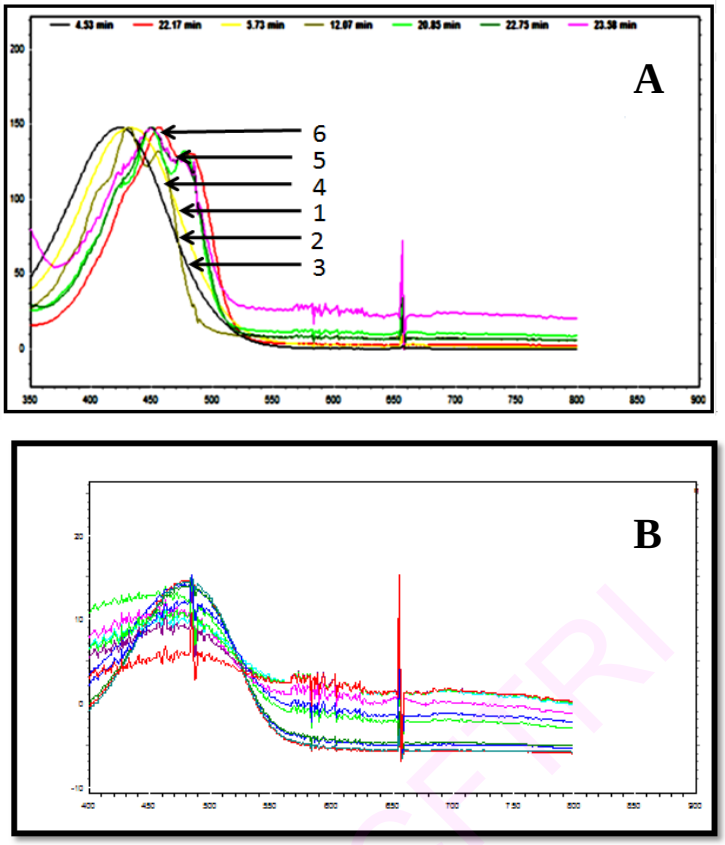


Fig. 36 Spectrum of different carotenoids, mono,di-ester of astaxanthin of *Haematococcus* extract by HPLC (λ_{max}). A. Different carotenoids and B. Astaxanthin esters. (1). Neoxanthin, (2). Violaxanthin, (3). Astaxanthin, (4). Lutein, (5). Zeaxanthin, (6.7). α, β-carotene.

3.3.8. Identification of astaxanthin & its esters by LC-MS (APCI)

Astaxanthin (2%) and its esters (78-80% of total carotenoids) were separated from total carotenoid extract of *H. pluvialis* with acetone: hexane (3:7) mobile phase using silica thin layer chromatography. Thin layer chromatography of total carotenoid showed astaxanthin (Rf-0.54), astaxanthin mono ester (Rf-0.77) and diester bands at relative front (Rf-0.54, 0.77 and 0.82). Astaxanthin and its esters were identified by the absorption spectra of the total carotenoid, astaxanthin and its esters were checked at 470-480 nm by HPLC (Fig. 45). These compounds were used for experiments.

Identified astaxanthin, mono and diester of astaxanthin by mass spectrum using APCI mode is shown in Fig. 37-39. The MS data used for the identification of FASX, MASX and DASX are summarized in Table 20. Mass spectrum was obtained from an astaxanthin monoester (ME $C_{16:0}$, ME $C_{17:2}$, ME $C_{17:1}$, ME $C_{17:0}$, ME $C_{18:4}$, ME $C_{18:3}$, ME $C_{18:2}$, ME $C_{18:1}$) in *H. pluvialis* extract. Because only mass differences between quasimolecular and fragment ions were used for assignment of acylchains, the location of double bonds could not be determined by the mass spectrum. Thus, many

isomers of astaxanthin esters in *H. pluvialis* could not be identified unequivocally. We have observed that the fragmentation pattern of astaxanthin esters was dominated by the loss of fatty acid and water. Protonated $[M+H]^+$ resulting from the positive ion mode. A total of 8 astaxanthin monoesters have been identified. Mass spectrum was obtained from an astaxanthin diester (DE $C_{16:0}/C_{16:0}$, DE $C_{16:0}/C_{18:2}$, DE $C_{18:1}/C_{18:3}$, DE $C_{18:1}/C_{18:2}$, DE $C_{18:1}/C_{18:1}$) in *H. pluvialis*. The basic peaks of other astaxanthin diesters showed characteristic fragment ions of losing one fatty acid, but their fragment ions of losing the second fatty acid had relative weaker intensity.

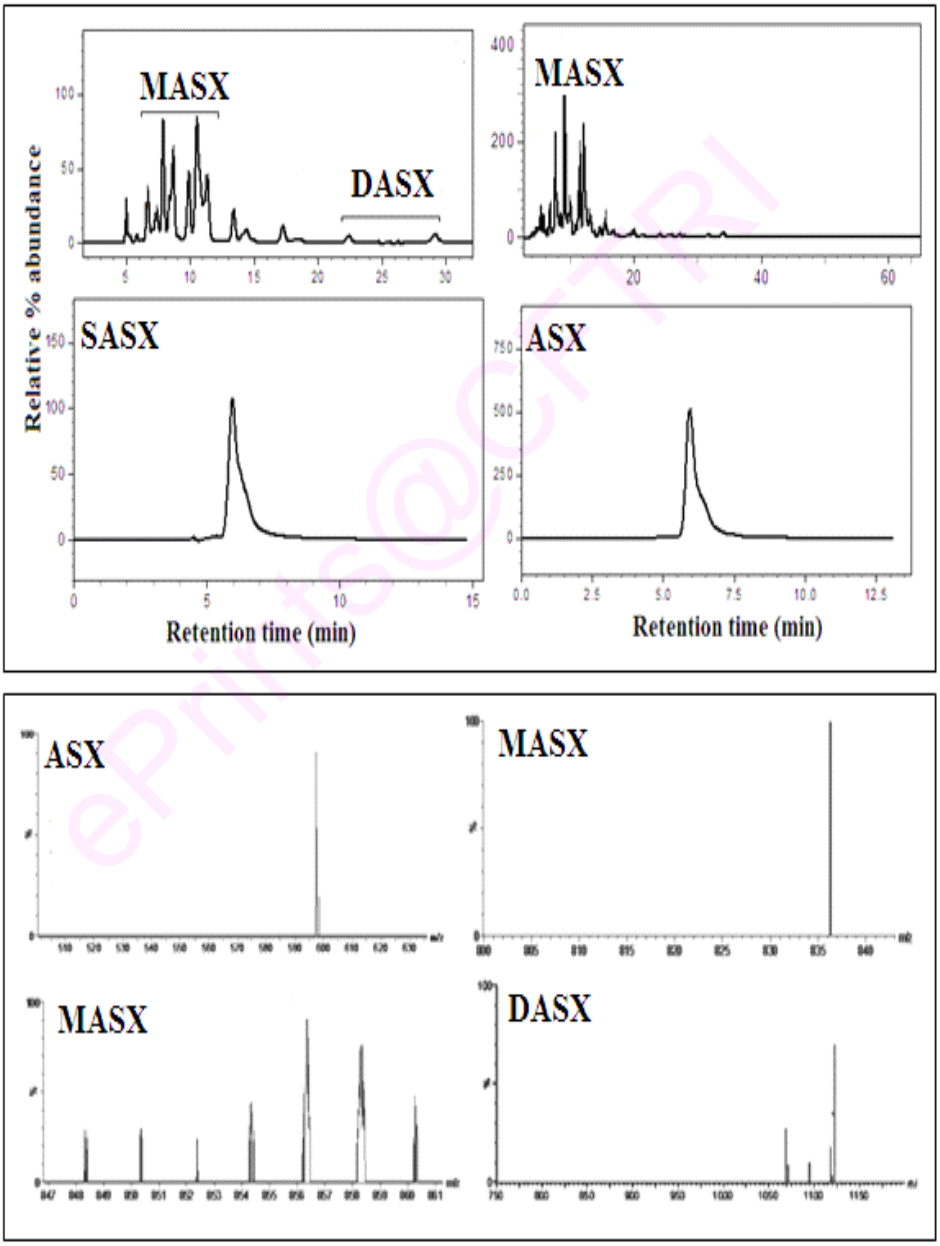


Fig. 37. HPLC and LC-MS profile of (ASX) astaxanthin, (MASX) mono ester of astaxanthin, (DASX) diester of astaxanthin from *H. pluvialis*.

Table 20. Positive ion LC-(APCI)-MS data used for carotenoids, astaxanthin and its esters from *H. pluvialis*

Sl.No	m/z			Compound
	$[M+H]^+$	$[M+2H-FA_1]^{+1}$	MS2	
1	551	$[M+H]^{+1}$	550	Echinenone
2	565	$[M+H]^{+1}$	564	Canthaxanthin
3	592	$[M+H_2O]$	574	7,8,7',8'-tetra dehydroastaxanthin
4	593	$[M+H_2O]$	575	7,8-didehydroastaxanthin
5	578	[M]	578	4-ketoalloxanthin
6	552	[M+H]+1	551	β-cryptoxanthin
7	597		579	Free astaxanthin
8	602		600	Neoxanthin
9	835	579	836	ME $C_{16:0}$
10	845	579	846	ME $C_{17:2}$
11	847	579	848	ME $C_{17:1}$
12	849	579	850	ME $C_{17:0}$
13	853	579	852	ME $C_{18:5}$
14	855	579	854	ME $C_{18:4}$
15	857	579	856	ME $C_{18:3}$
16	859	579	858	ME $C_{18:2}$
17	861	579	859	ME $C_{18:1}$
18	1072	579	1071	DE $C_{16:0}/C_{16:0}$
19	1096	579	1095	DE $C_{16:0}/C_{18:2}$
20	1120	579	1119	DE $C_{18:1}/C_{18:3}$
21	1122	579	1121	DE $C_{18:1}/C_{18:2}$
22	1124	579	1123	DE $C_{18:1}/C_{18:1}$

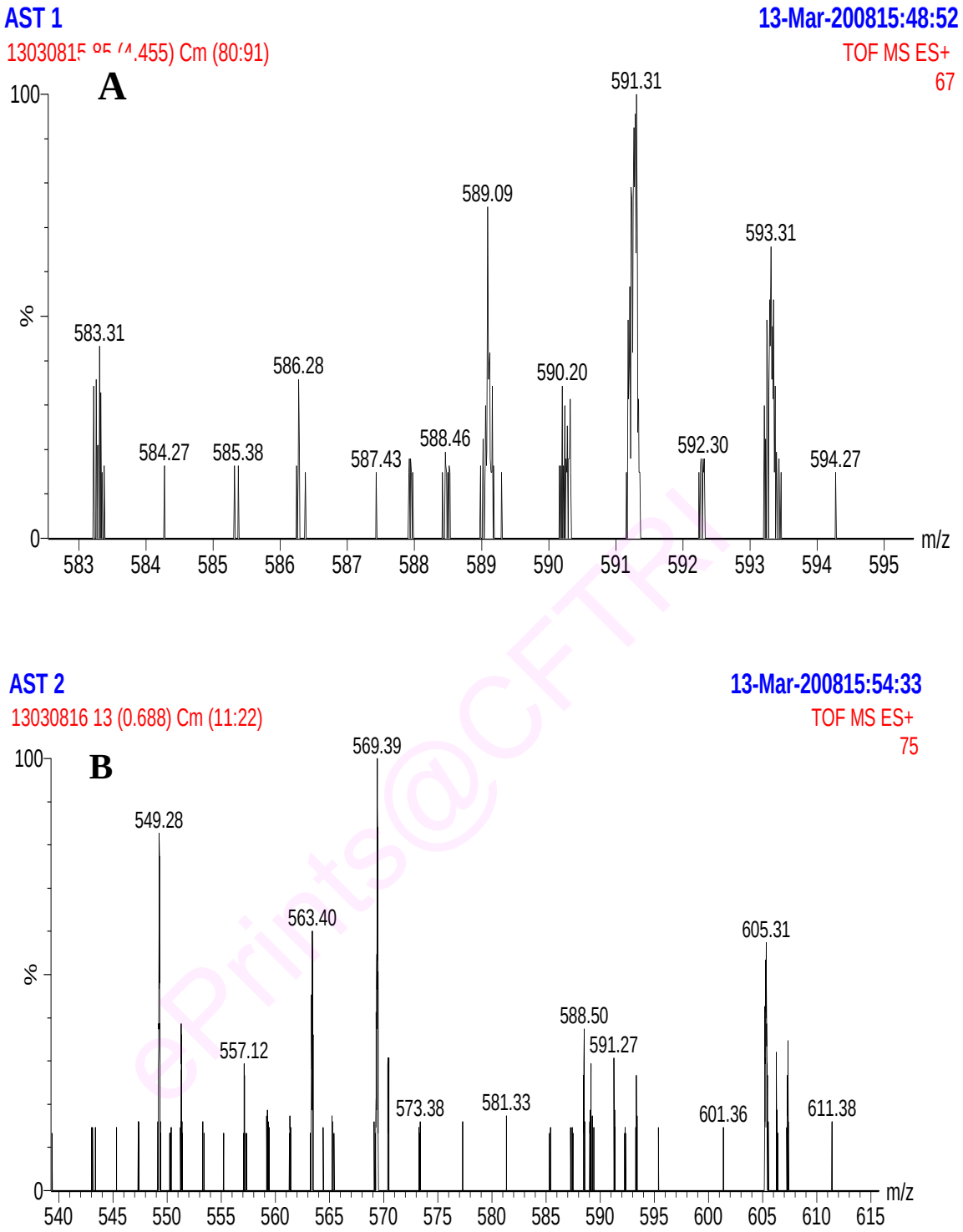


Fig. 38. Mass spectrum of carotenoids of *H. pluvialis* extact using MS-ESI positive mode. (A). Antheraxanthin, tetradehydroastaxanthin and didehydroastaxanthin, (B). Lutien and Zeaxanthin.

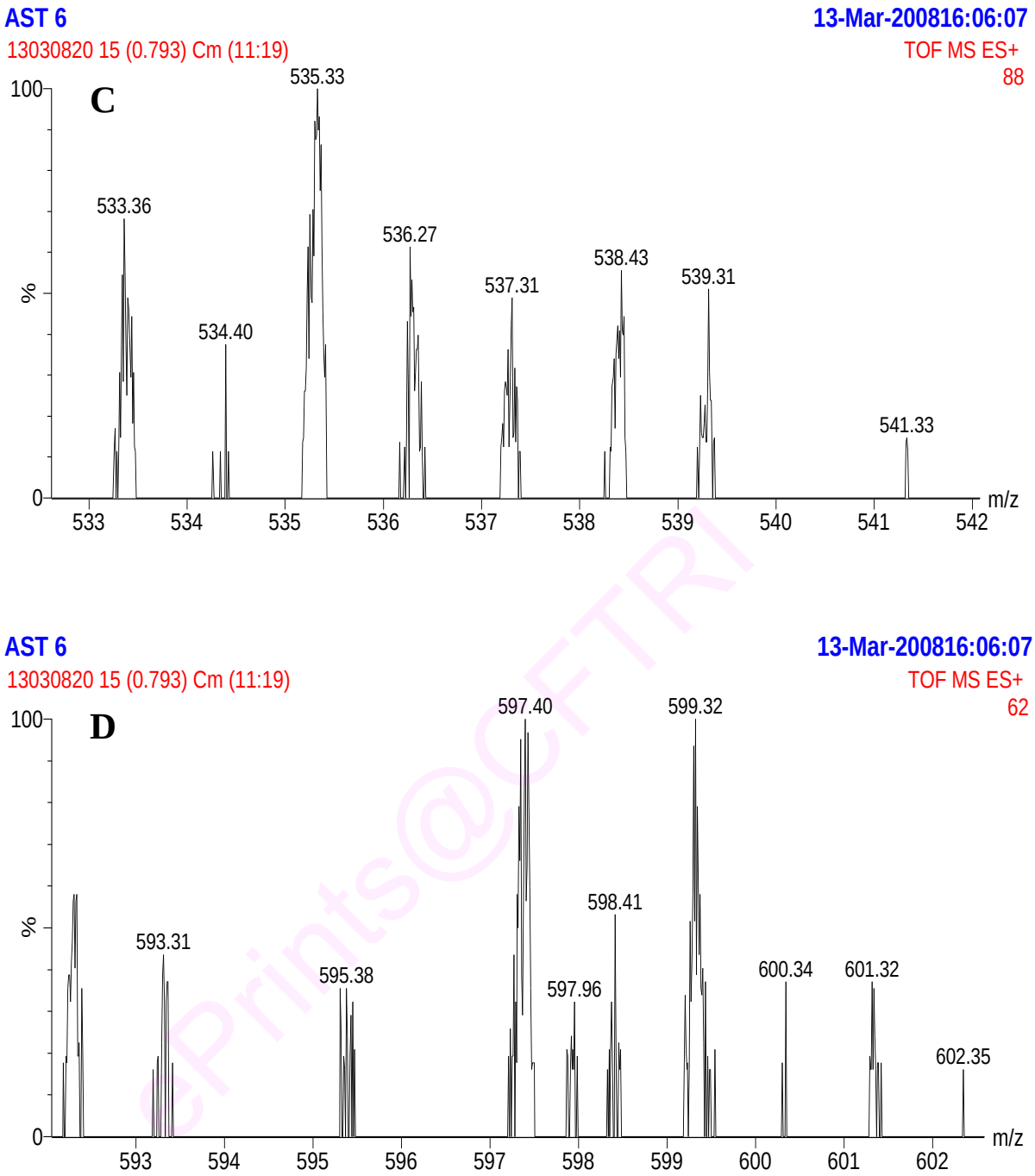


Fig. 39. Mass spectrum of carotenoids of *H. pluvialis* extract using MS-ESI positive mode. (C). α, β-carotene, (D) astaxanthin

3.3.9. Characterization of astaxanthin & its esters in *H. pluvialis* by NMR spectroscopy

All NMR experiments were recorded using a Bruker AMX 500 spectrometer (Brucker, Rheinstetten, Germany). The astaxanthin and its esters were assigned by recording unambiguous ^{13}C NMR, ^{1}H NMR, HSQC 2D, NOESY 2D and COSY 2D NMR.

3.3.9.1. Compound-I (Astaxanthin)

Basing on the NMR Signals 1HNMR, ^{13}C NMR from Fig. 40-43 and the HSQC NMR spectrum (Fig. 44-46) of the compound-I the assignment of signals has been made for compound-1 in the Table 21 for both hydrogen and the corresponding carbon as per the structure (Fig. 53) mentioned. The $^{13}CNMR$ spectrum displayed 49 carbon signals for 40 carbons that include a solvent peak ($CDCl_3$-76.2 ppm). The compound contained ten primary carbons, three each attached two rings, while the four attached to carbon chain. The carbon shifts are 20.01 (C_{16}), 20.32 ($C_{16`}$), 25.83 ($C_{17}$, $C_{17`}$), 25.83 ($C_{18,}$ $C_{18`}$) are attached to the each of the ring, while 13.83 ($C_{19}$), 13.64 ($C_{19`}$) and 12.25 (C_{20}), 12.49 ($C_{20`}$) that are attached to the carbon chain. There are 14 secondary methine carbons chemical shifts 126.52 ($C_7$), 125.91 ($C_{7`}$), 142.0$C_{8,}$) 141.87 ($C_{8`}$), 131.80 ($C_{10}$), 131.13 ($C_{10`}$), 129.00 (C_{11}), 129.40 ($C_{11`}$), 134.85 ($C_{12}$), 134.25 ($C_{12`}$), 131.80 (C_{14}), 131.13 ($C_{14`}$), 129.40 ($C_{15}$), 129.00 ($C_{15`}$), tertiary methine carbon 124.16 ($C_{5,}$), 124.30 ($C_{5`,}$) in the ring and four tertiary methine carbon 133.10 ($C_9$), 133.22 ($C_{9`}$),138.36 (C_{13}), 139.41 ($C_{13`,}$) in the side chain. The following 10 carbon shifts ( $\delta_{ppm}$) for 45.14, 45.17 ($C_2$, $C_{2`}$) are due to secondary methylene carbons in the ring and 68.88, 68.91 (C_1 $C_{1`}$) secondary methylene carbons attached to a hydroxyl group. The secondary carbons attached to oxygen (keto group) showed a shift 200.09 ($C_{6,}$ $C_{6`}$) the quaternary carbons shift on the ring 45.14 (C_3, $C_{3`}$) and tertiary methine β-ionone ring carbons has shifts 68.88 ($C_{4,}$ $C_{4`}$) that clearly confirmed the β-ionone ring.

In the case of ^1H NMR spectrum, 62 group signals are observed, the ^1H signals, the signals 1.23-1.36 are the proton signals of the primary carbon attached to the ring. The proton signals δ_H 1.10, 1.15, 1.17,1.23 (3H,s) and 2.17,2.18 (3H, dd, J=6Hz/3Hz), proton signals δ_H 1.34 (2H, dd, Hz=5); 1.86 (2H,d, Hz=25), δ_H 1.96 (3H, s), δ_H 2.02 (3H,s), δ_H 4.33 (1H,1-OH), δ_H 4.44 (1H, 1-OH), showing multiple signals for cis/trans isomerism protons, δ_H 6.22 (1H, m), 6.33 (1H, m), 6.66 (1H, m), 6.68 (1H, m), 6.85 (1H, m), 6.97 (1H, m), 6.99 (1H, m), 7.02 (1H, m), characteristic of mainly unsaturated double bonds in isoprene unit of the carbon chain. These observations support two β-ionone rings linked by the carbon chain of isoprene unit.

There are ten primary carbons, 6 attached to the ring and 4 on the carbon chain, 14 secondary methine carbons, tertiray methine 4 in the ring and 4 on the carbon chain, two quaternary carbons in the ring, two methylene carbons in the ring, 2 hydroxyl and two keto in each of the rings.

The assignment of protons each of the carbon atom is also based on the HSQC spectrum as per the (Fig. 44-46). The HSQC Fig. 46 shows clearly the mirror image structure and the proton coupling. The COSY experiments gives information regarding three find coupling (from proton to its carbon to the adjacent carbon, then to the carbons proton). The Fig. 47-49 give the COSY spectra provide the coupling of the protons 1, 1` to 2, 2`. 2, 2` with 17 and 18, and the coupling of the hydrogen at 7 with 8 and 19, the coupling of 10 with 11 and so all. The NOESY spectra (Fig. 50-52) in turn provide through space interaction of the methyl group 19, 20 with protons with the adjacent carbon atoms. These in there provide and confirm the compound-1 as astaxanthin. The long range spatial interacting of proton of the primary carbon with the hydrogen at the ring position is observed. The COSY and NOSEY spectrum confirms the existence of both *cis* and *trans* forms of astaxanthin. The molecule mass observed to be ($M+H^+$) 596 and the UV_{max} at 476 nm.

3.3.9.2. Compound-II (Monoester of astaxanthin)

Basing on the NMR Signals ^1H NMR, ^{13}C NMR from Fig. 54-57 and the HSQC NMR Spectrum (Fig. 58 and 59) of the compound-I the assignment of signals has been made for compound-II in the Table 21 for both hydrogen and the corresponding carbon as per the structure (Fig. 65) mentioned. The ^{13}C NMR spectrum displayed 66 carbon signals for 58 carbons that include a solvent peak ($CDCl_3$.76.9 ppm). The compound contained eleven primary carbons, six attached to a ring are 20.22 (C_{16}), 20.85 ($C_{16`}$), 26.87 ($C_{17}$), 26.87 ($C_{17`}$), 26.70 (C_{18}) 20.70 ($C_{18`}$),, three each attached two rings, while the five attached to carbon chain are 13.76 ($C_{19,\ 19`}$, $C_{20,\ 20`}$), and 13.92 (C_{38}). The Secondary methylene carbons in chain are 14 in a chain 29.36 (C_{22}), 29.01 (C_{23}), 29.16 (C_{24}), 28.83 (C_{25}), 28.78 (C_{26}), 29.05 (C_{28}), 29.36 (C_{31}), 29.16 (C_{32}), 30.01 (C_{33}), 29.36 (C_{34}), 29.16 (C_{35}), 31.59 (C_{36}), 20.22 (C_{37}) and two form part of β-ionone ring while that are attached to the carbon chain 71.68, 68.69 (C_1 C_1) and 42.41(C_2, $C_{2`}$). There are sixteen secondary methine carbon chemical shifts are 126.80 ($C_{7,}$ $C_{7`,}$), 131.62 ($C_{8,}$ $C_{8`,}$), 127.58 ($C_{10,}$ $C_{10`,}$), 127.44 ($C_{11,}$ $C_{11`,}$), 129.88 ($C_{12}$), 129.67 ($C_{12}$), 127.28 ($C_{14}$), 129.75 ($C_{14`}$), 127.44($C_{15,}$ $C_{15`}$ ), and 129.88 ($C_{29,}$ $C_{30}$). The tertiary methine carbons ($C_{13,}$ $C_{13`,}$) and (C_9, $C_{9'}$) does not show any carbon shifts in the spectrum.

The ^{13}C carbon shifts (δppm) for 42.41(C_2, $C_{2`}$) and 71.68, 68.69 ($C_1$ $C_{1`}$) is due to secondary methylene carbon in the ring and secondary methylene carbon linked to hydroxyl group. The secondary carbon attached to the group showed shift

202.21 (C_6), 196.11 ($C_{6`}$) the quaternary carbon shift on the ring 42.04 ($C_3$, $C_{3`}$) and tertiary methane carbon has shifts 68.81(C_4), 68.85($C_{4`}$) that clearly confirmed β-ionone ring. In case of ^{13}C NMR of spectrum displayed signals 48 for 40 carbons that include 11 primary (sp^3) carbons, 14 secondary sp3 carbons in a chain, 16 secondary methine sp^2 carbons, 4 tertiary methine sp^2 carbons, 4 quaternary sp^3 carbons. ^{1}H NMR spectrum of astaxanthin (compound-II) showed the signals in hydrogen attached to the ring in the rage δ_H 0.98 to 1.61. The proton signals ranged from 2 to 6.2. The proton signals δ_H 1.37, 1.36, 1.26, 1.31, 1.21, 1.21, 1.20, 1.20, 1.39, 1.48, 1.31, 1.20, 1.21, 1.26, 1.31, 1.35, 1.61, 0.98, 2.00, 2.01, 2.02, 2.03, 2.06, 2.08, 2.0, 2.02, 2.0, 2.0, 2.03 and 2.0. δ_H 5.1 (2), 5.33 (3), 5.35 (2), 5.37 (3), 5.38 (3), 5.39, 5.40 and 6.2 (2) Characteristic of mainly double bonds in isoprene unit of the carbon chain. These observations support two β-ionone rings linked by the carbon chain of isoprene units. The assignment of protons each of the carbon atom is also based on the HSQC spectrum as per the (Figs. 58 & 59). The HSQC Fig. 59 shows clearly the mirror image structure and the proton coupling. The COSY experiments gives information regarding three find coupling (from proton to its carbon to the adjacent carbon, then to the carbons proton). The Figs. (60-62) gives the COSY spectra provide the coupling of the protons 1,1` to 2,2` 2`,2` with 17 and 18 and the coupling of the hydrogen at 7 with 8 and 19, the coupling of 10 with 11 and so all. The NOESY spectra (Fig. 63 & 64) in turn provide through space interaction of the methyl group 19, 20 with protons with the adjacent carbon atoms. These in there provide and confirm the compound 2 as monoester of astaxanthin. COSY and NOESY spectra show the existence both the *cis*/*trans* forms of astaxanthin monoester.

3.3.9.3. Compound-III (Diester of astaxanthin)

Basing on the NMR Signals ^{1}HNMR, ^{13}C NMR from Figs. 66-69 and the HSQC NMR Spectrum (Figs. 70 & 71) of the compound-III the assignment of signals has been made for compound-III in the Table 21 for both hydrogen and the corresponding carbon as per the structure (Fig. 77) mentioned. The ^{13}C NMR spectrum displayed 66 carbon signals for 76 carbons that include a solvent peak ($CDCl_3$-76.9 ppm). The compound contained twelve primary carbons, six attached to a ring are 20.22 ($C_{16,}$ $C_{16`}$), 26.88 ($C_{17,}$ $C_{17`}$), 29.33 (C_{18}, $C_{18`}$), while that 13.76 ($C_{19,}$ $C_{19`}$, $C_{20,}$ $_{20`}$) and 13.92 ($C_{38}$) are attached to the carbon chain. The 24 secondary methylene carbon shifts are observed as follows. 29.02 ($C_{23}$ $C_{23`}$), 29.17, ($C_{24,}$ $C_{24`}$), 28.84 ($C_{25,}$ $C_{25`}$), 28.39 ($C_{26,}$ $C_{26`}$), 29.02 ($C_{28,}$ $C_{28`}$), 30.15 ($C_{31,}$ $C_{31`}$), 31.20 ($C_{32,}$ $C_{32`}$), 31.46 ($C_{33,}$ $C_{33`}$), 31.59 ($C_{34,}$

$C_{34`}$), 29.37 ($C_{35,}$ $C_{35`}$), 31.59 ($C_{36,}$ $C_{36`}$) and 20.22 ($C_{37,}$ $C_{37`}$) for the fatty acid side chain.

The tertiary methine carbons shifts are 122.75 ($C_{5,}$ $C_{5`,}$), 126.80 ($C_{7,}$ $C_{7`}$) in 131.63 ($C_{8,}$ $C_{8`}$), 133.38, 133.54 ($C_{9,}$ $C_{9`}$), 127.44 ($C_{10,}$ $C_{10`,}$), 129.89 ($C_{12,}$ $C_{12`}$), 127.29 (C_{14}, $C_{14`}$), 29.02 ($C_{28}$ $C_{28`}$), 129.89 ($C_{29,}$ $C_{29`}$).The tertiary methine carbons ($C_{13,}$ $C_{13`}$) and (C_9, $C_{9'}$) does not show any carbon shifts in the spectrum. All these signals are absorbed in the range of 26.52 to 30.19 amounting to sixteen secondary methylene carbon atoms. The carbon shifts (δppm) for 61.79 (C_2, $C_{2`}$) and 71.64, 68.69 ($C_1$ $C_{1`}$) is due to secondary methylene carbon in the ring and secondary methylene carbon linked to acyl $C_{1`}$ (68.89) and free hydroxyl group $C_1$ (71.64), the secondary carbon attached to the group showed shift 193.49, 193.41($C_6$) the quaternary carbon shift on the ring 37.22 ($C_3$, $C_{3`}$) and tertiary methane carbons shifts 68.81(C_4), 68.50 ($C_{4`}$) in the ring confirmed β-ionone ring. The ^{13}C NMR of spectrum (Table 21) displayed 66 for 76 carbons that include 12 primary (sp^3) carbons, 44 secondary methylene sp3 carbons (two in ring, 42 in the side chain), 8 secondary sp^3 carbons, 4 secondary carbons - four keto carbons (two in ring and two in carbon chain) and two acyl carbons linked to the ring), 4 tertiary methine carbons, 2 quaternary sp^3 carbons. ^1H NMR spectrum of astaxanthin (compound-III) showed the sequals in hydrogen attached to the ring in the rage δ_H 0.98 to 1.61.

The proton sequals ranged from 2 to 6.2. The proton sequals δ_H 1.37, 1.36, 1.26, 1.31, 1.21, 1.21, 1.20, 1.20, 1.39, 1.48, 1.31, 1.20, 1.21, 1.26, 1.31, 1.35, 1.61, 0.98, 2.00, 2.01, 2.02, 2.03, 2.06, 2.08, 2.0, 2.02, 2.0, 2.0, 2.03 and 2.0. δ_H 5.1 (2), 5.33 (3), 5.35 (2), 5.37 (3), 5.38 (3), 5.39, 5.40 and 6.2 (2) Characteristic of mainly double bonds in isoprene unit of the carbon chain. These observations support two β-ionone rings linked by the carbon chain of isoprene units. The assignment of protons each of the carbon atom is also based on the HSQC spectrum as per the (Fig. 70 & 71).

The HSQC Fig. 71. shows clearly the mirror image structure and the proton coupling. The COSY experiments gives information regarding three find coupling (from proton to its carbon to the adjacent carbon, then to the carbons proton). The Fig. 72-74 gives the COSY spectra provide the coupling of the protons 1,1` to 2,2` 2`,2` with 17 and 18 and the coupling of the hydrogen at 7 with 8 and 19, the coupling of 10 with 11 and so all. The NOESY spectra (Fig. 75 & 76) in turn provide through space interaction of the methyl group 19, 20 with protons with the adjacent carbon

atoms. These in there provide and confirm the compound-III as diester of astaxanthin. COSY and NOSEY spectra confirm the occurence of both *cis/trans* astaxanthin diesters in the mixture.

Table 21. ^{1}H (500 MHz) and ^{13}C NMR data of astaxanthin, astaxanthin monoester,astaxanthin diester from *H. pluvialis*

Position	Compound-I (Astaxanthin)		Compound-II (Astaxanthin monoester)		Compound-III (Astaxanthin diester)	
	δ_H mult (J in Hz)	δ_c (ppm)	δ_H mult (J in Hz)	δ_c (ppm)	δ_H mult (J in Hz)	δ_c (ppm)
1	4.32 (m, 1-OH), 4.33 (m, 1H)	68.88	5.1	71.64	5.1 (-OH, H)	71.64
1’	4.35,4.36 (OH)	68.88	5.10	68.89	5.1 (-OH, H)	71.64
2	1.85 (m, 1H)	61.85	1.37	61.78	1.37 (m, H)	61.79
2`	1.83	61.85	1.36	61.78	1.36 (m, H)	61.79
3	--	45.14	--	42.41	--	37.22
3`	--	45.14	--	42.41	--	37.17
4	1.23	68.76	1.26	68.81	1.26	68.81
4`	1.32	68.76	1.31	68.55	1.31	68.50
5	--	127.77	--	127.79	--	127.75
5`	--	127.77	--	127.79	--	127.75
6	--	200.09	--	202.21	--	193.49
6`	--	200.09	--	196.11	--	193.17
7	6.48 1H, m)	126.52	5.40	126.80	5.40(1H, m)	126.80
7`	6.46 (1H, m)	125.91	5.39	126.80	5.40(1H, m)	126.80
8	6.45(1H, m)	142.00	5.38	131.62	5.38	131.63
8`	6.43 (1H, m)	141.87	5.37	131.62	5.37	131.63
9	--	133.10	--	143.32	--	133.38
9`	--	133.22	--	142.65	--	133.38
10	6.21	131.80	5.38	127.38	5.28	127.44
10`	6.25	131.13	5.37	127.38	5.33	127.44
11	6.70	129.00	5.35	127.44	5.40	127.58
11`	6.70 (1H, m)	129.40	5.35	127.44	5.40	127.58
12	6.26 (j=13Hz, 6Hz)	134.85	5.33	129.88	5.38	129.89
12`	6.23 (dd, J=13Hz/6Hz)	134.25	5.33	129.67	5.37	129.89
13	--	--	--	--	--	---
13`	--	--	--	--	--	--
14	5.31	131.80	5.38	127.28	5.40	127.29
14`	5.35	131.13	5.37	127.75	5.40	127.29

15	6.25	129.40	6.2	127.44	5.38	127.44
15`	6.21	129.00	6.2	127.44	5.37	127.44
16	2.02	20.01	2.00	20.22	2.00	20.22
16`	2.01	20.32	2.01	20.85	2.01	20.22
17	1.31 (3H, m)	25.83	1.21	26.87	1.27(3H, m)	26.37
17`	1.31 (3H,m)	25.83	1.21	26.87	1.27 (3H, m)	26.37
18	1.32 (3H, m)	25.83	1.20	26.70	1.29(3H, m)	26.70
18`	1.32 (3H,m)	25.83	1.20	26.70	1.29 (3H, m)	26.70
19	1.96 (3H, m)	13.83	2.02	13.76	2.04 (3H, m)	13.76
19`	1.97 (3H,m)	13.64	2.03	13.76	2.04 (3H, m)	13.76
20	1.99 (3H, m)	12.25	2.06	13.76	2.05 (3H, m)	13.76
20`	1.99 (3H,m)	12.49	2.08	13.76	2.05 (3H, m)	13.76
21				36.01	--	37.22
21`			--	--	--	37.22
22			2.0	29.36	2.331	29.37
22`			--	--	2.331	29.37
23			1.39	29.01	1.384	29.02
23`			--	--	1.371	29.02
24			2.02	29.16	2.006	29.17
24`			--	--	2.019	29.17
25			1.48	28.83	1.48	28.84
25`			--	--	1.48	28.84
26			1.31	28.78	1.316	28.39
26`			--	--	1.357	28.39
27			2.00	28.38	2.006	28.84
27`			--	--	2.006	28.84
28			2.00	129.67	2.019	29.02
28`			--	--	2.019	29.02
29			2.00	129.88	2.031	129.89
29`			--	--	2.031	129.89
30			2.03	129.67	2.043	129.85
30`			--	--	2.043	129.85
31			2.00	29.36	2.071	30.15
31`			--	--	2.071	30.15
32			1.20	29.86	1.270	31.20
32`			--	--	1.270	31.20
33			1.21	30.01	1.295	31.46
33`			--	--	1.295	31.46
34			1.26	29.36	1.270	31.59
34`			--		1.270	31.59
35			1.31	29.16	1.316	29.37
35`			--	--	1.316	29.37
36			1.35	31.59	1.357	31.59
36`			---	--	1.357	31.59
37			1.61	20.22	1.625	20.22
37`			---	---	1.625	20.22
38			0.98	13.92	0.991	13.92
38`			--	--	0.976	13.92

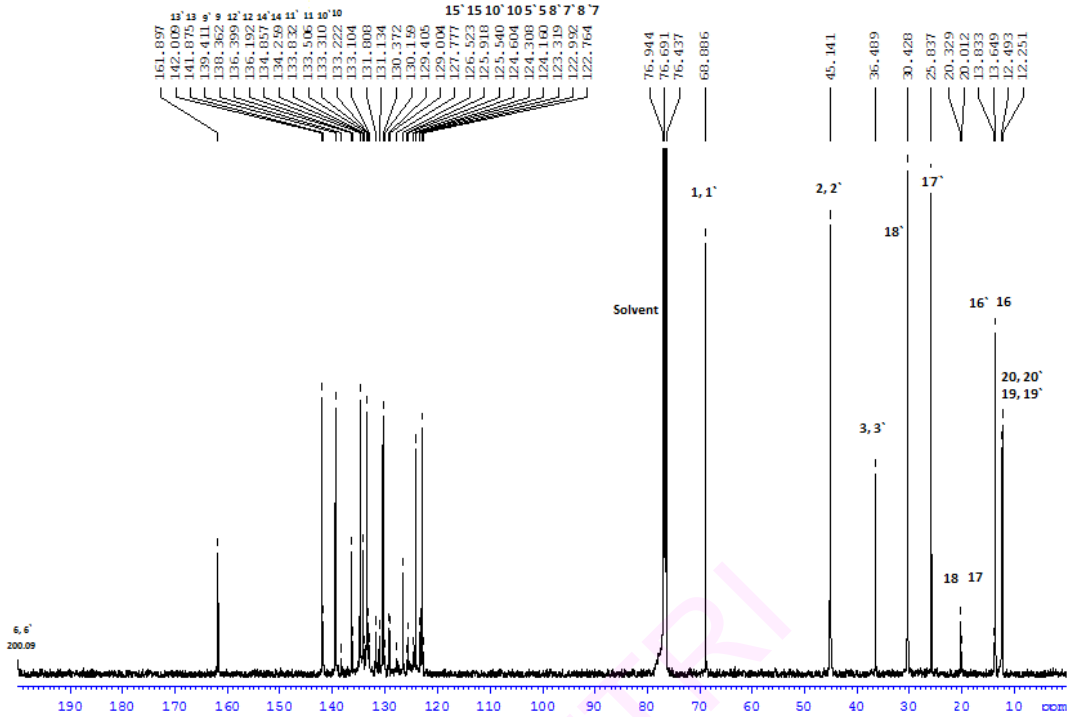


Fig. 40. The ^{13}C NMR spectrum of the compound-I with carbon shift signals from (0-190) ppm

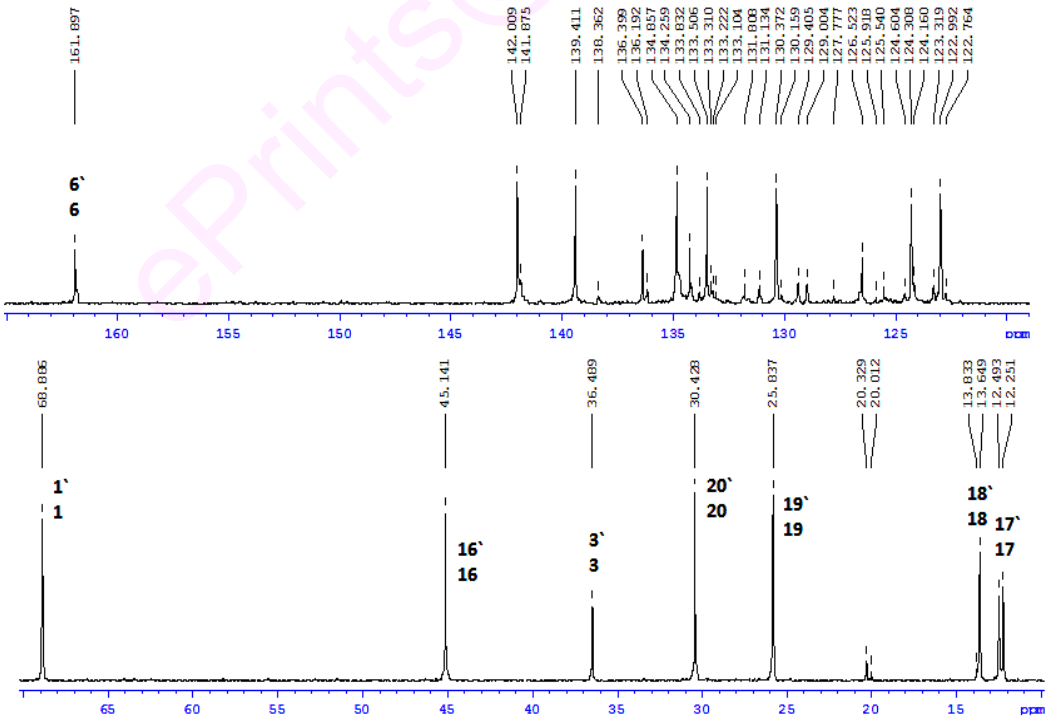


Fig. 41. The ^{13}C NMR spectrum of the compound-I with split carbon shift signals from (0-70 ppm) and (120-160 ppm).

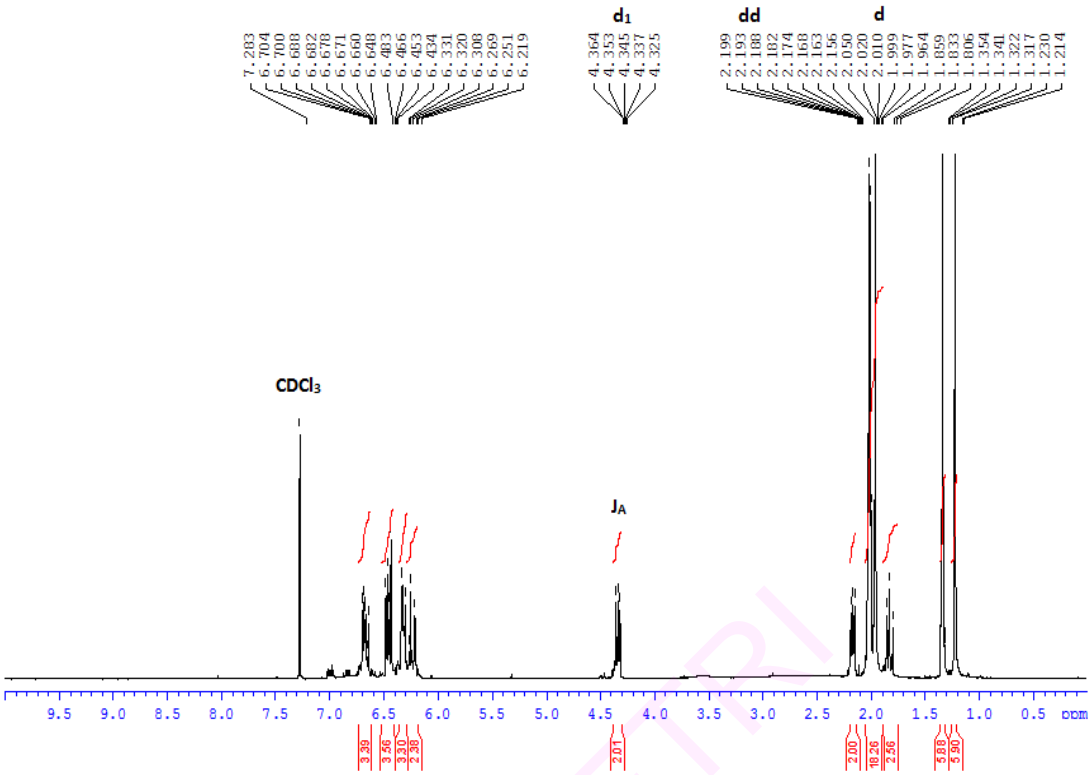


Fig. 42. The ^{1}H NMR spectrum of the compound-I describing hydrogen shifts signals from 0-10 ppm

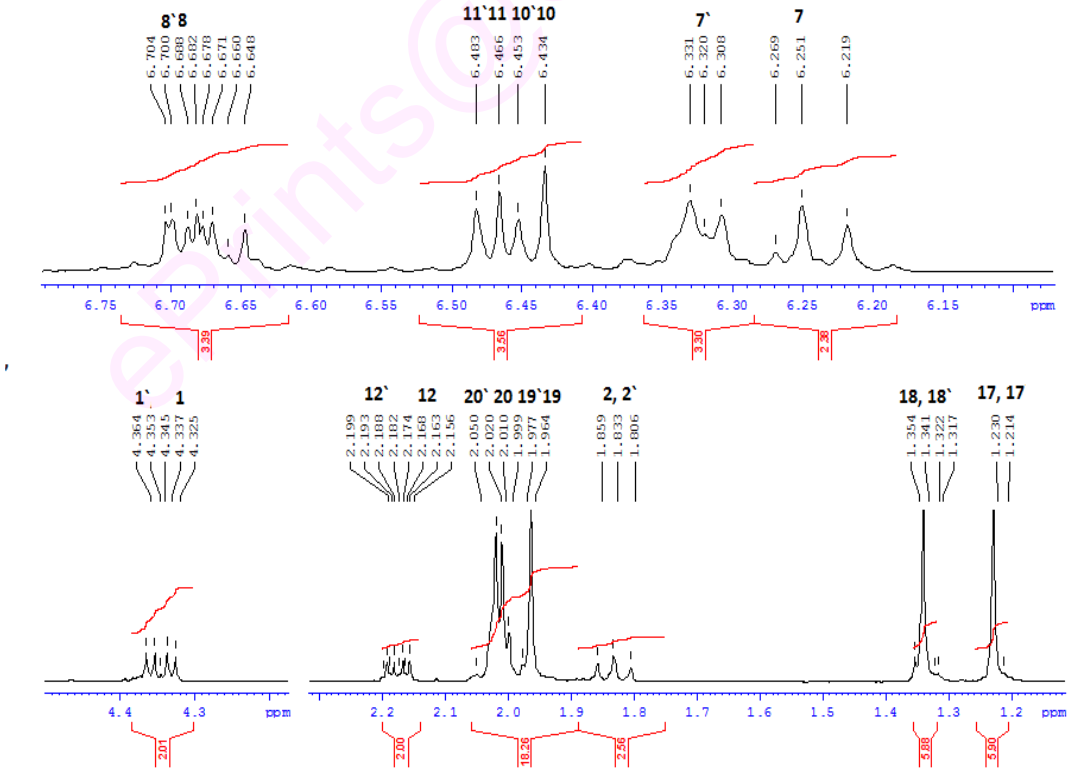


Fig. 43. The ^{1}H NMR spectrum of the compound-I describing hydrogen shift signals from 1-5 ppm and 6.0-7.0 ppm.

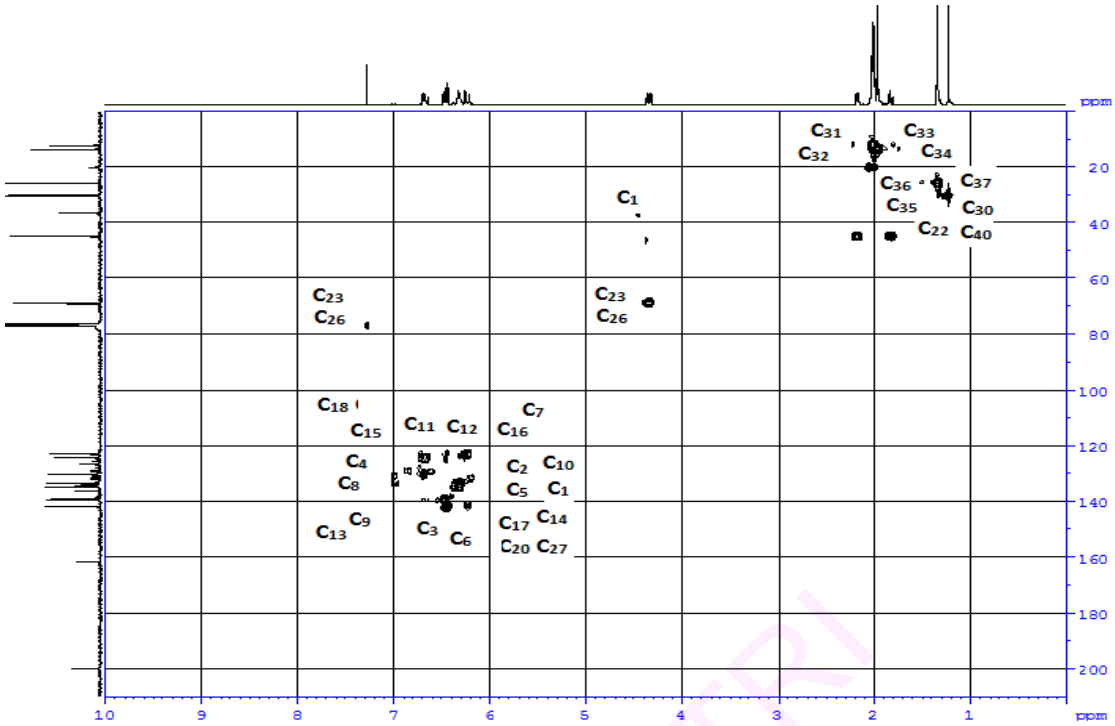


Fig. 44. The HSQC 2D NMR spectrum of compound-I correlating the ^{1}H proton shift signals with ^{13}C proton shift signals (1-10 ppm proton), (0-200 ppm carbon).

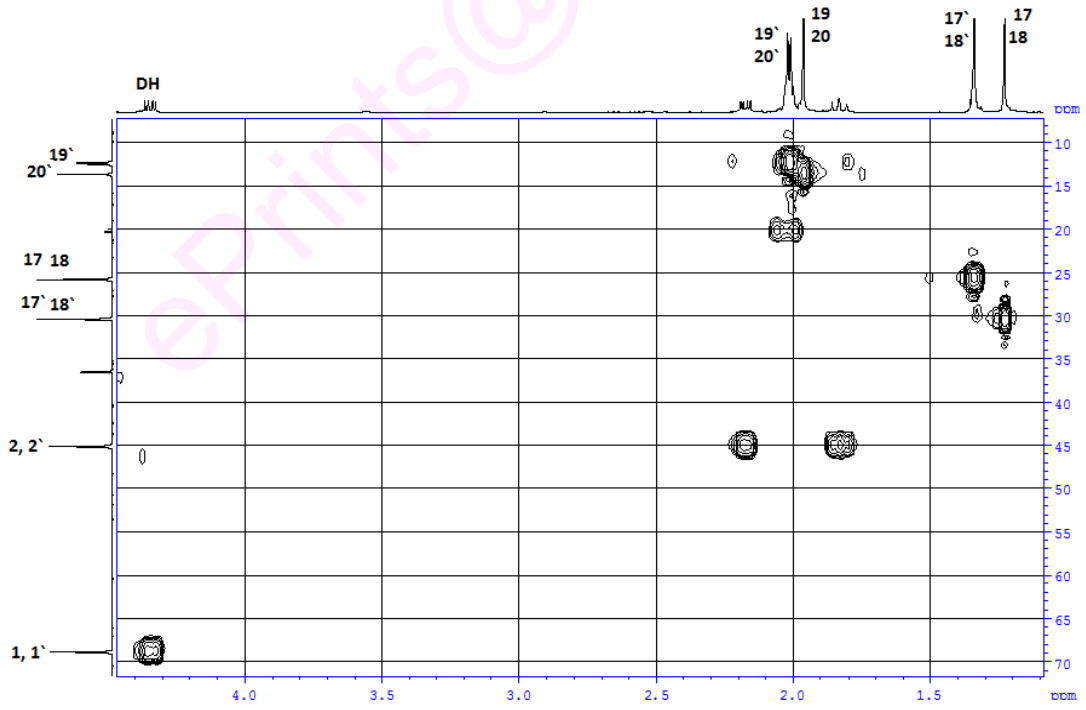


Fig. 45. The HSQC NMR spectrum of compound-I correlating the ^{1}H proton shift (0-5 ppm) signals with ^{13}C carbon shifts (0-70 ppm)

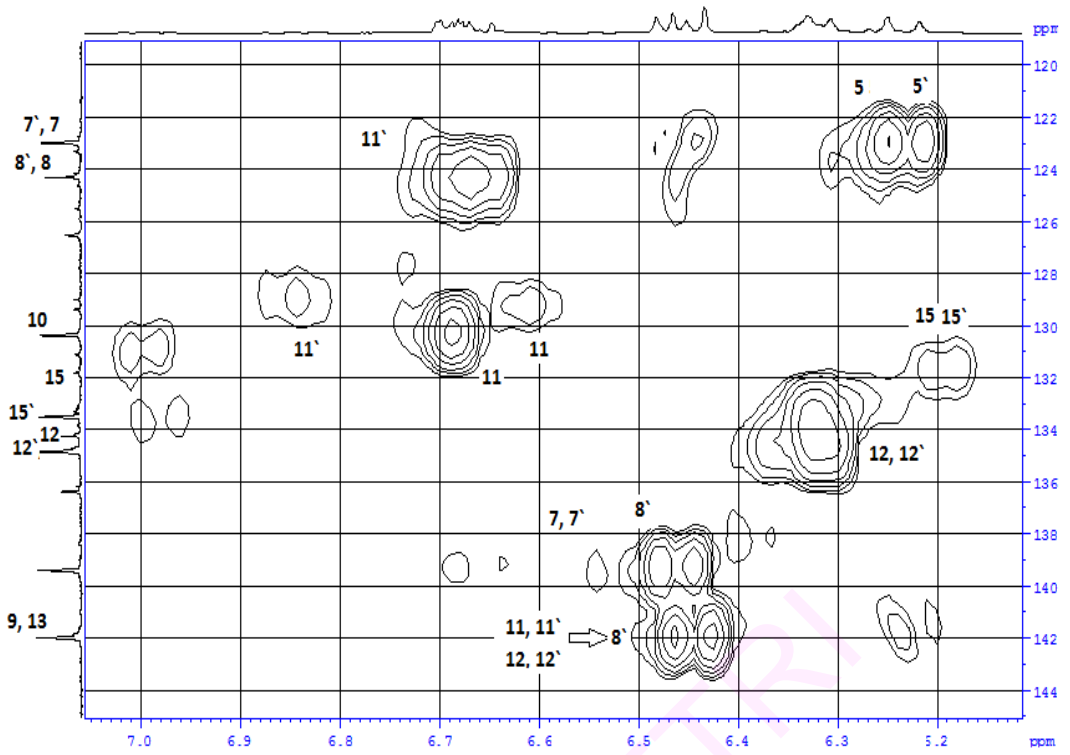


Fig. 46. The HSQC 2D NMR spectrum of compound-I correlating the ^{1}H proton shift signals (6-7.5 ppm) and ^{13}C carbon shift signals (120-146 ppm)

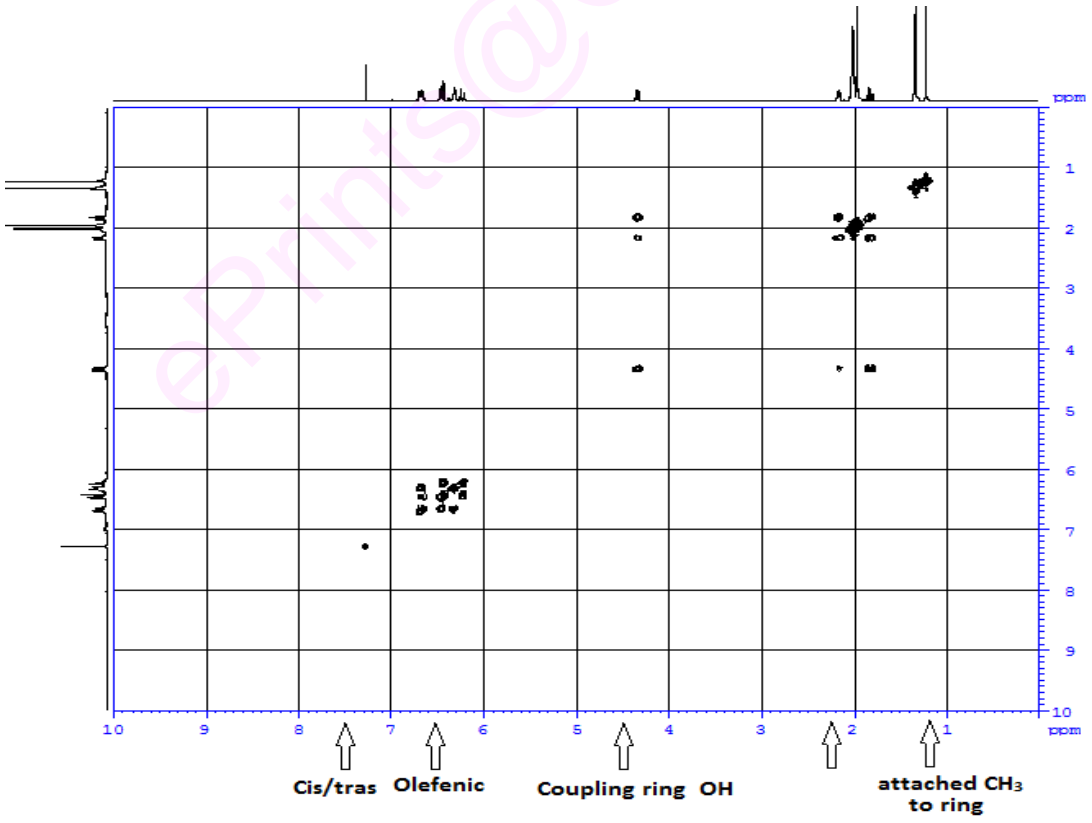


Fig. 47. The COSY 2D NMR spectrum of compound-I $^{1}HNMR$ of compound-1 with shifts (0-10 ppm).

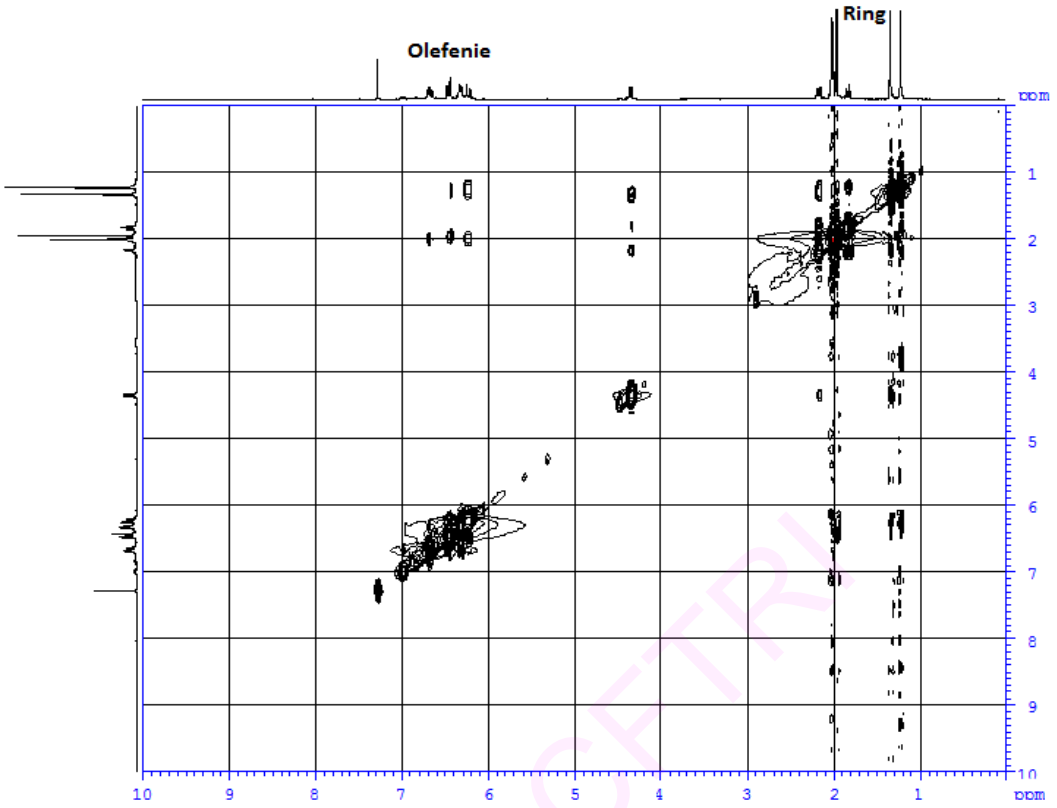


Fig. 48. The ^{1}H proton 2D NOESY NMR spectrum of compound-I with shift ranges (1-10ppm).

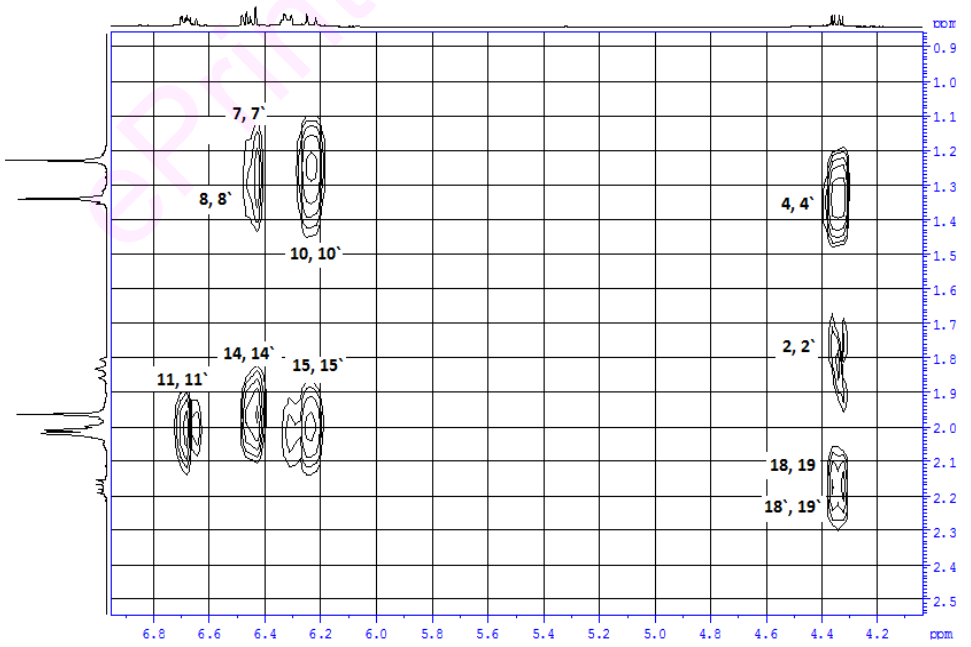


Fig. 49. The ^{1}H proton NOESY 2D NMR spectrum of the compound-I with shift ranges (4.0-7.0) for X-axis and (0.9-2.5 ppm) on the Y-axis.

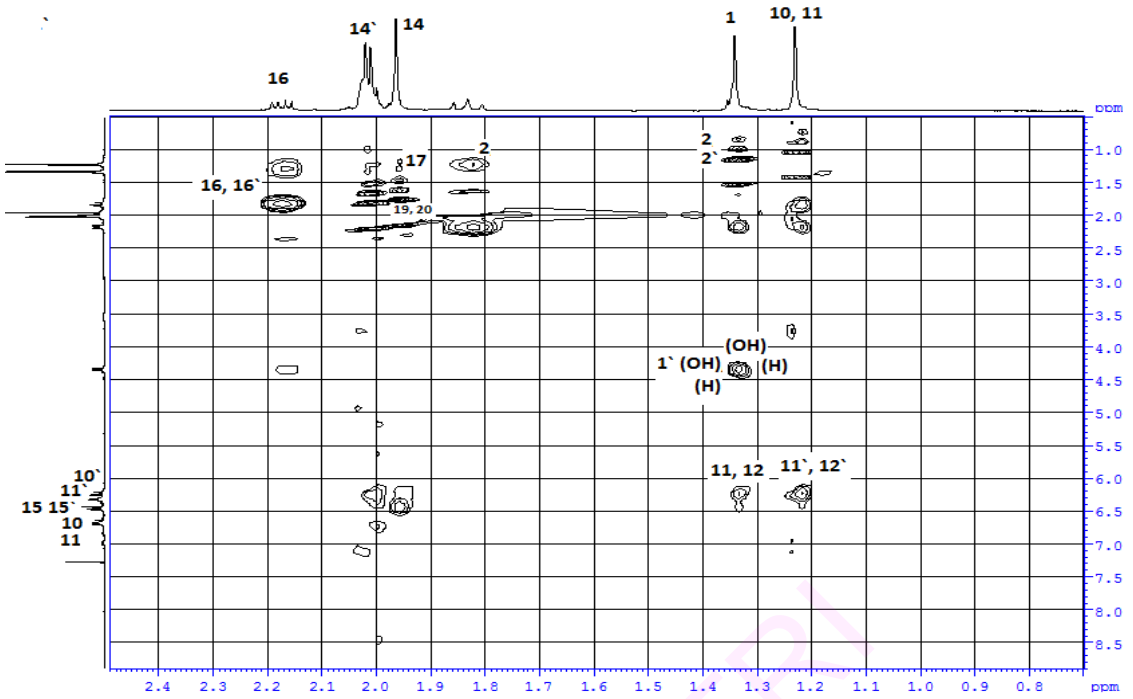


Fig. 50. The ^{1}H proton NOESY 2D NMR spectrum of the compound-I with shift ranges (0-2.4 ppm) on the X-axis and (0-9.0 ppm) on the Y-axis.

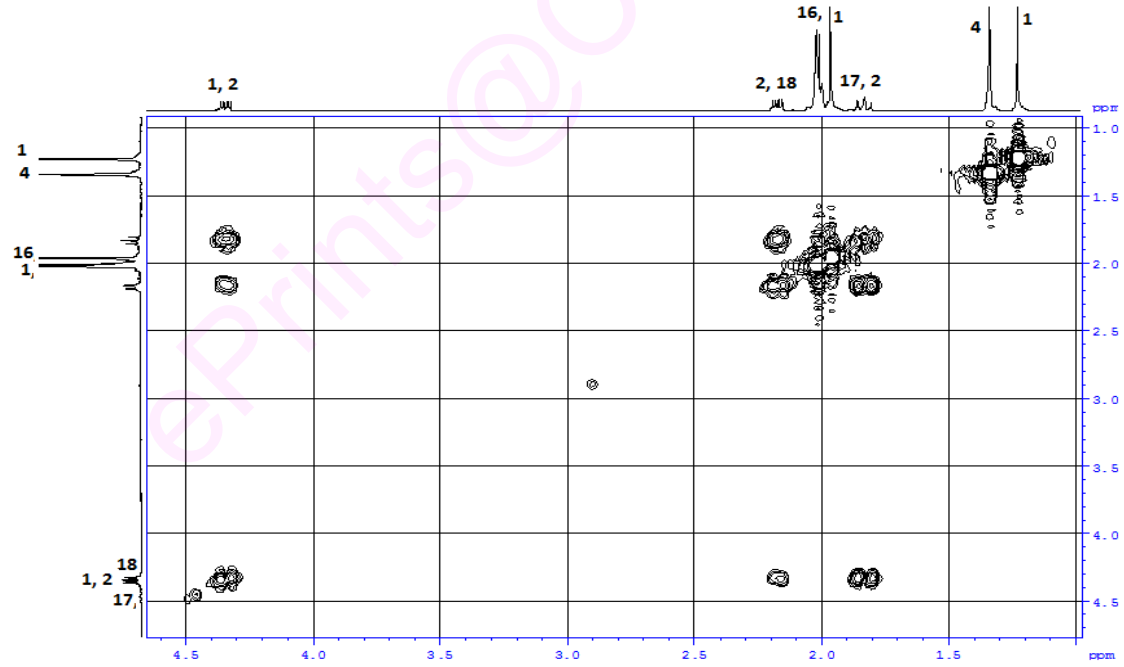


Fig. 51. The ^{1}H proton COSY 2D NMR spectrum of the compound-I with shift ranges (1.0 - 4.5 ppm) on the X-axis and (0.8-4.5 ppm) on the Y-axis.

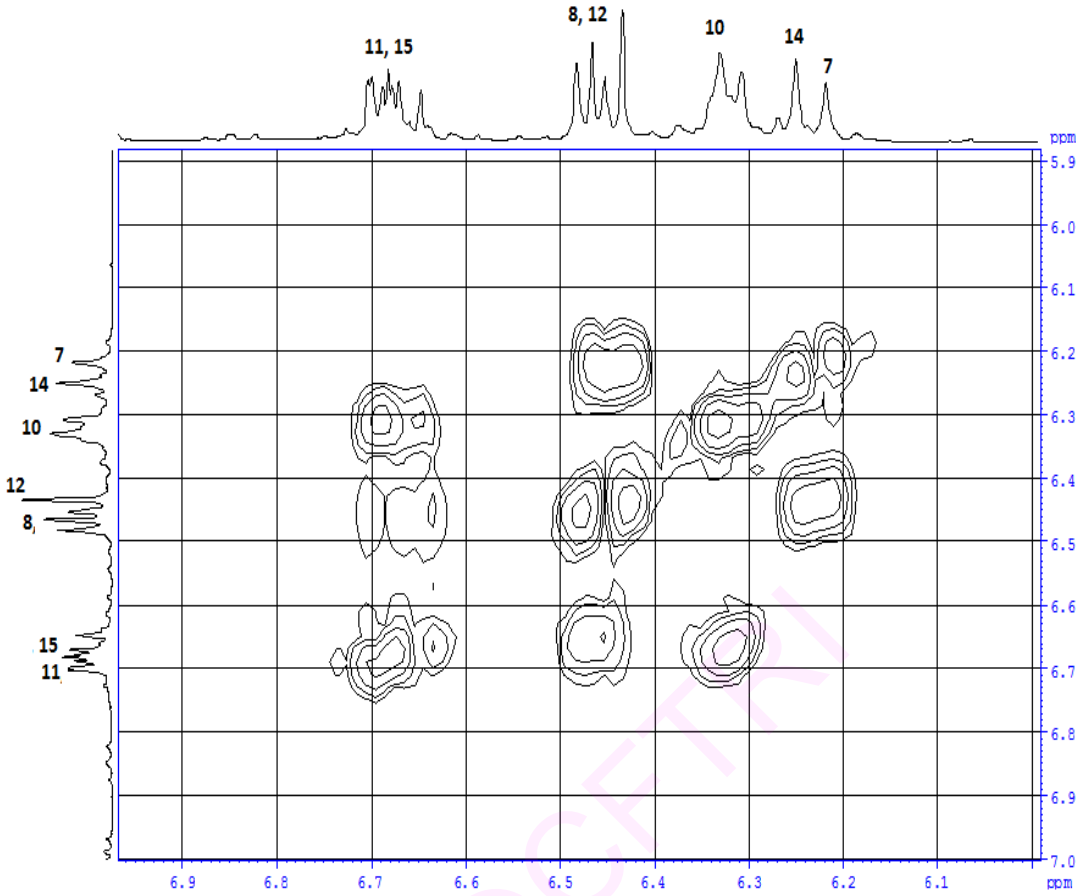


Fig. 52. The ^{1}H proton COSY 2D NMR spectrum of the compound-I with shift ranges (6.0-6.9 ppm) on the X-axis and (5.9-7.0 ppm) on the Y-axis.

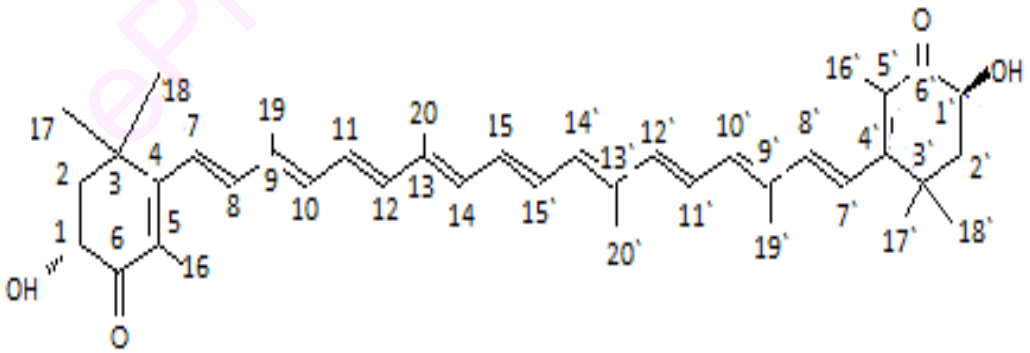


Fig. 53. Structure of astaxanthin

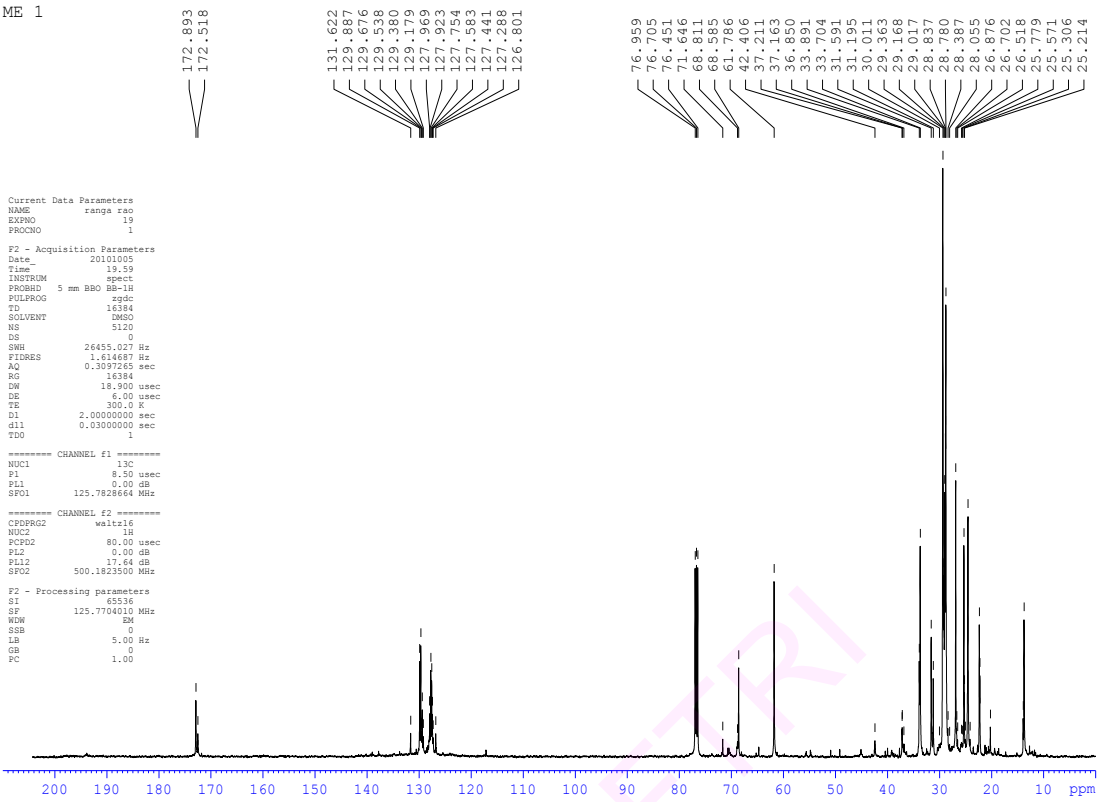


Fig. 54. The ^{13}C NMR spectrum of the compound-II with carbon shift signals from 0-200 ppm

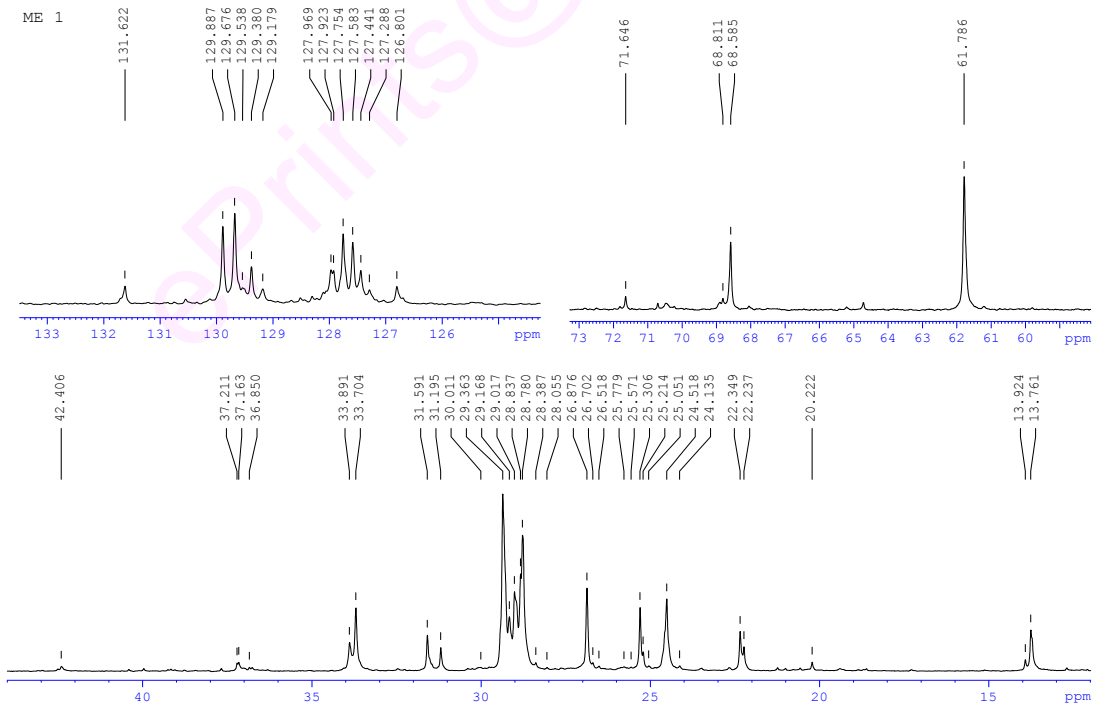


Fig. 55. The ^{13}C NMR spectrum of the compound-II with split carbon shift signals from (0-40 ppm) and (50- 140 ppm).

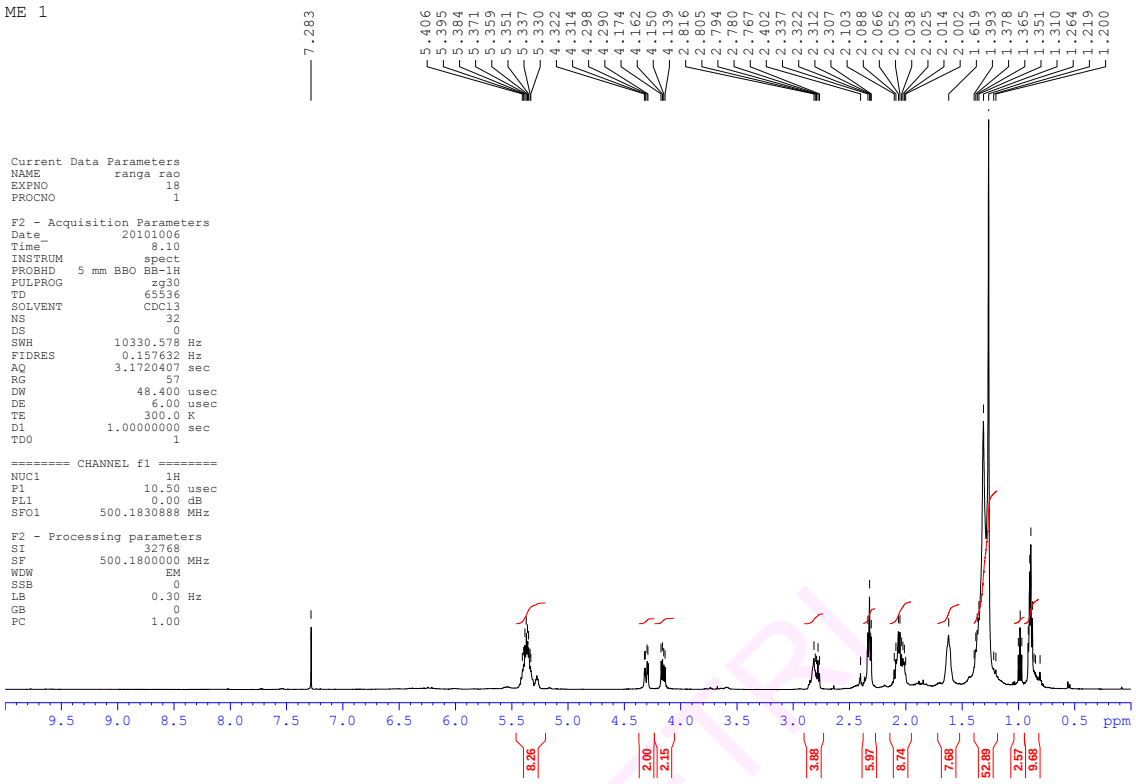


Fig. 56. The ^{1}H NMR spectrum of the compound-II describing hydrogen shifts signals from 0-10 ppm.

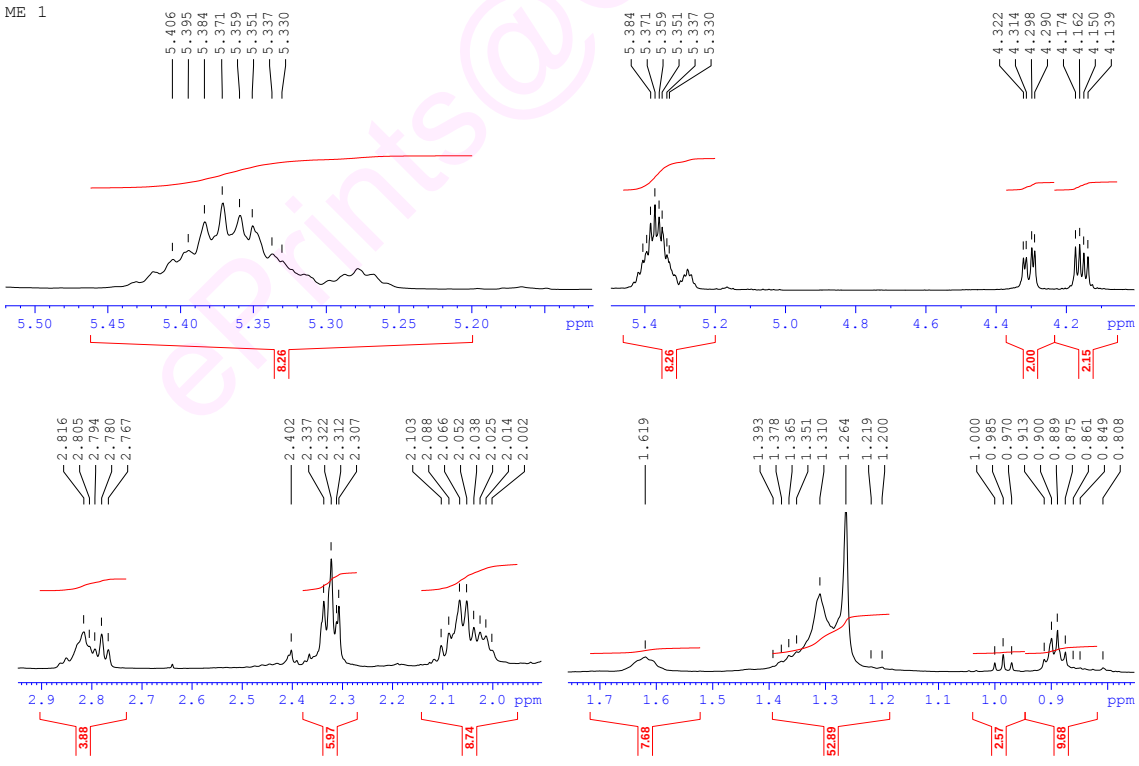


Fig. 57. The ^{1}H NMR spectrum of the compound-II describing hydrogen shift signals from 0- 3 ppm and 4-6 ppm.

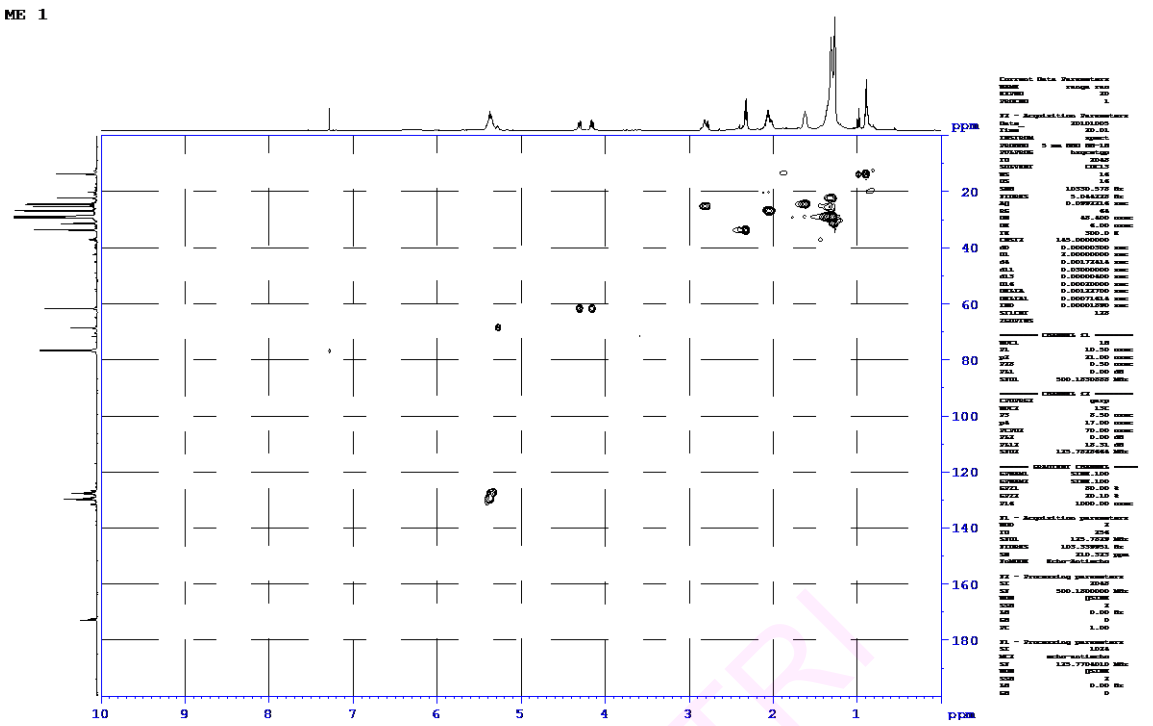


Fig. 58. The HSQC 2D NMR spectrum of compound-II correlating the ^{1}H proton shift signals with ^{13}C proton shift signals (0-10 ppm proton), (0- 190 ppm carbon).

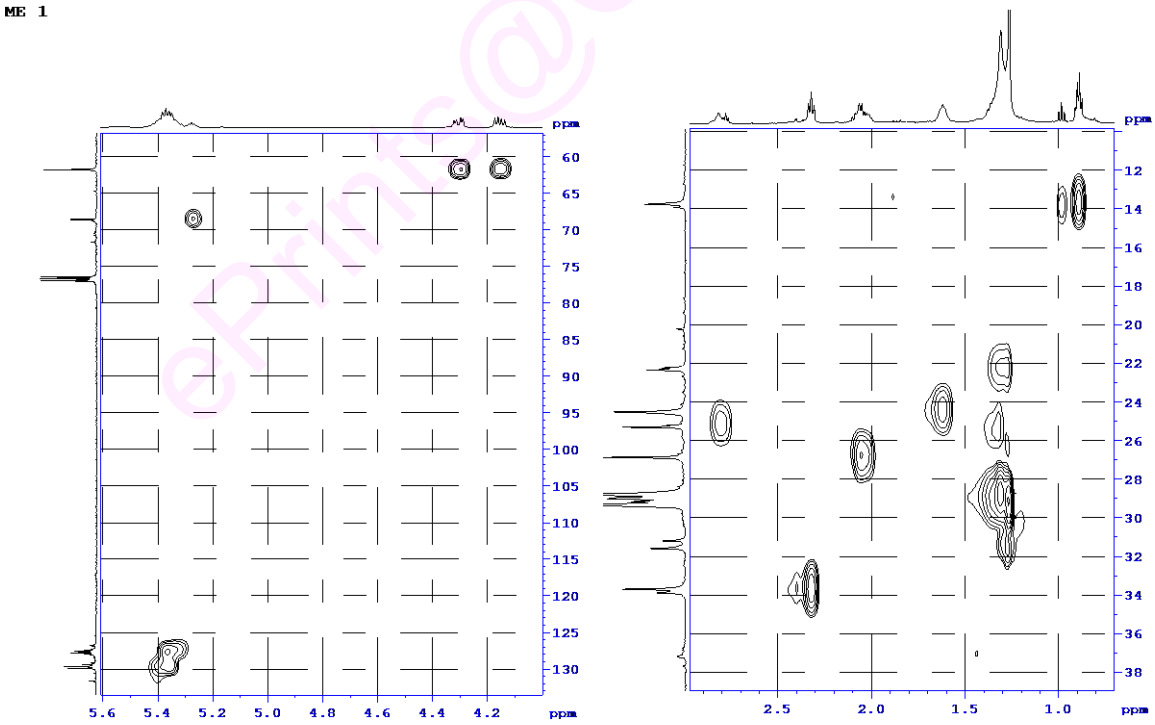


Fig. 59. The HSQC 2D NMR spectrum of compound-II correlating the ^{1}H Proton shift (0-3 and 4-5.6 ppm proton) signals with ^{13}C carbon shifts (0-40 ppm,0-135 ppm)

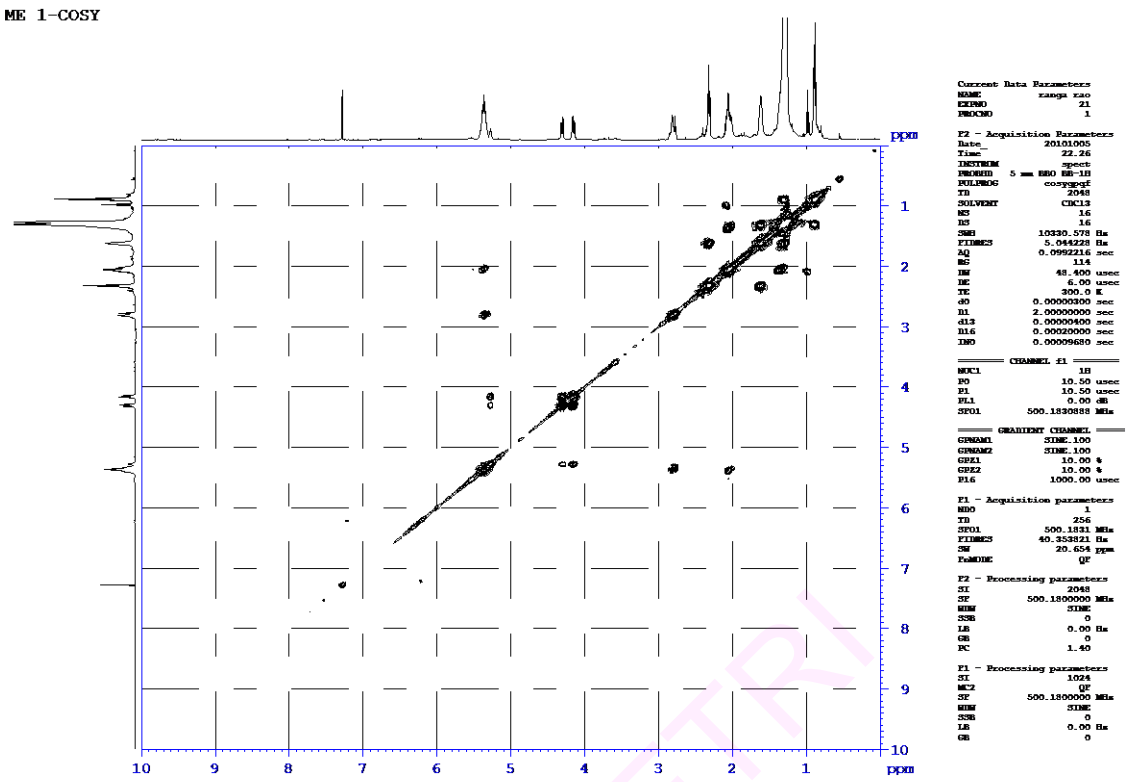


Fig. 60. The COSY 2D NMR spectrum of compound-II ^{1}H NMR of compound -11 with shifts (0-10 ppm).

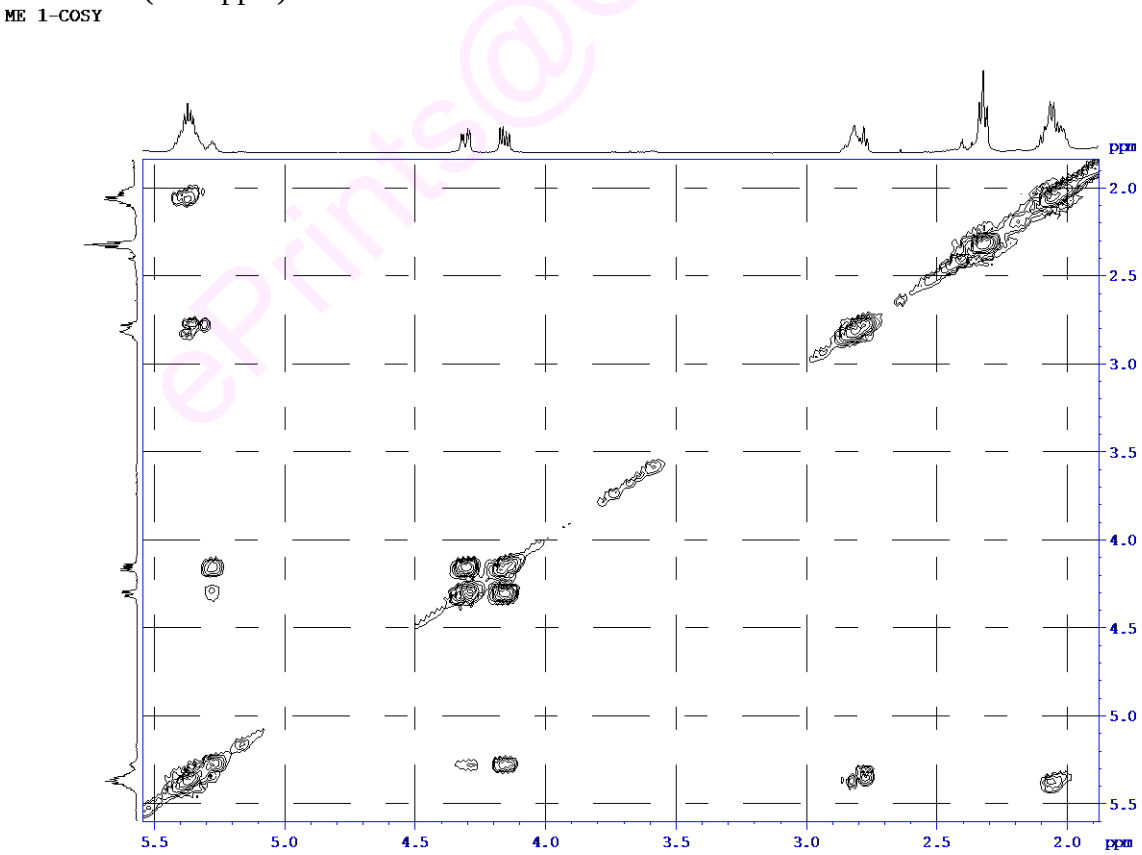


Fig. 61. The ^{1}H proton COSY 2D NMR spectrum of the compound-II with shift ranges (0.5 ppm)

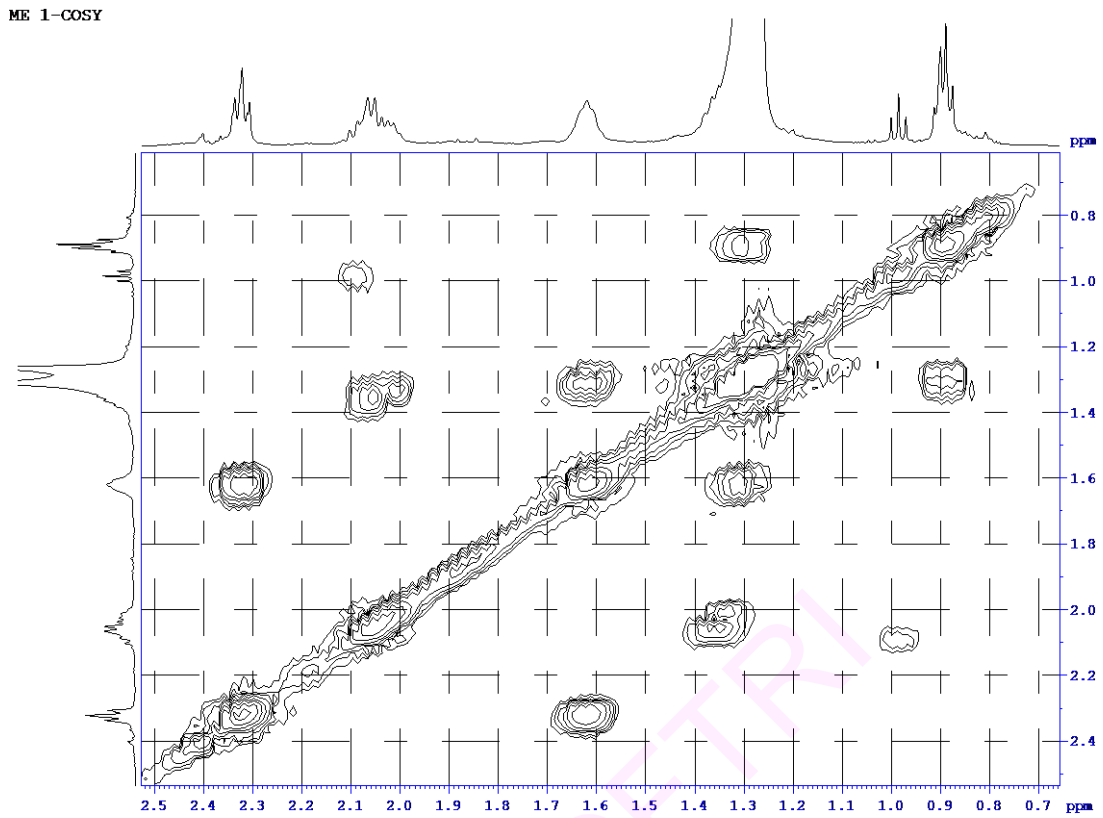


Fig. 62. The ^{1}H proton COSY 2D NMR spectrum of the compound-II with shift ranges (0-2.5 ppm) on the X-axis and (5.9-7.0 ppm) on the Y-axis.

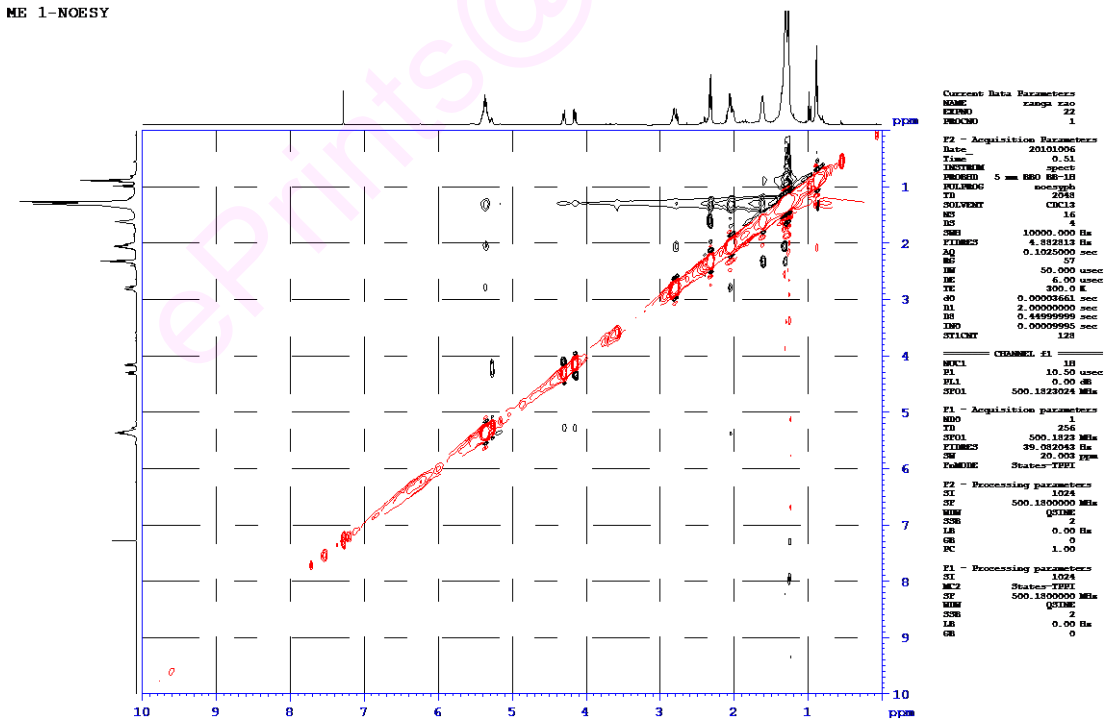


Fig. 63. The ^{1}H proton 2D NOESY NMR spectrum of compound-II with shift ranges (1-10 ppm).

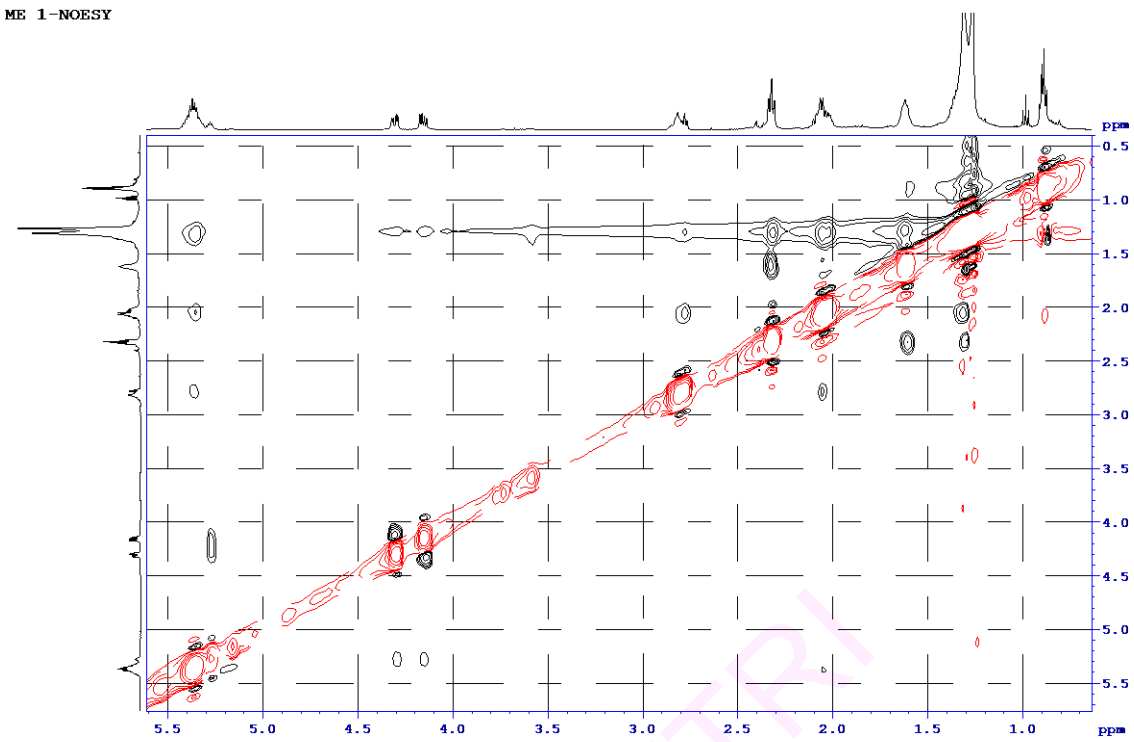


Fig. 64. The ^{1}H proton NOESY 2D NMR spectrum of the compound-II with shift ranges (0-5.5 ppm) for X-axis and (0.5-5.5 ppm) on the Y-axis.

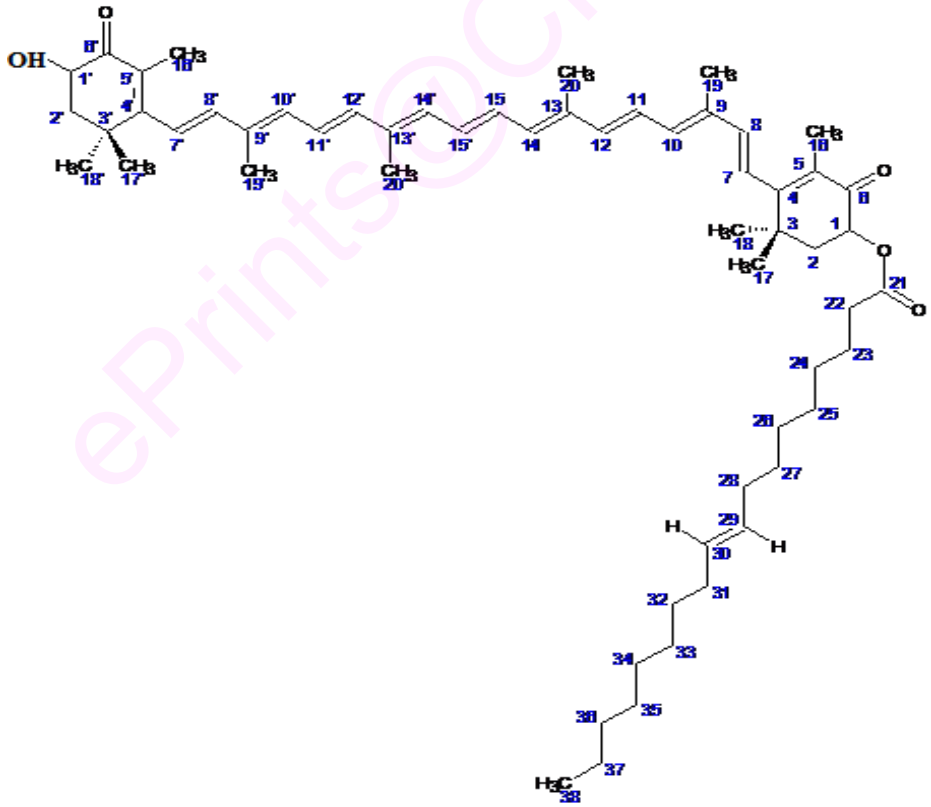


Fig. 65. Structure of monoester of astaxanthin

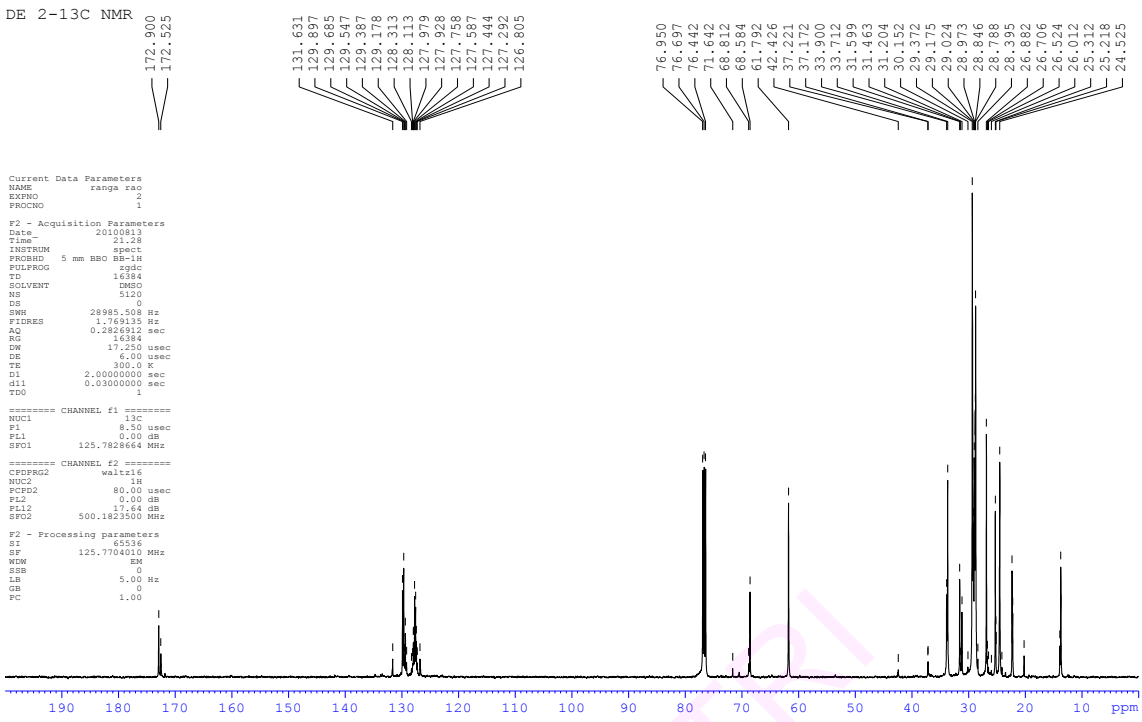


Fig. 66. The ^{13}C NMR spectrum of the compound-III with carbon shift signals from 0-200 ppm

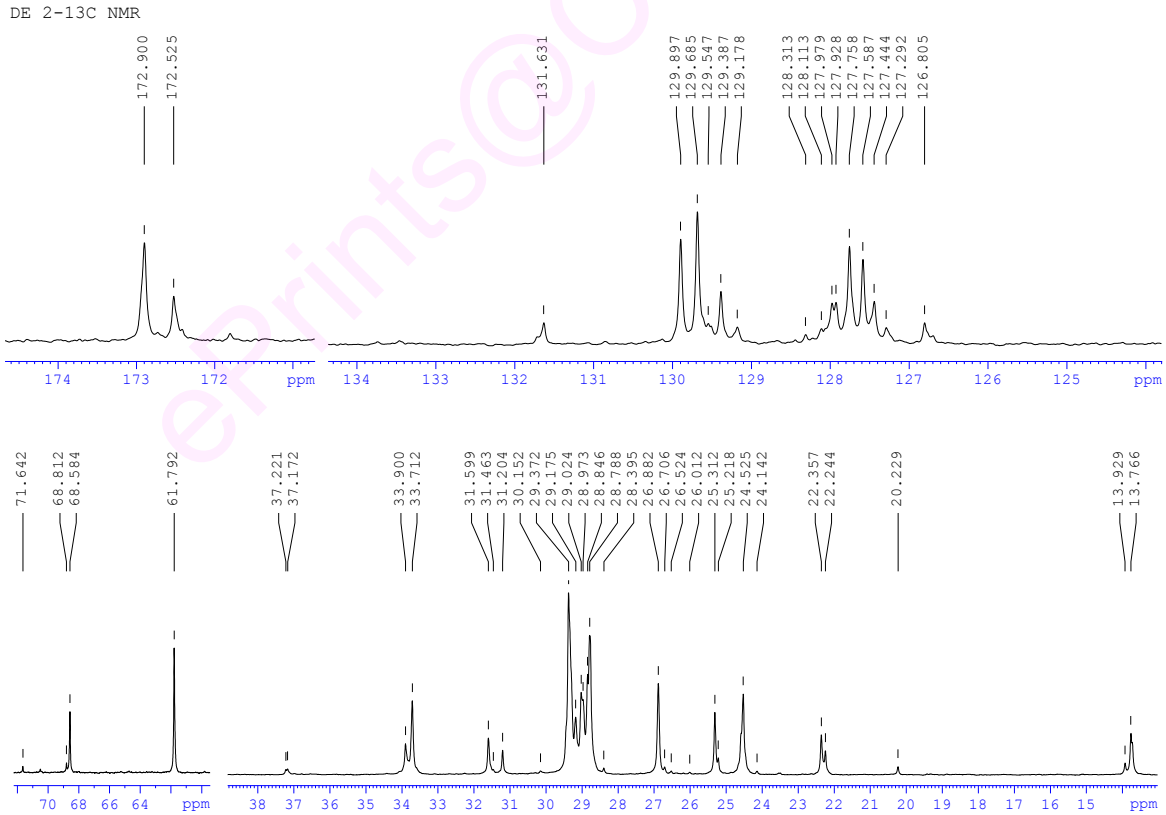


Fig. 67. The ^{13}C NMR spectrum of the compound -III with split carbon shift signals from (0-40 ppm) and (50- 140 ppm).

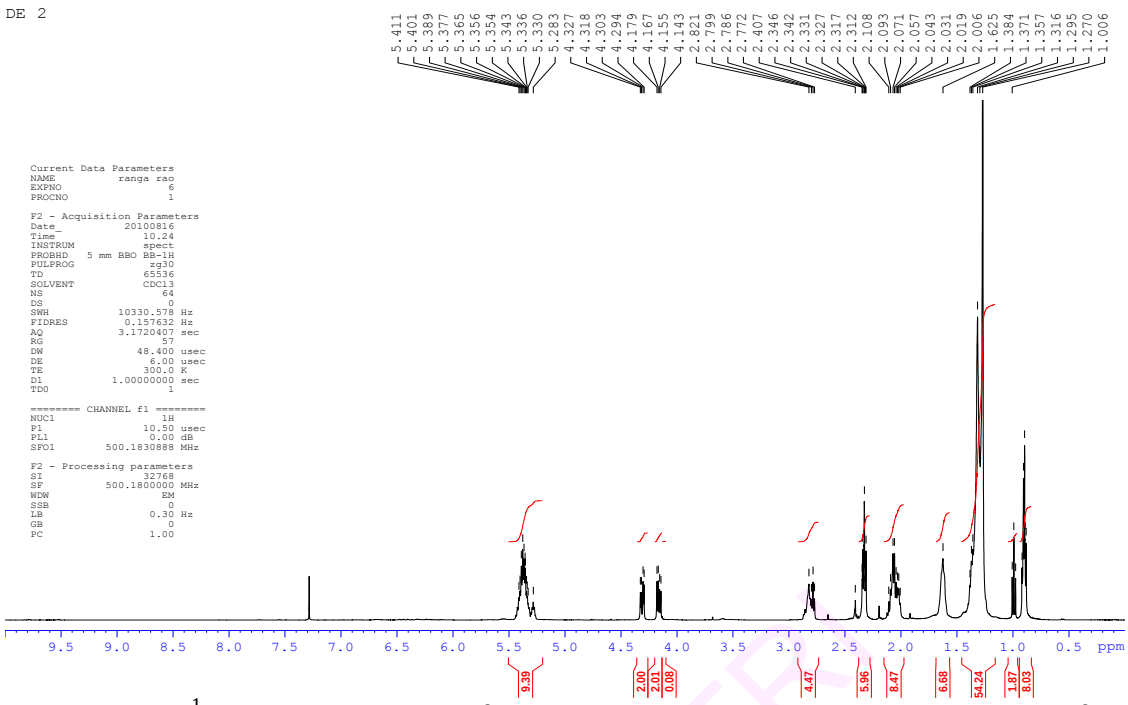


Fig. 68. The ^{1}H NMR spectrum of the compound-III describing hydrogen shifts signals from 0-10 ppm.

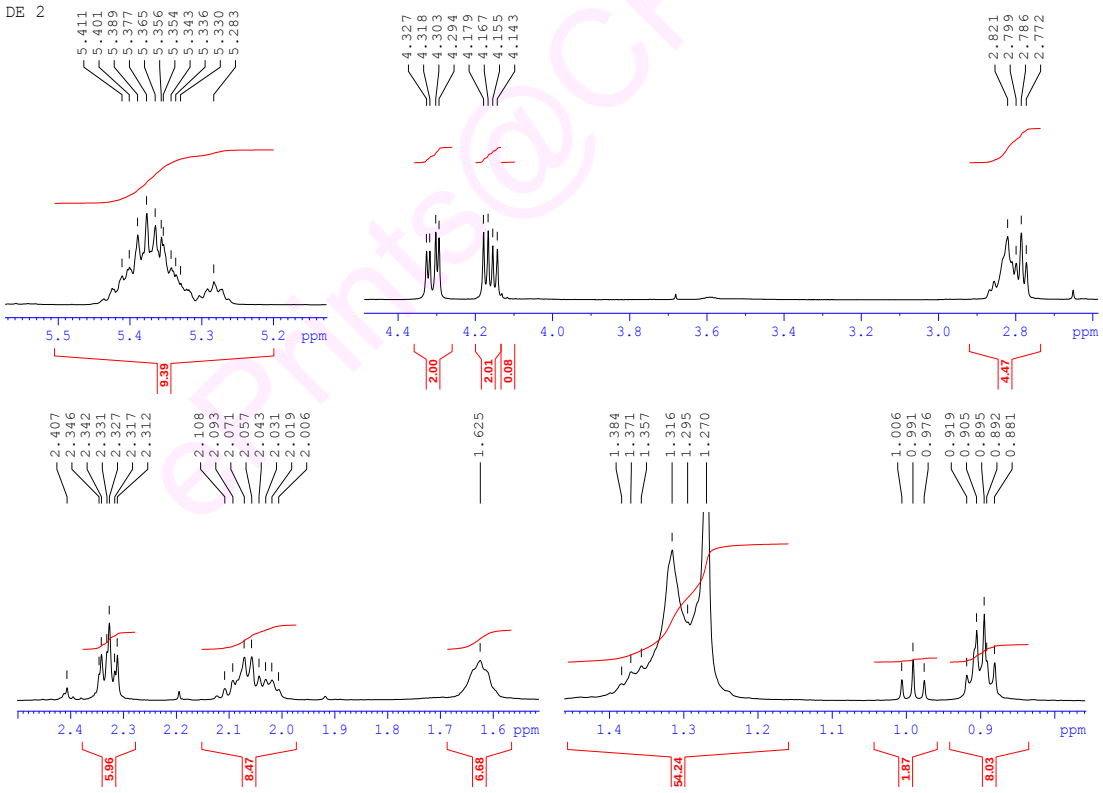


Fig. 69. The ^{1}H NMR spectrum of the compound-III describing hydrogen shift signals from 0- 3 ppm and 4-6 ppm.

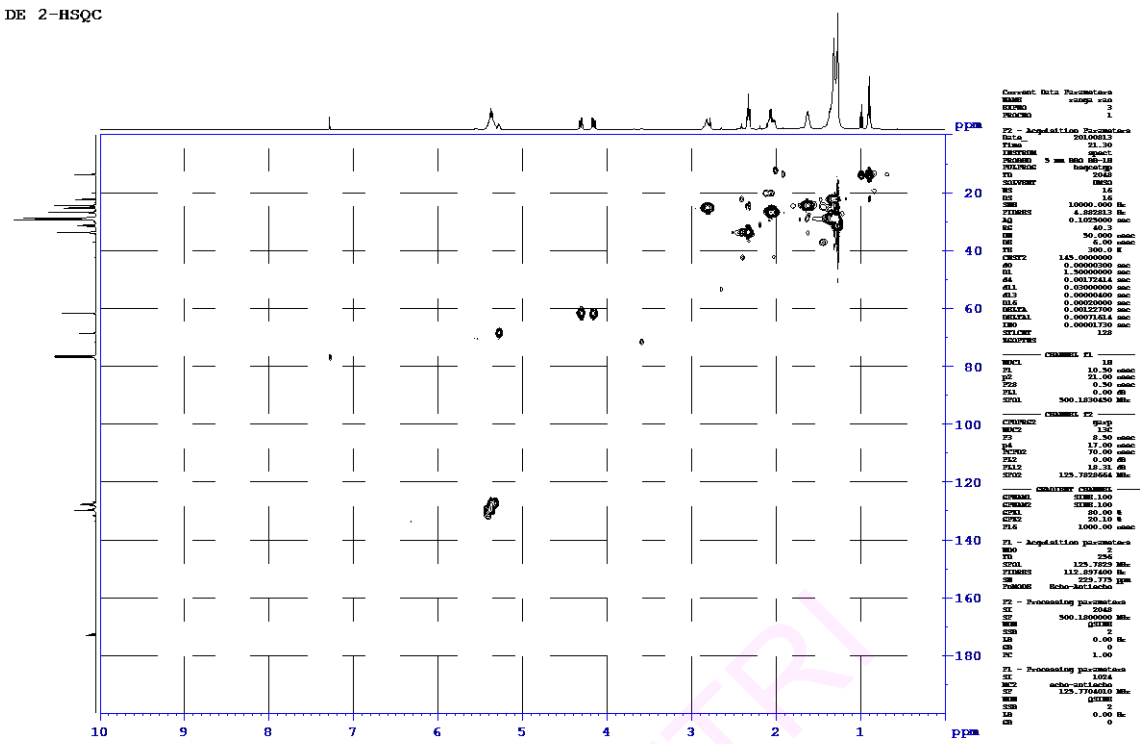


Fig. 70. The HSQC 2D NMR spectrum of compound-III correlating the ^{1}H proton shift signals with ^{13}C proton shift signals (0-10 ppm proton), (0-200 ppm carbon).

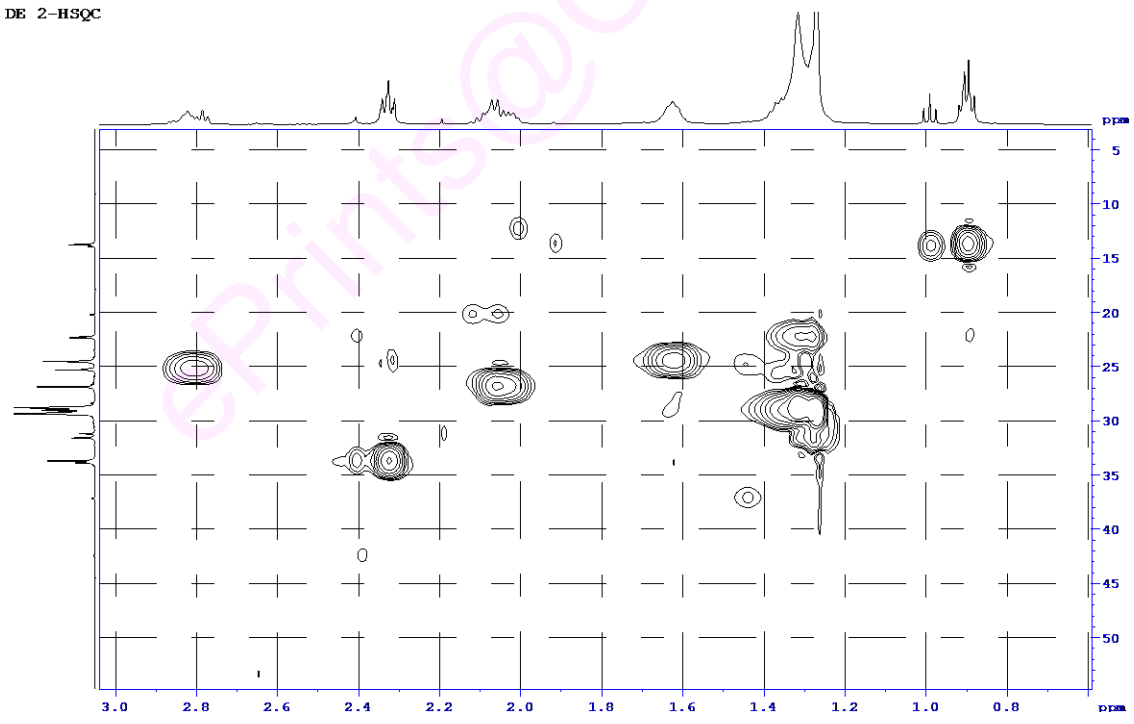


Fig. 71. The HSQC 2D NMR spectrum of compound -III correlating the ^{1}H Proton shift (0-3.0 ppm proton) signals with ^{13}C carbon shifts (0-55 ppm).

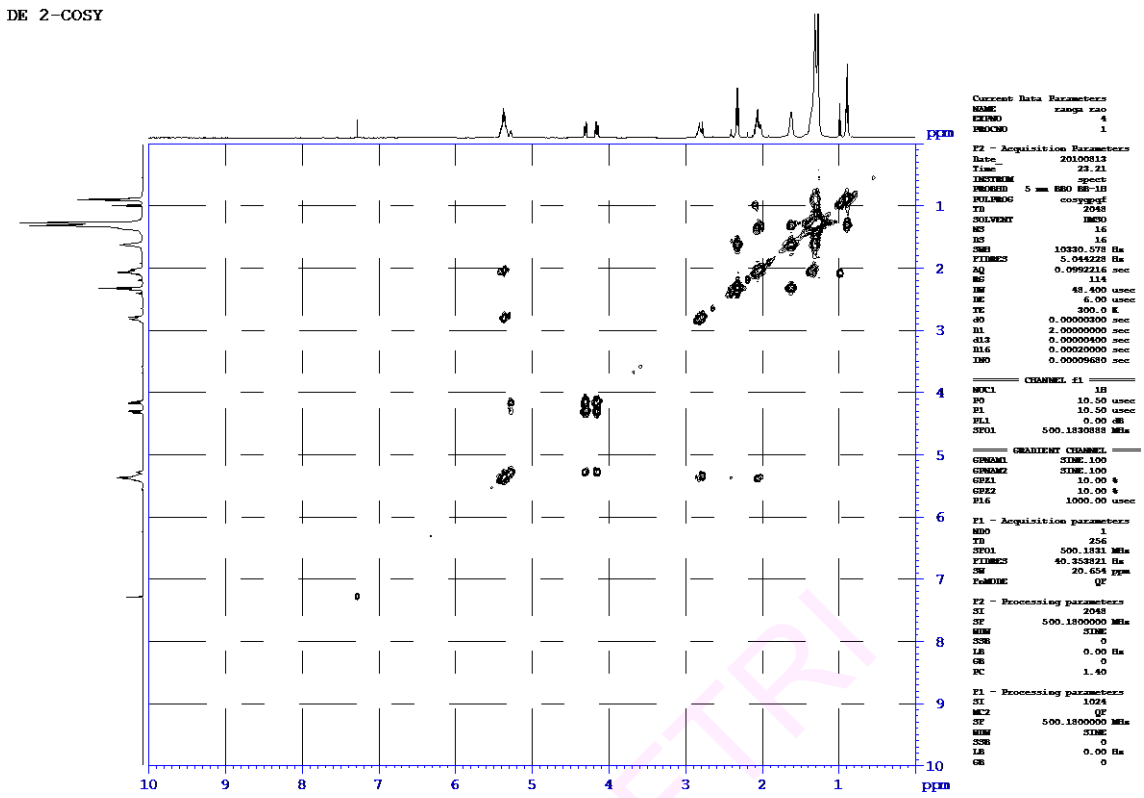


Fig. 72. The COSY 2D NMR spectrum of compound ^{1}HNMR of compound –III with shifts (0-10 ppm).

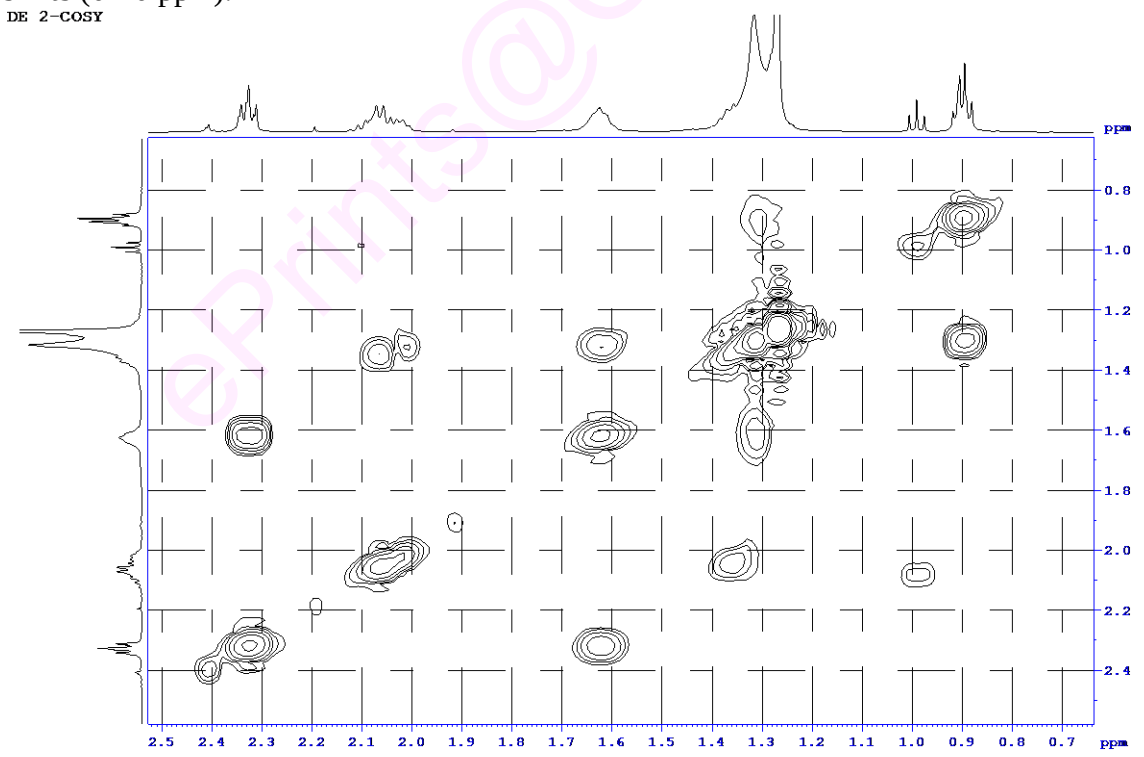


Fig. 73. The ^{1}H proton COSY 2D NMR spectrum of the compound –III with shift ranges (0-2.5 ppm).

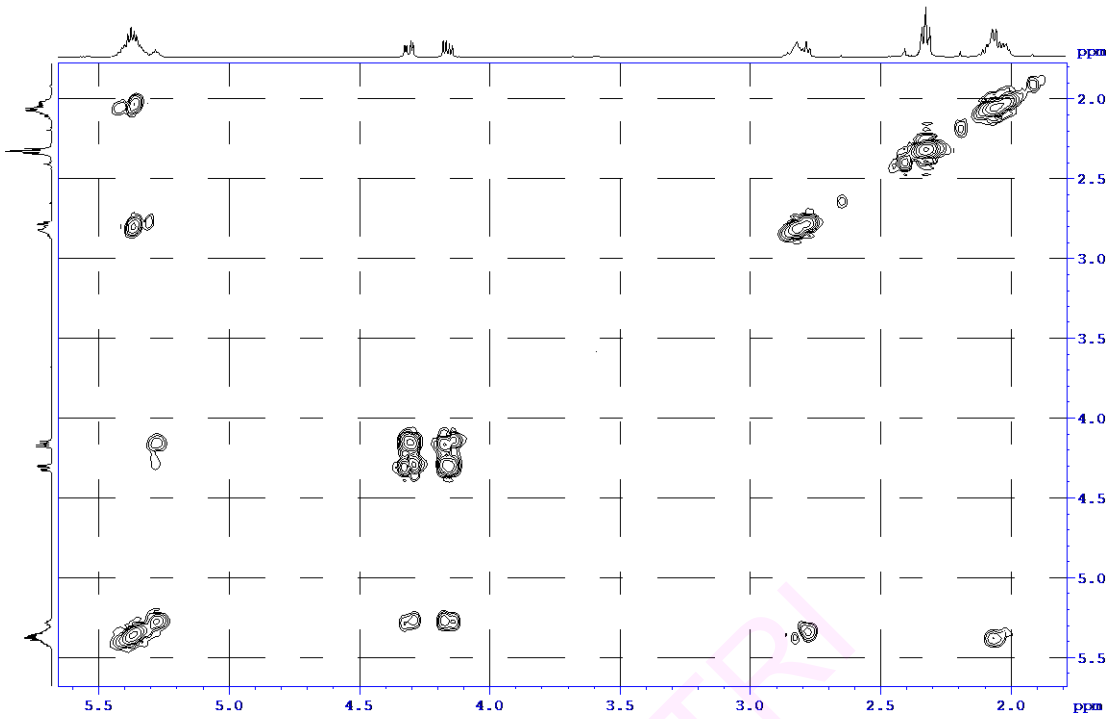


Fig. 74. The ^{1}H proton COSY 2D NMR spectrum of the compound -III with shift ranges (0-5.5 ppm).

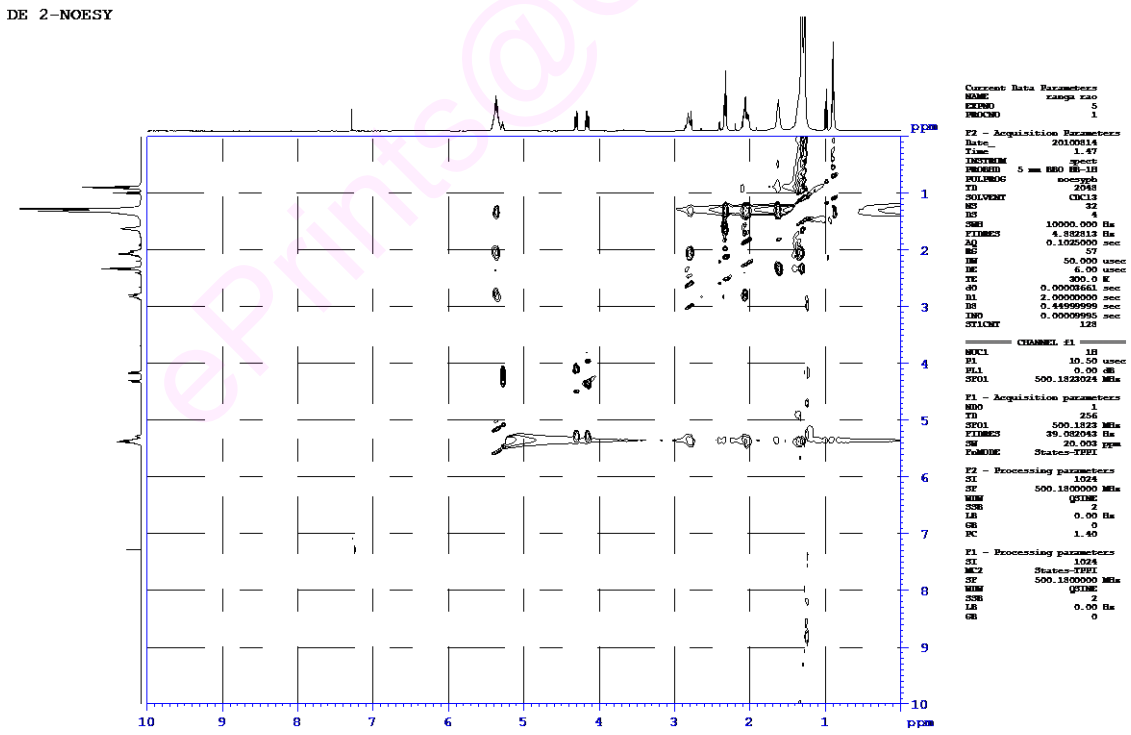


Fig. 75. The ^{1}H proton 2D NOESY NMR spectrum of compound -III with shift ranges (1-10ppm).

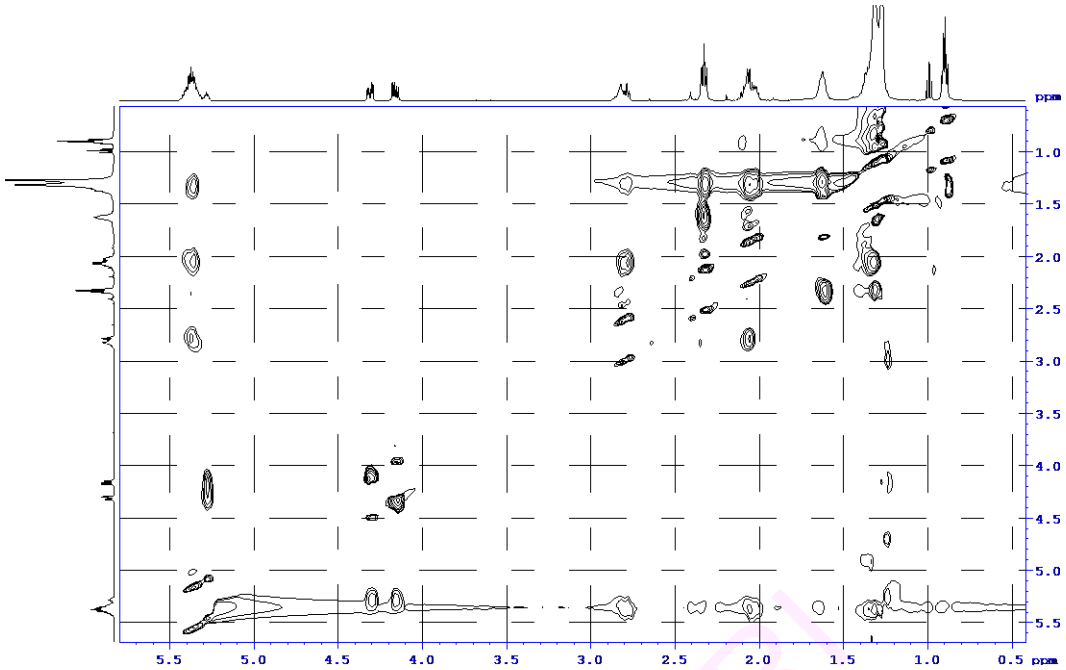


Fig. 76. The ^{1}H proton NOESY 2D NMR spectrum of the compound –III with shift ranges (0-5.5 ppm) for X-axis and (0.5-5.5 ppm) on the Y-axis.

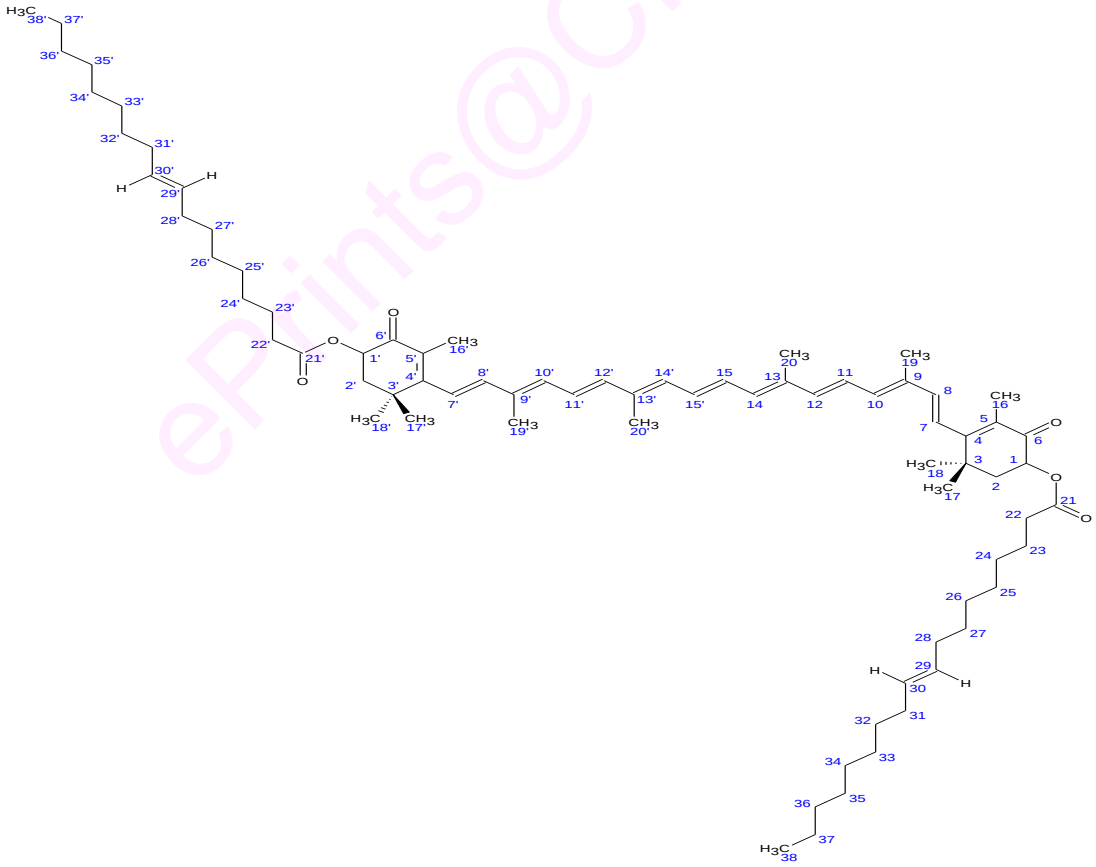


Fig. 77. Structure of di-ester of astaxanthin

3.4. Stability of astaxanthin in various edible oils

3.4.1. Characterization of carotenoid in oils

The carotenoid preparation from *H. pluvialis* constituted 85% of total astaxanthin and its esters, 2.2% chlorophyll and rest β-carotene, lutein and other carotenoids to a minor proportion. The absorption spectra of the carotenoid extract from *H. pluvialis* ells as given in Fig. 78 showed major absorption peak at 474 nm (carotenoid) and a minor at 661 nm (chlorophyll). The carotenoid profile of the extract of *Haematococcus* cells as analyzed by HPLC is presented in Fig. 79 showed the predominance of astaxanthin esters.

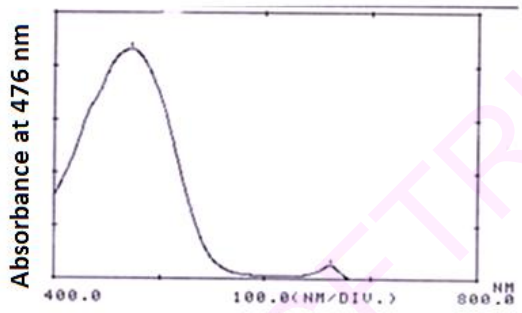


Fig. 78 . The absorption spectra of the carotenoid extract from *Haematococcus* cells

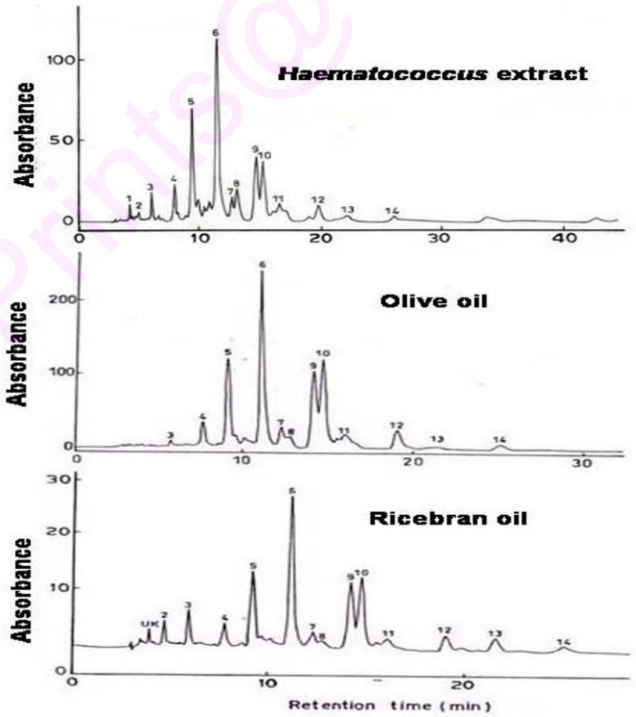


Fig. 79. HPLC analysis of carotenoid extract enriched edible oils stored at 70-90^{o}C for 8h. Absorbance of the peaks was measured at 470nm. Peak No.1.astaxanthin (free); Uk. Unknown, 2.lutein; 3.canthaxanthin 4 to 10, 12, 14 astaxanthin esters, 11. chlorophyll; 13. β- carotene

3.4.2. Estimation of tocopherol and phenolic content in edible oils

Tocopherols and phenolics were estimated in various edible oils such as mustard, groundnut, rice bran, gingelly, sunflower, palm, coconut and olive data (Table 22). Among the oils, gingelly oil contain maximum γ-tocopherol (28.77 μg/g), whereas 3.87 (mg/g) phenolic content was found. The absorption spectra of rice bran, sunflower, ground nut and gingelly oils indicated a major peak in the range of 340-346 nm. This may be due to the presence of tocopherol like or phenolic compounds (Brien, 1998). The data presented in Table 23 supports this observation. Coconut and olive oils did not show absorption in this region whereas mustard oil has showned prominent absorption in the region of 350-500 nm (Fig. 80). Although there are no sharp peaks in 400-500 nm range, the above mentioned oils exhibited absorbance in this range which is an indication of presence of some pigments in the oils.

Table 22 Tocopherol and phenolic contents in edible oils.

Edible oil	α-tocopherol μg/g	γ-tocopherol μg/ g	δ-tocopherol μg/g	Phenolics mg/g
Mustard oil	1.48 ± 0.01^{e}	8.45 ± 0.05^{e}	13.64 ± 0.05^{a}	4.31 ± 0.02^{b}
Groundnut oil	18.02 ± 0.18^{a}	21.00 ± 0.11^{b}	1.68 ± 0.01^{c}	1.90 ± 0.13^{a}
Rice bran oil	1.74 ± 0.03^{e}	12.35 ± 0.03^{d}	0.73 ± 0.01^{d}	3.97 ± 0.07^{c}
Gingelly oil	4.67 ± 0.04^{d}	28.77 ± 0.02^{a}	0.75 ± 0.14^{d}	3.82 ± 0.16^{c}
Sunflower oil	13.52 ± 0.04^{b}	3.29 ± 0.02^{f}	--	1.88 ± 0.10^{d}
Palm oil	10.12 ± 0.34^{c}	15.26 ± 0.23^{c}	4.16 ± 0.024^{b}	5.32 ± 0.03^{a}
Coconut oil	--	--	--	1.49 ± 0.09^{d}
Olive oil	0.92 ± 0.02^{e}	--	--	1.74 ± 0.11^{d}

Values are mean ± SD (n=3). Values are not sharing a similar superscript within the same column are significantly different (P<0.05) as determined by ANOVA.

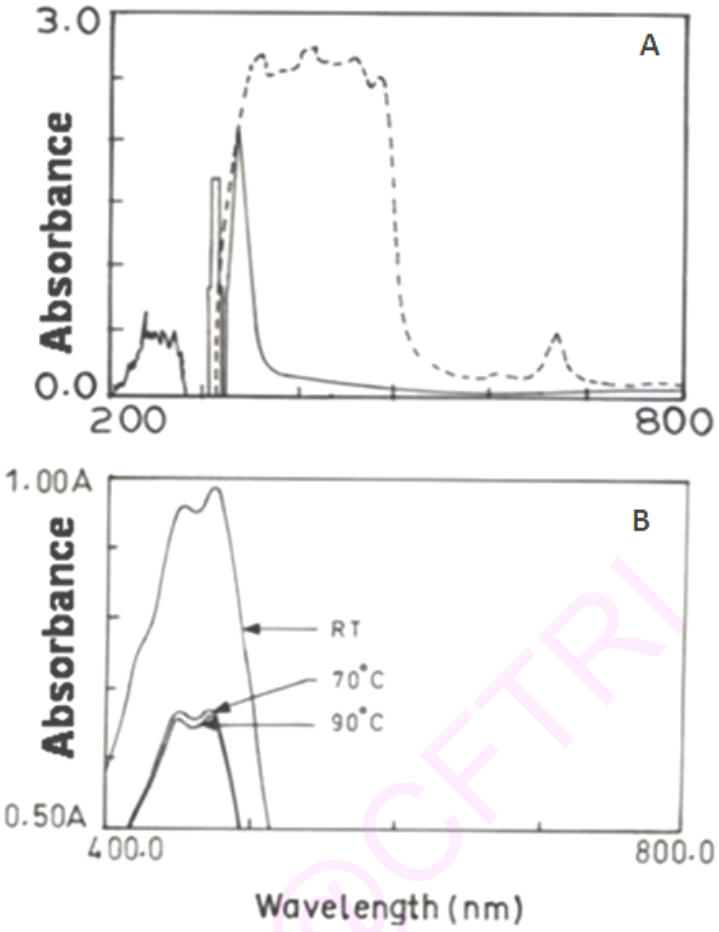


Fig. 80. Absorption spectra of carotenoid extract from *H. pluvialis* cells and edible oils. A. Absorption spectra of Gingelly (—), Mustard (---), B. Absorption spectra of carotenoid in mustard oil at room temperatures treated at 70°C and 90°C for 8h.

3.4.3. Stability of carotenoid extract in different oils

The astaxanthin stored in oils at room temperature was fairly stable while the astaxanthin in oils subjected to heat treatment at 70 °C and 90 °C differed in the extent of stability. Rice bran, gingelly and palm oil retained 84-90% of astaxanthin when heated at 70 °C for 8 h and subsequently stored at room temperature for 3 months. Only palm oil was effective in retaining 90% of astaxanthin even after 8 h treatment at 90 °C. The spectra profile of astaxanthin in mustard oil did not change due to heating but decrease in quantity was evident. There was no difference in the HPLC profile of carotenoid extract from *H. pluvialis* cells and the carotenoid extract stored in oils exposed to 70 - 90 °C (Fig. 81). The astaxanthin which existed as ester in encysted cells of *Haematococcus* remained same after storage and also after heating at 70 and 90 °C for 8 h. The heat treated sample when analysed on TLC indicated presence of astaxanthin ester spots (Fig. 82). This was also clearly evident from

HPLC analyses . Under similar conditions, astaxanthin in aqueous medium when heated to 70-90 ^{o}C there was significant loss in astaxanthin content. The TLC analyses of the sample showed no astaxanthin ester spot.

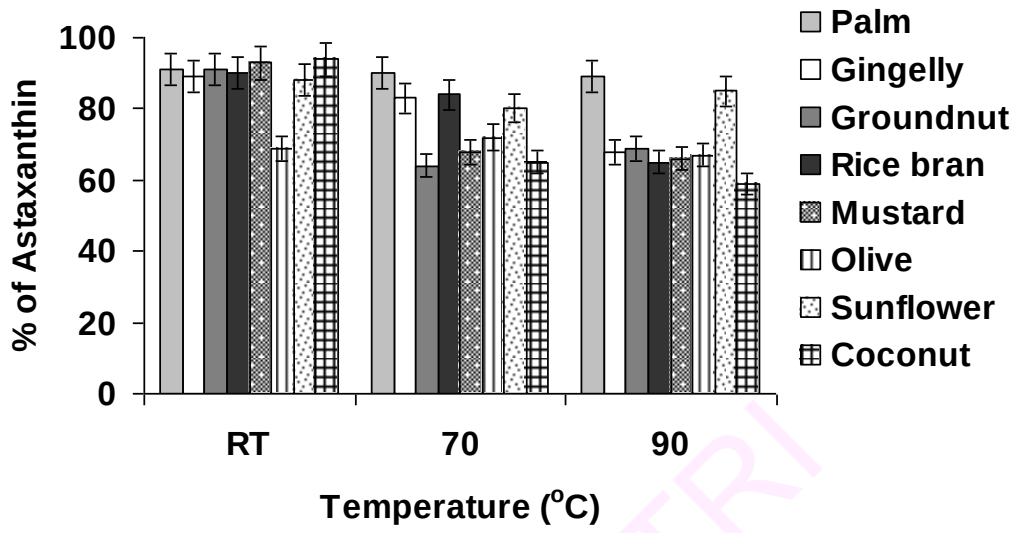


Fig. 81. Stability of astaxanthin in edible oils at different temperatures. Data represents an average of 3 replicates. Bars indicates ± SD

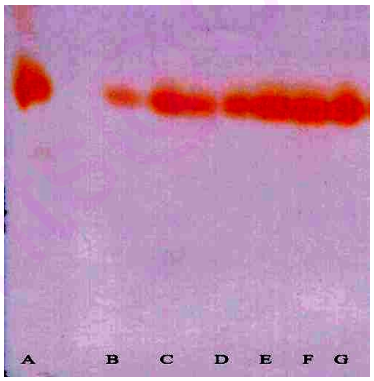


Fig. 82. TLC separation of carotenoid extract from *Haematococcus* and oils. A: Astaxanthin ester in *Haematococcus* extract, B - G: Astaxanthin ester from oil (separated by column)

The high stability of astaxanthin in the edible oils at room temperature may be attributed to the presence of flavonoid polyphenols (olive), tocopherols (mustard), vitamin E (sunflower), tocopherols and gamma oryzanol (rice bran oil), which are known to have stabilizing activity (Brien, 1998) and prevents rancidity of oils which in turn enhanced the stability of astaxanthin. Data presented in Table 23 indicates presence of different tocopherols and phenolic compounds in different oils. The oils differed in the quantity and quality of tocopherols (Fig. 82) and in the amount of phenolics. The inherent stability of oils depends on the fatty acid composition therefore it would have also contributed to the stability of astaxanthin besides the

presence of tocopherol and phenolic compounds. The relative decrease in stability of astaxanthin at high (90^{o}C) temperatures especially in coconut, ground nut, olive and mustard oils when compared to the stability in palm oil may be attributed to the differences in the inherent stability of oils offered by its fatty acid composition (Narula, 1995) Thus the present data substantiates the stability of carotenoids in edible oils.

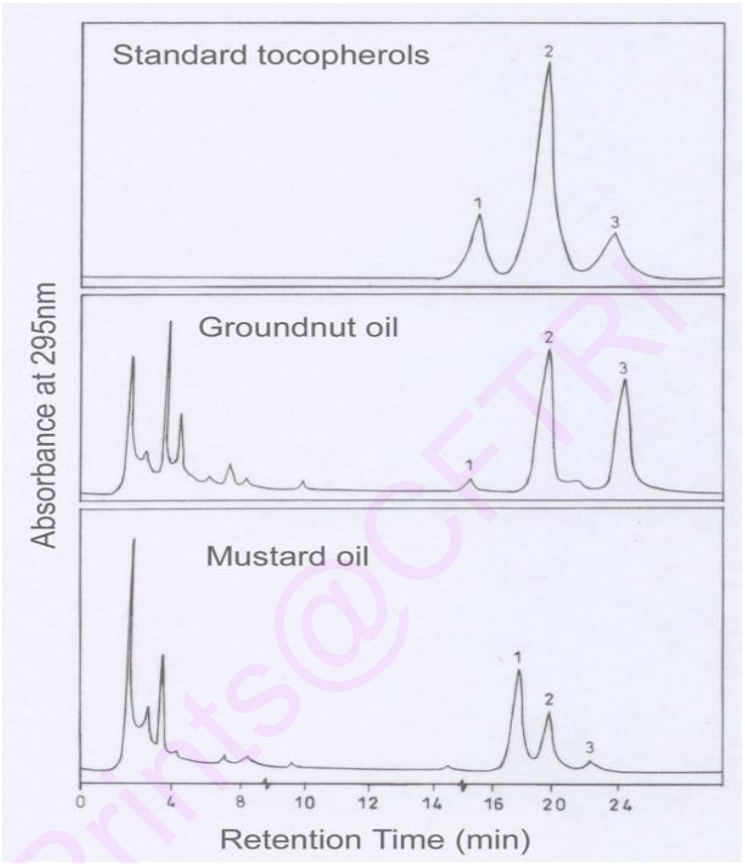


Fig. 83. HPLC analysis of tocopherols from edible oils, 1 = δ - tocopherol 2 = γ - tocopherol 3 = α - tocopherol.

3.4.4. Effect of temperature

In order to see the effect of high temperatures on astaxanthin content, gingelly and palm oils with astaxanthin extract were subjected to 120 and 150 ^{o}C for a period of 6-8 h. The stability profile of astaxanthin in these oils at different temperatures is given in Fig. 84 & 85. The rate of carotenoid loss at 150 ^{o}C was very high compared to that at 120 ^{o}C. The astaxanthin content decreased by 50-75% at 120 ^{o}C and 86-93% at 150 ^{o}C in gingelly and palm oils respectively at the end of 8 h treatment (Fig. 86 A-C). It is clearly evident that above 90 ^{o}C degradation of astaxanthin is faster which is also reflected in absorption spectra. The TLC analyses also substantiated the degradation of astaxanthin as there was no clear spot corresponding to astaxanthin

ester spot. The fatty acid profile of palm oil and gingelly oil when analyzed before and after heat treatments did not show significant changes or alterations (data not shown). This is similar to earlier report of Leszkiewicz and Kasperek, (1988) no significant changes in the fatty acids and nutritive value of rape seed oil was observed when heated in the temperature range of 110- 150 ^{o}C for 8 h.

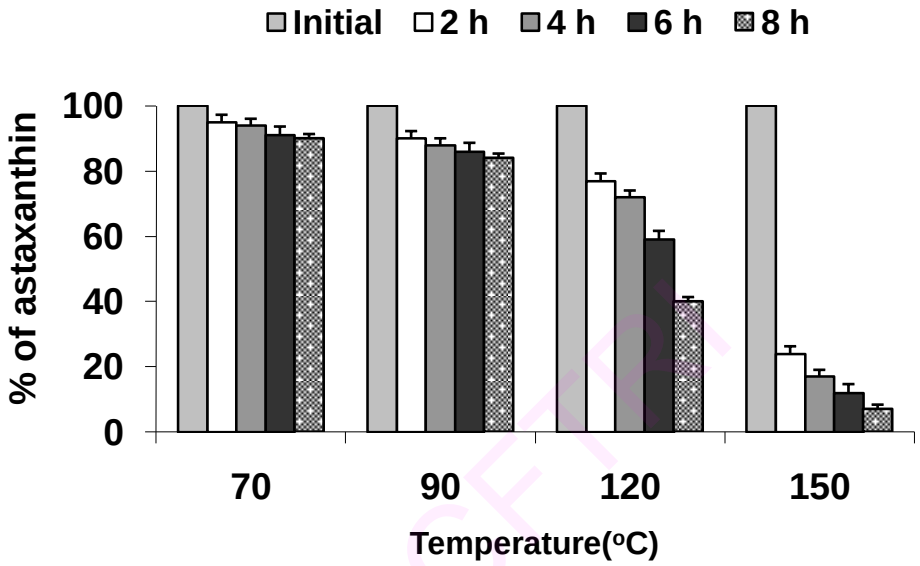


Fig. 84. Stability of astaxanthin at different temperatures in palm oil. Data represents an average of 3 replicates. Bars indicates ± SD.

3.4.5. Influence on color value

The colour values measured in terms of hunter a, b, L and DE are shown in Table 23. The positive value for 'a' indicates the redness of the samples. From a very red sample with an initial 'a' values at 7.5 and 16.24 units in palm and gingelly oils respectively, the redness of the oils drastically reduced by heating at 150 ^{o}C. L value which indicates the lightness of the sample increased and DE total or overall colour value decreased in the samples treated at high temperatures indicating the sensitivity of the astaxanthin to high temperatures. The decrease in 'a' value is not significant in oils at room temperature. Similarly the total colour change expressed in terms of DE was found to be same during the 4 months study at room temperature.

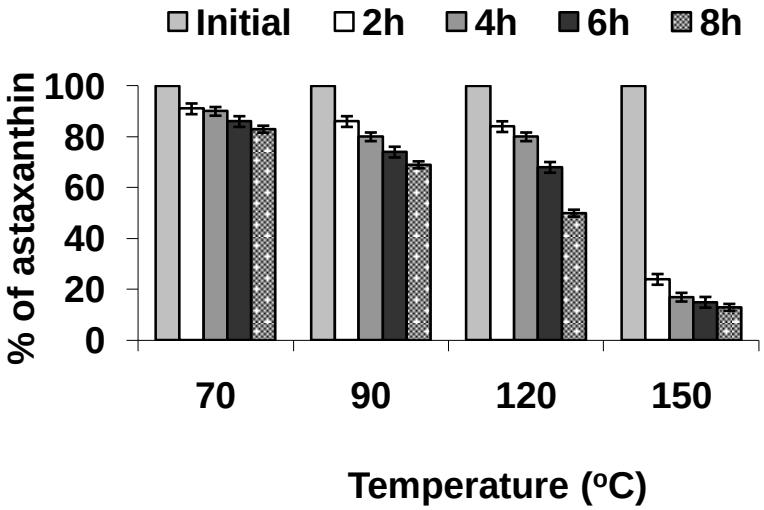


Fig. 85. Stability of astaxanthin at different temperatures in gingelly oils. Data represents an average of 3 replicates. Bars indicates ± SD.

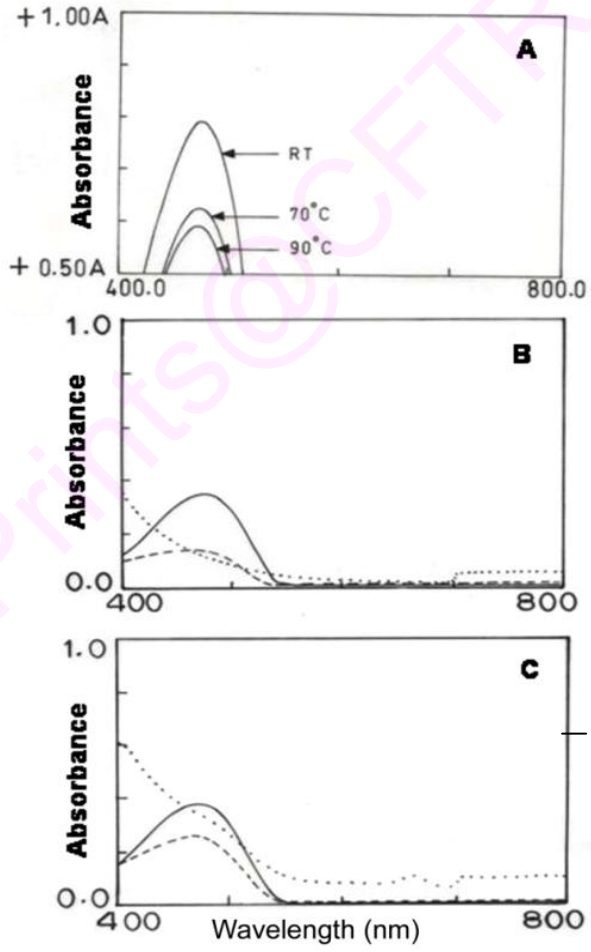


Fig. 86. Absorption spectra of carotenoids in edible oils. Sunflower oil at room temperature, treated at 70°- 90°C for 8h. Palm oil (—) control; (---) 120 °C; (···) 150 °C Gingelly oil (—) control; (---) 120 °C; (···) 150 °C.

Table 23. Hunter colour values for carotenoid in edible oils at different conditions.

Sample	L	a	b	DE
Palm oil[a1]	35.20±0.41[b]	7.30±0.14[c]	14.54±0.06[c]	65.85±0.25[c]
Palm oil[2]	35.73±0.35[b]	7.06±0.08[c]	14.90±0.14[c]	65.50±0.21[c]
Aqueous[1]	33.04±0.15[c]	8.60±0.10[b]	7.73±0.06[e]	66.79±0.15[b]
Aqueous[3]	32.39±0.19[c]	5.63±0.17[d]	7.98±0.05[e]	67.08±0.17[b]
Gingelly oil[1]	33.86±0.33[c]	16.02±015[a]	15.49±0.11[b]	69.12±0.18[a]
Gingelly oil[4]	41.24±0.17[a]	5.79±0.08[d]	17.52±0.12[a]	60.52±0.26[d]
Palm oil[4]	44.03±0.14[a]	3.49±0.37[e]	11.95±0.19[d]	56.04±0.13[e]

1 refers to Initial; 2 refers to 3 months after at room temperature; 3 refers to 8 h after treatment at 90 ^{o}C; 4 refers to 8 h after treatment at 150 ^{o}C; Values are mean ± SD (n=3). Values are not sharing a similar superscript within the same column are significantly different ($P<0.05$) as determined by ANOVA.

3.4.6. Peroxide value

The peroxide values in different oils increased with increase in temperature compared to controls at room temperature, whereas in the oils with carotenoid extract, the peroxide values were found to be 40 to 45% lesser (Fig. 87) than that in control oils (without carotenoid). This further more indicates carotenoid protective action against peroxides formation. Similarly, decrease in peroxide formation when ethanol extracts of the leafy vegetables were added to ground nut and sunflower oils during heating (frying temperatures) and subsequent storage was reported by Shyamala et al., (2005)**.** Peroxide formation in edible oils at normal storage conditions occurs very slowly but it is triggered when exposed to heat, air and light reported by Naz et al., (2004). The results in Fig. 88 are in accordance with the statement, peroxide formation in edible oils at different temperatures and storage conditions were reported (Naz et al., 2004). The extent of oxidation varied among different oils based on their inherent stability which depends on the fatty acid composition (Naz et al., 2004). In addition to antioxidant activity of astaxanthin, the decrease in peroxide value in astaxanthin added oils at different temperatures also implies protective effect on peroxide formation in the edible oils studied.

3.4.7. Free fatty acids

The free fatty acid formation in heat treated samples showed marginal increase. However there was no significant difference in treated and control samples with regard to free fatty acid formation.

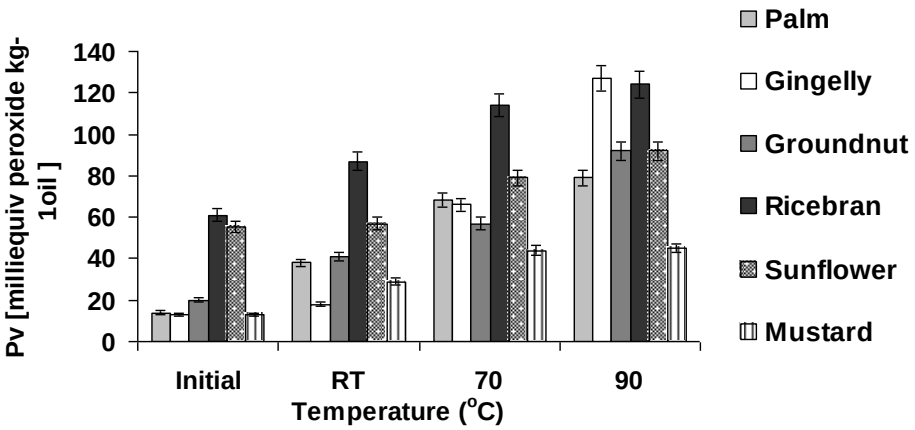


Fig. 87. Peroxide value in control edible oils treated at different temperatures. Data represents an average of 3 replicates. Bars indicates ± SD

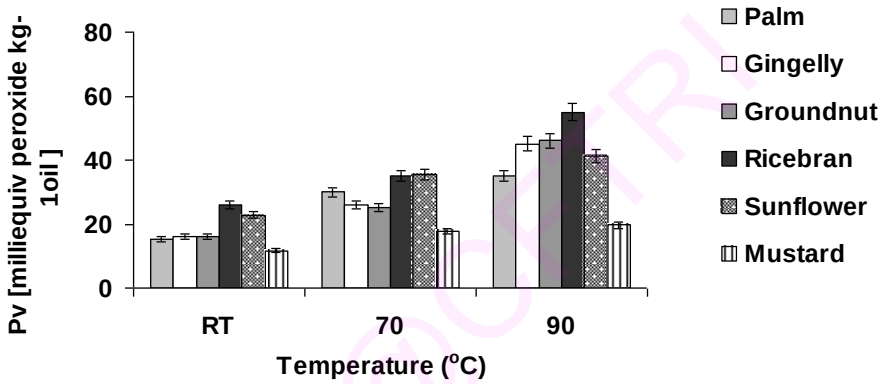


Fig. 88. Peroxide value in oils with astaxanthin treated at different temperatures. Data represents an average of 3 replicates. Bars indicates ± SD.

3.5. Antioxidant properties of astaxanthin containing edible oils

3.5.1. DPPH method

The antioxidant potential of the carotenoids from oils is evaluated in *in vitro* models. Antioxidant activity was found in all the carotenoid extracts stored in different oils. In both the models studied, antioxidant activity was found to be comparable with standard BHA which is at nearly 95% of BHA activity in all the carotenoid samples stored in different oils at room temperature. The carotenoids from oils exposed to various temperatures also exhibited antioxidant activity, sometimes with a less activity. Astaxanthin exhibited 50%, 40% and 25% of antioxidant activity at 70, 90 and 120 ^{o}C at 20 ppm level of carotenoid by using DPPH method as shown in Fig. 89.

3.5.2. Hydroxyl radical scavenging activity

Astaxanthin exhibited 75%, 50% and 45% of antioxidant activity at 70, 90 and 120 ^{o}C at 20 ppm level of carotenoid by using hydroxyl radical scavenging activity as shown in Fig. 90. In general the hydroxyl radical scavenging activity was found to be more

in the carotenoid samples from different oils. This shows that the antioxidant potential of the astaxanthin is stable if stored in oils at room temperature and also after exposure to 8 h at 70 and 90 ^{o}C temperatures. The results are in conformity with the report that astaxanthin has high antioxidant activity than α- tocopherol and *β*-carotene (Lorenz et al., 2000)

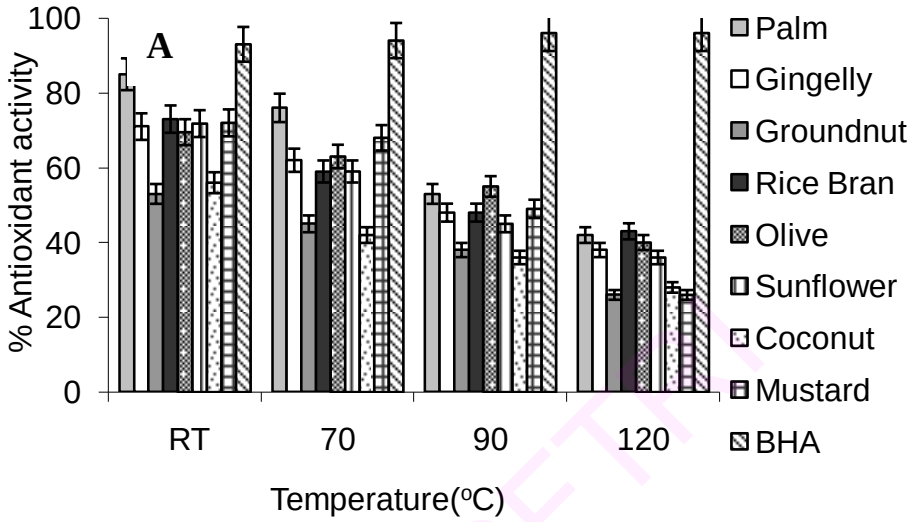


Fig. 89. Antioxidant activity of carotenoids from edible oils by DPPH method. Data represents an average of 3 replicates. Bars indicates ± SD.

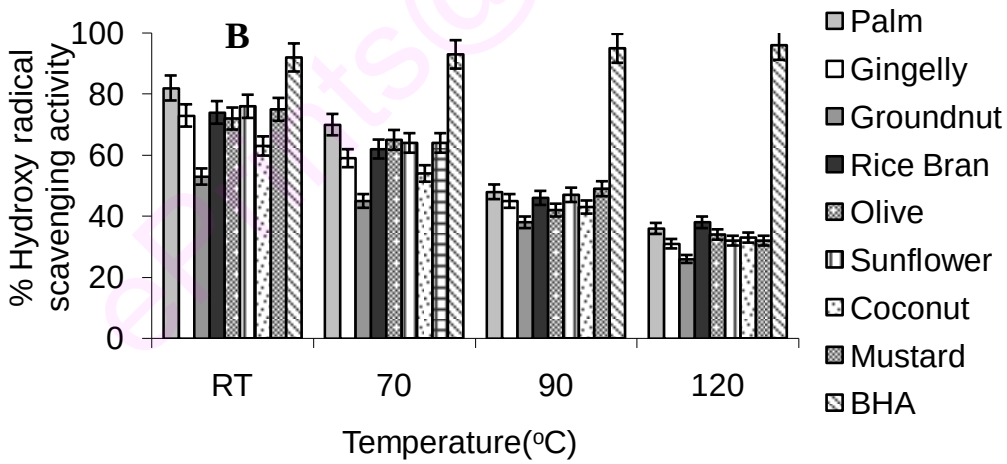


Fig. 90. Antioxidant activity of carotenoids from edible oils using hydroxyl scavenging activity. Data represents an average of 3 replicates. Bars indicates ± SD.

3.6. Discussion

H. pluvialis carotenoids were quantified in terms of total carotenoids by measuring optical density at 450-476 nm and found to yield 2.2% w/w of total carotenoids in terms of dry weight. This has identified by comparison of spectral patterns of the carotenoids with authenticated samples (Fig. 28). These carotenoids were subjected to

identification using TLC adopting various solvents on silica gel coated plate. Among the different solvents employed, n-hexane:acetone:ethylacetate (7:3:0.5) has shown high resolution in separation of carotenoids. Based on the TLC separation it was found that *H. pluvialis* contain a total of nine pigments including astaxanthin esters and chlorophyll a and b (Fig. 33). The carotenoids were separated on silica and alumina column using various solvents and were identified based on their absorption as astaxanthin, lutein, zexanthin, violaxanthin, neoxanthin, astaxanthin esters and α, β-carotene (Table 18 & 19, Fig. 36). This observation was further strengthened by means of isocratic HPLC analysis developed by us using acetonitrile, methanol and dichloromethane as mobile phase. Number of HPLC reports identified the carotenoids from various biological materials using C_{18} column employing various solvents including method an acetonitrile, dichloromethane, methylene chloride, hexane in isocratic and gradient elution (Khachik *et al.*, 1986). Another method using C_{18} column has been reported using reverse phase chromatography in acetonitrile, methanol and dichloromethane in gradient elution pattern and isocratic elution (Darko et al., 2000, Lakshminarayana et al., 2005). Most of the methods are applicable for either carotenoids containing hydrocarbon caroteniods or oxygenated carotenoids (xanthophylls). After a number of attempts to have a suitable solvent system which can resolve all the pigments of algae, an isocratic solvent system consisting of acetonitrile, methanol, dichloromethane was developed to get better resolution. HPLC confirmed the carotenoids along with astaxanthin & its esters which were initially identified by TLC. It was also found that aceonitrle: methanol (1:1) can also resolve the pigments and the same was employed for LC-MS. Different methods used for identification of astaxanthin and its esters by liquid chromatography-mass spectrometry have been reported (Miao et al., 2006, Lim et al., 2002, Yuan et al., 1997, 1998,.Richard et al., 1996, Renstrom et al., 1981, Grung et al., 1992, Takaichi et al., 2003). Astxanthin & its esters, violaxanthin, neoxanthin, lutein, zeaxanthin, α-carotene, β-carotene, echinenone, 7, 8, 7', 8'-tetradehydroastaxanthin, and antheraxanthin were identified in *H. pluvialis* by LCMS (APCI) with their characteristric molecular peaks (Fig. 40). It also revealed the association of astaxanthin with monoester (ME $C_{16:0}$, ME $C_{17:2}$, ME $C_{17:1}$, ME $C_{17:0}$, ME $C_{18:4}$, ME $C_{18:3}$, ME $C_{18:2}$, ME $C_{18:1}$) and diester (DE $C_{16:0}/C_{16:0}$, DE $C_{16:0}/C_{18:2}$, DE $C_{18:1}/C_{18:3}$, DE $C_{18:1}/C_{18:2}$, DE $C_{18:1}/C_{18:1}$) as show in (Fig. 45**)**. Structure and composition of these compounds are summarized in Table 24. Because of only mass differences

between quasimolecular and fragment ions they were used for assignment of acylchains, the location of double bonds could not be determined by the mass spectrum. Thus, many isomers of astaxanthin esters in *H. pluvialis* could not be identified unequivocally. We have observed that the fragmentation pattern of astaxanthin esters was dominated by the loss of fatty acid and water. Protonated $[M+H]^+$ resulted from the positive ion mode. The basic peaks of other astaxanthin diesters showed characteristic fragment ions of losing one fatty acid, but their fragment ions of losing the second fatty acid had weaker relative intensity. For the first time we have reported details of all the carotenoids present in the algae, which can extend its utility for a wider range of sources containing astaxanthin and its esters. Analysis of various carotenoids indicate that *Haematococcus* is not only rich in astaxanthin, which is major projected bioactive molecule, it has also shown the presence of oxygenated carotenoids namely xanthophylls like lutein, β-cryptoxanthin, zeaxanthin, α, β-carotene, which have great significance in enhancement of bioavailability in eye health and other health benefits.

Table 24. Comparison of various methods employed for estimation of carotenoids by liquid chromatography

Method	Column	Mobile phase	Carotenoids and its esters detected	Reference
Gradient	C_{18}	Acetone/water	Astaxanthin, echineone, canthaxanthin, Siphonaxanthin, monesters and diesters	Miao et al., 2006
Gradient	C_{30}	Methanol/ TBME/water	Astaxanthin and it esters	Breithaupt, 2004
Isocratic	C_{18}	Methanol	Astaxanthin, monoester of astaxanthin and diester of astaxanthin	Takaichi et al., 2003
Gradient	C_{30}	Methanol/water/ triethylamine /MTBE	Violaxanthin, antheraxanthin Lutein, and zeaxanthin, β-cryptoxanthin, β -carotene	Breithaupt et al., 2002
Isocartic	C_{18}	Methanol/acetonitrle/ dichloromethane	Neoxanthin , violaxanthin, lutein, zeaxanthi, and then the chlorophylls and α, β-carotene	Lakshminarayana et al., 2005
Gadient	C_{30}	Methanol/TBME/ water	Astaxanthin, monoester, diesters	Holtin et al., 2009
Gradient	C_{18}	Acetone/water	Astaxanthin, monester, diester	Miao et al., 2008

Nishimura (2003) in a Japanese patent disclosed astaxanthin formulations in the form of solutions, gels, capsules etc along with other ingredients for improving the health of eyes. Spiller and Dewell (2003) reported safety of *Haematococcus* algal extract

when given to humans in gel caps containing 2 mg astaxanthin in safflower oil for a period of 8 weeks. Mari and Jiro (2003) in a Japanese patent disclosed that astaxanthin formulation in palm oil and tocotrienol along with rape seed oil, glycerin and gelatin to be useful for skin-conditioning, skin –moisturizing and wrinkle–preventing effects. Therefore astaxanthin stabilization in lipophilic and hydrophilic forms will be useful for nutraceutical and pharmaceutical applications. The edible oils used in the present study are all known for their nutritional, health, pharmaceutical and cosmetic applications (Brien, 1998). The study clearly indicated that astaxanthin is stable in its ester form in almost all the edible oils at room temperature than in aqueous form. Astaxanthin esters could withstand 70 - 90 ^{o}C temperature in oils with 90% retention (in terms of its content, profile and colour) which implies its use in food, pharmaceutical and nutraceutical applications. The drastic decrease in astaxanthin content (loss of characteristic absorption spectra) and colour (significant decrease in hunter 'a' value) at 120 and 150 ^{o}C temperatures indicates its sensitivity to high temperatures. This shows its potential application during the last stages of processing in the processed foods and its suitability for capsule/gel making using different edible oils for different formulations. Presence of tocopherols and polyphenols in different oils shows their possible role in the stability of carotenoids besides the inherent stability of oils. The high or comparable antioxidant activity of astaxanthin in edible oils and standard BHA was obtained in this study. Our results show that edible oils can be used for stabilization of astaxanthin for its varied applications such as food colorant, antioxidant, nutraceutical and pharmaceutical.

3.7. Salient features

In this study, isolation and characterization of carotenoids, astaxanthin & its esters from *H. pluvialis* and its stability studies were carried out. Analysis of carotenoids of *Haematococcus* using various analytical techniques has shown that algal biomass contained three different classes of pigments such as xanthophylls, chlorophylls, hydrocarbon carotenoids. *H. pluvialis* biomass contained astaxanthin esters (77.58%) as the major carotenoids followed by neoxanthin (0.9%), violaxanthin (0.3%), astaxanthin (3.8%), lutein (1.4%), zeaxanthin (4.2%), β-cryptoxanthin (5.5%) and β-carotene (11.7%). They were identified by their retention times in HPLC and the absorption spectra of the respective reference standards. Astaxanthin and its esters were confirmed by mass spectrum using APCI mode. Based on mass spectral

fragmentation data, mono esters of astaxanthin (ME $C_{16:0}$, ME $C_{17:2}$, ME $C_{17:1}$, ME $C_{17:0}$, ME $C_{18:4,}$ ME $C_{18:3}$, ME $C_{18:2}$, ME $C_{18:1}$) and diesters of astaxanthin (DE $C_{16:0}/C_{16:0}$, DE $C_{16:0}/C_{18:2}$, DE $C_{18:1}/C_{18:3}$, DE$C_{18:1}/C_{18:2}$, DE $C_{18:1}/C_{18:1}$) were identified in *H. pluvialis*. Astaxanthin in its ester forms was found to be fairly stable in all edible oils such as rice bran, mustard, groundnut, gingelly, coconut and palm oil at room temperature with variation in terms of its loss in content and colour during storage for 4 months period. Rice bran, gingelly and palm oil retained 84–90% of astaxanthin when heated at 70 ◦C and 90 ^{o}C for 8 h.

CHAPTER-III

BIOLOGICAL ACTIVITIES OF ASTAXANTHIN & ITS ESTERS FROM *H. PLUVIALIS* USING IN *IN VITRO* & *IN VIVO* MODELS OF EXPERIMENTAL RATS AND SAFETY EVALUATION OF BIOMASS

4.0. Background

This section deals with the evaluation of biological activity of *H. pluvialis* biomass as well as astaxanthin and its esters, with emphasis on free radical mediated antioxidant, antibacterial, hepatoprotective activity, nutritional quality and bioavailability. In order to evaluate these properties, biomass and carotenoids obtained from our process was subjected to successive extraction using solvents of varied polarity. The extracts of different solvents were subjected to analysis of radical scavenging ability by various *in vitro* models**.** Further the evaluation of efficacy of biomass was studied in rat models. Hexane, chloroform, ethyl acetate, acetone and methanol extracts of *H. pluvialis* were tested against important bacteria such as *Bacillus subtilus, Bacillus cereus, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus luteus, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Streptococcus fecalis* and *Yersinia enterocolitica.* The antibacterial activity was determined by agar-well diffusion assay and minimum inhibitory concentration (MIC). Effect of biomass feeding on the gain of body weight, hematological profile and weight of different organs was studied. A study was also under taken to know the possible antioxidant properties of biomass fed at 100 and 250 µg/kg body weight, upon challenging animals with carbon tetra chloride, which is known to generate high amount of free radicals. The same group was examined for possible protection of liver, which is damaged upon administration of carbon tetrachloride as measured by various biochemical markers in serum. Similarly *in vivo* studies on antioxidant and hepatoproective activity were undertaken by treating the rats with astaxanthin & its esters of *H. pluvialis* at 100 and 250 µg/kg. These results were compared with control group fed with normal diet rats and animals supplemented with 100 µg/kg of synthetic astaxanthin. Further the nutritional quality of *H. pluvialis* cells were evaluated using albino rats. Humans are unable to synthesize carotenoids and must acquire these essential compounds through the diet. Research to understand and enhance the carotenoid bioavailability is also important. Hence in this section the importance of *Haematococcus pluvialis* biomass as source of vitamin A supplement is dealt with *in vivo* model studies. However, little is known about the bioavailability of these carotenoids from whole foods. The present study

was undertaken to evaluate the bioavailability of astaxanthin and their antioxidant properties from *H. pluvialis* biomass in experimental rat model.

4.1. Materials and methods

4.1.1. Chemicals

HPLC grade acetonitrile, methanol and dichloromethane were purchased from Rankem Chemicals Ltd (Mumbai, India). Analytical grade acetone, hexane, chloroform, methanol and petroleum ether were purchased from Sisco Chemicals laboratorary (Mumbai, India). Ascorbic acid, ferrous sulphate, hydrochloric acid, hydrogen peroxide, α-tocopherol, potassium ferric cyanide, sodium carbonate, potassium iodide, potassium chloride, hydroxylamine hydrochloride, sodium acetate and ferrous chloride were obtained from Loba Chemicals Ltd (Mumbai, India). NBT, TCA, TBA, BHA, BHT, Tris-HCl buffer, standard astaxanthin, *β*-carotene were obtained from Sigma Chemicals, Co (St. Louis, Mo).

4.1.2. Cultivation of *H. pluvialis*

H. pluvialis (19-1a) was obtained from Sammlung von Algenkulturen, Pflanzen Physiologisches Institut, Universitat Gottingen, and Gottingen, Germany and was maintained on autotrophic medium agar slants (Sarada et al., 2002b).

4.1.3. Growth and carotenoid formation of *H. pluvialis*

H. pluvialis culture was grown in modified autotrophic Bold basal medium (BBM) as reported by Usha et al., (1999) and carotenoid formation was obtained under salinity stress as reported earlier by Sarada et al., (2002a). The encysted red cells rich in carotenoids were harvested, freeze-dried and stored at (4^{o} C).

4.1.4. Extraction and estimation of total carotenoid and chlorophyll

Known quantity (0.5 gram) of encysted cells of *H. pluvialis* were homogenized with motor and pestle in dark room and extracted repeatedly with acetone. Total carotenoid and chlorophyll contents were analyzed as per the procedure of Lichtenthaler (1987) by measuring the absorbance at 470 nm for carotenoids, 645 and 661.5 nm for chlorophyll (Shimadzu UV-Vis Spectrophotometer UV- 160-A). The total carotenoid and astaxanthin were expressed in terms of percent dry weight. Astaxanthin content was determined at 480 nm by using an extinction coefficient of 2500 at 1% level (Davies, 1976).

4.1.5. *In vitro* antioxidant activity of crude extracts of *H. pluvialis*

Known quantity of freeze-dried *H. pluvialis* biomass was extracted with acetone repeatedly, the pooled acetone extract was concentrated by rotary evaporation (Buchi, Germany) and re dissolved in ethanol and taken for different *in vitro* assays. The acetone extract was analysed for carotenoid content, chlorophyll content, total phenolics and reducing power.

4.1.6. Determination of total phenolic compounds

Estimation of total phenolic content procedure is given in details in Chapter-II methodology.

4.1.7. Reducing power of *H. pluvialis*

The reducing power of alga *H. pluvialis* extract was determined according to the method of Oyaizu (1986). The acetone extract (1 mg/ml) was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferric cyanide [$K_3Fe(CN)_6$, 1%] then incubated at 50 ^oC for 20 min. To this mixture, 2.5 ml of TCA (10%) was added and centrifuged at 3,000 rpm for 20 min. The upper layer (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%), and read at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

4.1.8. Determination of protein

Protein was estimated in animal tissues and algal sample by using the method of Lowry et al., (1951).

4.2. Antioxidant assays

4.2.1. Assay for β-carotene linoleate model system (β-CLAMS)

The antioxidant activities of *H. pluvialis* extract was evaluated by the procedure of Fayaz et al., (2005). In brief, β-carotene (0.2 mg), 20 mg of linoleic acid and 200 mg of tween-40 (polyoxyethylene sorbitan monopalmitate) were mixed in 0.5 ml of chloroform. Chloroform was removed at 40 ^oC under vacuum using a rotary evaporator (Buchi, Germany). The resulting mixture diluted with triple distilled water to 10 ml, mixed well for 1-2 min and made up to 50 ml with oxygenated water. Aliquots (4 ml) of this emulsion were transferred to different test tubes containing (5, 7 and 9 ppm of total carotenoids) test samples. Butylated hydroxy anisole (BHA) was used for comparative purposes. A control and 4 ml of the above emulsion without β-carotene were prepared. The tubes were placed in water bath maintained at 50 ^oC.

Absorbance of all the samples at 470 nm were taken at zero time (t=0). Measurement of absorbance was continued until the color of β-carotene disappeared in the control group (t=180 min) at 15 min intervals. A mixture prepared as above without β-carotene served as blank. All determinations were carried out in triplicates. A dose-response relationship of antioxidant activity for *H. pluvialis* extract was determined at different concentrations. The antioxidant activity (AA) of the *H. pluvialis* extract was evaluated in terms of bleaching of the β-carotene using the following formula.

$$AA = 100[1 - (A_0 - A_t)/(A^0{}_0 - A^0{}_t)]$$

Where A^0 and $A^0{}_0$ are the absorbance values measured at zero time incubation for test sample and control, respectively, where A_t and $A^0{}_t$ after incubation for 180 min.

4.2.2. Assay for DPPH and hydroxyl radical scavenging activity

The extracts of *H. pluvialis* were assayed for radical scavenging activity using DPPH (1, 1-diphenyl-2-picrylhydrazyl) by the procedure of Duh and Yen (1997)**.**

The hydroxyl radical scavenging activity of *H. pluvialis* extract was determined according to the procedure of Fayaz et al., (2005). In brief, different concentrations (5, 7 and 9 ppm total carotenoids) of *H. pluvialis* extracts were taken and the solvent was evaporated to dryness under a stream of nitrogen. The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.4), 167 µM iron-EDTA mixture (1:2 w/w), 0.1 mM EDTA, 2 mM ascorbic acid and 33 mM Me_2SO in a final volume of 3.0 ml. The reaction mixture was incubated at 37 ^oC for 30 min and the reaction was terminated by the addition of 1 ml of TCA (17.5%) and 2 ml of Nash reagent and left at room temperature for 15 min. The intensity of yellow colour was measured spectrophotometrically (Shimadzu 160A) at 412 nm against reagent blank. The percentage hydroxyl radical scavenging activity of sample was calculated as % inhibition relative to the control.

4.2.3. Antioxidant activity of astaxanthin and its esters in *in vivo* model

Albino rats of Wistar strain weighting 200-220 grams were used for studies. The animals were grouped into six groups (n=5), The 1st group served as the control, which received only olive oil, the 2nd group received normal diet and was administered with toxin (CCl_4), The 3rd, 4th and 5th groups were treated with astaxanthin, monoester, and diester of astaxanthin. 6th group treated with synthetic

astaxanthin orally at dose of 100 µg/kg (dissolved in olive oil) for 14 days. The animals of 1st and 2nd groups were simultaneously administered with olive oil until 14 days. The animals of 2nd, 3rd, 4th, 5th and 6th group were given a single oral dose of CCl_4 (1:1 in olive oil) at dose of 2.0 g/kg b.w. after the 6 h of last dose of administration of astaxanthin & its esters/olive oil on 14th day. After 24 h animals were sacrificed and liver from each animal was isolated to prepare the liver homogenate. 5% (w/v) liver homogenate was prepared with 0.15M KCl & centrifuged at 1000 x g for 10 min. The cell free supernatant was used for the estimation of lipid peroxidation, catalase, peroxidase, and SOD. All the biochemical markers of antioxidant activity were estimated by standard protocols.

4.2.4. Assay for lipid peroxidation using kidney homogenates

The kidneys of normal rats were homogenized with a Polytron (speed setting 7-8) in 10 ml of ice-cold Tris-HCl buffer (20 mM, pH 7.4) by the procedure of Chidambaramurthy et al., (2005). The homogenate was centrifuged at 14,000 rpm for 15 min. The supernatants (1 ml) were incubated with different levels of *H. pluvialis* extracts (5, 7 and 9 ppm total carotenoids) in the presence of 10 µM $FeSO_4$ and 0.1 mM ascorbic acid at 37 °C for 1 h. The reaction was terminated by addition of 1.0 ml TCA (28%) and 1.5 ml TBA (l %). The solution was heated at 100 °C for 15 min, cooled to room temperature, centrifuged at 4,696 x g or 15 min and the color of the MDA-TBA complex in the supernatant was read at 532 nm using a spectrophotometer. BHA was used as a positive control. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = $(A-A_l)$ /A x 100 %, where, A is the absorbance of the control, and A_l is the absorbance of the test sample.

4.2.5. Assay for lipid peroxidation using brain homogenates

The brain of normal rats were homogenized with a Polytron (speed setting 7-8) in 10 ml of ice-cold Tris-HCl buffer (20 mM, pH 7.4) by the procedure of Chidambaramurthy et al., (2005). The homogenate was centrifuged at 14,000 rpm for 15 min. The supernatants (1 ml) were incubated with different levels of *H. pluvialis* extracts (5, 7 and 9 ppm total carotenoids) in the presence of 10 µM $FeSO_4$ and 0.1 mM ascorbic acid at 37 °C for 1 h. The reaction was terminated by addition of 1.0 ml TCA (28%) and 1.5 ml TBA (l %). The solution was heated at 100 °C for 15 min, cooled to room temperature, centrifuged at 4, 696 x g for 15 min and the color of the

MDA-TBA complex in the supernatant was read at 532 nm using a spectrophotometer. BHA was used as a positive control. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = $(A-A_1)/A \times 100$ %, where, A is the absorbance of the control, and A_1 is the absorbance of the test sample.

4.2.6. Assay for lipid peroxidation using liver homogenates

Inhibitory effect of *H. pluvialis* extract on lipid peroxidation was carried out as given in Ranga Rao et al., (2006). Normal rats were anesthetized with diethyl ether and sacrificed by exsanguinations. The perfused liver was isolated and homogenized with 9 parts of isotonic phosphate buffer saline using Potter-Elvehjem homogenizer at 4 ^{o}C. The homogenate was centrifuged at 2, 800 x g for 15 min and supernatant was used for the *in vitro* lipid peroxidation assay. In brief, to different concentrations of sample extracts (5, 7 and 9 ppm total carotenoids), 1 ml of 0.15 M potassium chloride and 0.5 ml of rat liver homogenate were added. Peroxidation was initiated by adding 100 µl of 0.2 mM ferric chloride. After incubation at 37 ^{o}C for 30 min, the reaction was stopped by adding 2 ml of ice-cold HCl (0.25 N) containing 15% TCA, 0.38% TBA and 0.5% BHT. The reaction mixture was heated at 80 °C for 60 min, cooled, centrifuged at 2, 800 x g and the supernatant was read at 532 nm. A control without added sample extract was also run simultaneously. The percentage of lipid peroxidation inhibitory activity (%LP) was calculated as lipid peroxidation =1-(sample OD/blank OD) X 100.

4.2.7. Assay for antioxidant activity on liposome model system

The lipid peroxidation-inhibitory activity of the *H. pluvialis* extracts in a liposome model system was determined according to the method of Duh and Yen (1997). Egg lecithin (3 mg/ml phosphate buffer, pH 7.4) was sonicated in ultrasonic homogenizer (Son plus HD 2200). *H. pluvialis* extracts at different concentrations (5, 7 and 9 ppm total carotenoids) were added to 1 ml of liposome mixture and to the control (without test samples). Lipid peroxidation was induced by adding 10 µl of $FeCl_3$ (400 mM) and 10 µl of L-ascorbic acid (200 mM). After incubation at 37^{o}C for 1h, the reaction was stopped by adding 2 ml of 0.25N HCl containing 150 mg/ml TCA and 3.75 mg/ml TBA. The reaction mixture was subsequently boiled for 15 min, cooled to room temperature, centrifuged at 2, 800 x g for 15 min and the absorbance of the supernatant was read at 532 nm by spectrophotometer.

4.2.8. Antioxidant activity on human low-density lipoprotein (LDL) oxidation

Plasma was separated from blood drawn from human volunteers and stored at 4 ^{o}C until used. Isolation of LDL from the plasma and antioxidant activity of the algal extract was done according to procedure of Ranga Rao et al., (2006). The *H. pluvialis* extract (5, 7 and 9 ppm total carotenoids), 40 µl of copper sulfate (2 mM) was added, and made upto 1.5 ml with phosphate buffer (50 mM, pH 7.4). A tube without extract and copper sulfate served as negative control and another tube without copper sulfate served as positive control. All the tubes were incubated at 37 ^{o}C for 45 min. To the aliquots of 0.5 ml drawn at 2 h, 4 h and 6 h intervals from each tube, 0.25 ml of TBA (1% in 50 mM NaOH) and 0.25 ml of TCA (2.8%) were added. The tubes were incubated again at 95 ^{o}C for 45 min, cooled to room temperature and centrifuged at 4, 600 x g for 15 min. A pink chromogen was extracted and read at 532 nm by spectrophotometer.

4.3. Antibacterial properties of *H. pluvialis* extracts

4.3.1. Preparation of microalgal extracts

Known quantity of freeze dried algal biomass (2 grams) was ground well in a mortar and pestle and carotenoids were extracted with different solvents (each 10 ml) acetone, methanol, chloroform, and ethyl acetate (Akinyemi et al., 2000). The extraction was repeated until the sample became color less (total volume 50 ml). The crude extract (60 ml) was taken, mixed well, concentrated to dryness by flash evaporation (Buchi rota vapor R205, Germany) at 30-35 ^{o}C and redissolved in ethanol (20 ml) and each extract was tested for antibacterial activity.

4.3.2. Bacterial strains and culture conditions

The antibacterial activity was tested against *Bacillus subtilus, Bacillus cereus, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus luteus, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Streptococcus fecalis and Yersinia enterocolitica.* The above bacterial strains were obtained from the stock cultures of Microbiology laboratory, Department of Microbiology, Mysore Medical College, Mysore, India. The bacterial stock cultures were maintained on nutrient agar (HiMedia lab. Pvt., Ltd, Bombay, India) slants at 37 ± 1°C.

4.3.3. Agar-well diffusion assay

In vitro antibacterial activity of four crude extracts was determined by agar well diffusion method (Owais et al., 2005). Extracts were dissolved in respective solvents (5 mg /ml) were used for the assay. About 50 µl of the sample was placed in the wells and allowed to diffuse for 2 h. Plates were incubated at 37 ^{o}C for 48 h and the activity was determined by measuring the diameter of inhibition zones. DMSO alone was used as a control and amoxycillin as a positive control. The assay was carried out in triplicate.

4.3.4. Minimum inhibitory concentration (MIC)

The MIC was determined by the modified method developed by Dufour *et al.,* (2003) and Gary *et al.,* (2003). Different concentrations (50 ppm to 300 ppm) of test sample and 100 µl of the bacterial suspension (10^5 CFU/ml) was placed aseptically in10 ml of nutrient broth and incubated for 24 h at 37 ^{o}C. The growth was observed both visually and by measuring O.D. at 600 nm at regular intervals followed by pour plating with nutrient agar. The lowest concentration of test sample showing no visible growth was recorded as the minimum inhibitory concentration. Triplicate sets of tubes were maintained for each concentration of test sample and the mean readings were plotted against O.D. at 600 nm as growth curves.

4.4. Estimation of hepatoprotective activity of carotenoids in *in vivo* model

Rats were anesthetized with diethyl ether after 24 h of the hepatotoxin administration. The blood was collected in heparinized tubes from each animal by cardiac puncture (3 ml). The blood samples were allowed to clot for 1-2 h at room temperature. Serum was separated by centrifugation at 2,500 rpm for 15 min and measured various biochemical parameters. Activities of the enzymes SGOT (Bergmeyer et al., 1976), SGPT (Bergmeyer and Horder, 1980), ALP (Szasz *et al.,* 1974) Albumin (Wooton et al., 1964) were estimated by using standard enzymes kits.

4.4.1. Histopathological studies

Liver samples were fixed in 10% buffered formalin for 24 h. The processed tissues were embedded in paraffin blocks and sections made were stained with hematoxylin and eosin dye (Lillie, 1965). The sections were analysed by observing under light microscope (Leitz, Germany) at 40x magnification.

4.5. Estimation of nutritional quality of *H. pluvialis* cells in *in vivo* model

Albino rats of the Wister strain weighing 225-250g were used for the studies. The animals were grouped into three groups containing five animals in each group, first group served as normal without algal biomass treatment, the second and third received *Haematococcus* whole algal cells at 2.5 and 5.0 µg/kg b.w. respectively. Biomass was fed by forced feeding using oral catheter after making a fine powder and suspending in oil, once a day for two weeks. Weight of animals was measured on 7th and 14th day. On 14th day animals were sacrificed after anaesthetizing using diethyl ether, blood was collected in a vial containing 10% EDTA (anticoagulant) and stored in cold temperature until further analysis. Another 1 ml was collected without any anticoagulant, serum was separated as upper layer by keeping the tube in slanting position and used for analysis of protein. Weight of vital organs viz liver, heart, brain, spleen, kidney, adrenals, lungs, thymus and testis from male animals were noted. Blood was subjected for analysis of % hemoglobin, white blood cells (WBC) and serum protein manually. Other parameters like red blood cells (RBC) count, Mean corpuscular hemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC), Mean corpuscular volume (MCV) were analysed at Ravi diagnosis laboratory, Mysore, using automatic hematocrit analyzer (ABX) micro blood count analyzer.

4.5.1. Estimation of haemoglobin content

Haemoglobin content of different animals was measured by cyanomethhaemoglobin method using Drabkinis reagent (Beacon diagnostic Ltd. Navsari, India). In alkaline condition haemoglobin and its derivatives were oxidized in presence of potassium ferricyanide and converted into methemoglobin, which react with potassium cyanide to form purple red colored cyanmethemoglobin, which were monitored colorimetrically at 546 nm. Content of the samples were calculated comparing with the optical density of standard and expressed as % Hb content.

4.5.2. Estimation of white blood cells count

WBC count was done by diluting 20 µl of blood with diluting fluid and allowing it to reach for 2-3 minutes and counting was done using hemocytometer and results were expressed as number of cell/ml blood.

4.5.3 Estimation of serum protein

Serum separated from blood was analysed for protein content using method of Lowry et al., (1951). Protein content was calculated using standard curve prepared using Bovine serum albumin and expressed as mg of protein per dL of serum.

4.6. Bioavailability and antioxidant properties of *H. pluvialis* biomass in *in vivo* model

4.6.1 Animals

All animal experiments were performed after due clearance from the institutional animal ethics committee. Male Wistar rats [Out B - Wistar, IND-Cft (2C)] weighing 41 ± 2 g were housed individually in steel cages at room temperature (28 ± 2^{o}C). A 12 h light dark/cycle was maintained and the rats received daily fresh pellet diet (Amrut feeds, Sangli, India) and had free access to tap water. The left over diets were weighed and discarded. After 7 days of acclimatization, rats were deprived of food for 12 h and administered a single dose of *H. pluvialis* biomass as source of astaxanthin respectively. Diet samples were processed for the analysis of carotenoids by HPLC (Lakshminarayana et al., 2007).

4.6.2 Single dose time course study

Group of rats (n=25) were administered by intubation to the stomach a single dose (0.2 ml/rat) of *H. pluvialis* biomass solubilized in olive oil as source of 200 µM equivalent of astaxanthin. Each group was divided into five subgroups (n=5/subgroup) to measure the time course plasma and tissues response of carotenoids for 9 h. A separate group (n=5) not fed either of the biomass was considered as 0 h. Rats in 0 h and in each treatment group (n=5/time point) at 2, 4, 6, and 9 h after gavage were exsanguinated under mild ether anesthesia, blood was collected from heart into heparinized tubes, and plasma was separated immediately by centrifuging at 1000 x g for 15 min at 4^{o}C. Liver and eyes were removed and washed with ice cold saline and immediately stored at -70 ^{o}C until analyzed.

4.6.3. Repeated dose study

Group of rats (n=12) were administered by intubations to the stomach a repeated dose (0.2 ml/rat) of *H. pluvialis* biomass solubilized in olive oil as source of 200 µM equalent of astaxanthin for 14 days. Each group was divided into two subgroups (n=5 subgroup) to measure the plasma and tissues response of astaxanthin for 14 days. A

separate group (n=5) not fed either of the biomass was considered as zero-time control (base line). After 14th day the rats were exsanguinated under mild ether anesthesia and blood was collected from heart into heparinized tubes, and plasma was separated immediately by centrifuging at 1000 x g for 15 min at 4 ^{o}C. Liver and eyes were removed and washed with ice cold saline and immediately stored at -70 ^{o}C until analyzed.

4.6.4 Extraction of astaxanthin and retinol from plasma of rats

Astaxanthin from plasma of rats were extracted and analyzed by HPLC (Baskaran et al., 2003). In brief, to the plasma (0.8 ml), 3 ml of dichloromethane: methanol (1:2, v/v) containing α-tocopherol (2 mM) was added. After mixing, hexane (1.5 ml) was added, mixed well and centrifuged at 1000 x g for 15 min, and the resulting upper hexane/dichloromethane phase was collected. This extraction was repeated for the lower phase 2 more times using 1 ml of dichloromethane and 1.5 ml of hexane. The pooled extracts were evaporated to dryness under a stream of nitrogen. The residue was redissolved in 100 µl of mobile phase, and used for HPLC analysis of astaxanthin.

4.6.5. Extraction of astaxanthin and retinol from liver of rats

Astaxanthin from liver of rats were extracted and analyzed by HPLC (Baskaran et al., 2003). Liver samples (1 g) were homogenized separately with 9 parts ice-cold isotonic saline with a Potter-Elvehjem homogenizer, astaxanthin was extracted from 0.8 ml of the homogenate and analyzed by HPLC with the same procedure used for plasma. The liver extract was further saponified by incubating in 2 ml of 10 M KOH at 60 ^{o}C for 45 min. In brief, to the liver (0.8 ml), 3 ml of dichloromethane: methanol (1:2, v/v) containing α-tocopherol (2 mM) was added. After mixing, hexane (1.5 ml) was added, mixed well and centrifuged at 1000 Xg for 15 min, and the resulting upper hexane/dichloromethane phase was collected. This extraction was repeated for the lower phase 2 more times using 1 ml of dichloromethane and 1.5 ml of hexane. The pooled extracts were evaporated to dryness under a stream of nitrogen. The residue was redissolved in 100 µl of mobile phase, and used for HPLC analysis of astaxanthin. Samples were handled on ice under dim yellow light to minimize isomerization and oxidation of carotenoids by light irradiation.

4.6.6. Extraction of astaxanthin and retinol from eyes of rats

Astaxanthin from eye of rats were extracted and analyzed by HPLC (Baskaran et al., 2003). Eye samples were homogenized separately with ice-cold isotonic saline with a Potter-Elvehjem homogenizer, astaxanthin was extracted from 0.8 ml of the homogenate and analyzed by HPLC with the same procedure used for plasma. The eye extract was further saponified by incubating in 2 ml of 10 M KOH at 60 ^{o}C for 45 min. In brief, to the eye (0.8 ml), 3 ml of dichloromethane: methanol (1:2, v/v) containing α-tocopherol (2 mM) was added. After mixing, hexane (1.5 ml) was added, mixed well and centrifuged at 1000 xg for 15 min, and the resulting upper hexane/dichloromethane phase was collected. This extraction was repeated for the lower phase 2 more times using 1 ml of dichloromethane and 1.5 ml of hexane. The pooled extracts evaporated to dryness under a stream of nitrogen. The residue was redissolved in 100 µl of mobile phase, and used for HPLC analysis of astaxanthin. Samples were handled on ice under dim yellow light to minimize isomerization and oxidation of carotenoids by light irradiation.

4.6.7. HPLC analysis of astaxanthin in plasma, liver, eyes and retinol of rats

Astaxanthin levels in plasma, liver and eyes were analyzed using HPLC (Shimadzu 10AS, Kyoto, Japan) reverse phase 25 cm × 4.6 mm, 5 μm, C_{18} column (Wakosil 11 5C 18RS) with an isocratic solvent system consisting of dichloromethane: acetonitrile: methanol (20:70:10, v/v/v) at a flow rate of 1.0 ml/min (Ranga Rao et al., 2006). Astaxanthin was monitored at 476 nm and 325 nm with UV- visible detector (Shimadzu, Kyoto, Japan). The peak identification and λ_{max} values of these components were confirmed by their retention times and characteristic spectra of standard chromatograms recorded with a Shimadzu model LC-10AVP series equipped with (SPD–10AVP) photodiode array detector. They were quantified from their peak areas in relation to the respective reference standards.

4.6.8. Antioxidant enzymes

4.6.8.1 Catalase assay

The catalase assay was carried out by the method of Aebi (1984). Plasma (0.2 ml) from each groups was diluted with 1.9 ml of phosphate buffer (125 mM, pH-7.4). The reaction was initiated by the addition of 1 ml of hydrogen peroxide (30 mM). Blank without plasma was prepared with 2.9 ml of phosphate buffer and 1 ml of

hydrogen peroxide (30 mM). The decrease in optical density due to decomposition of hydrogen peroxide was measured at the end of 1 min against the blank at 240 nm. Units of catalase activity was expressed as the amount of enzyme that decomposes 1 μM H_2O_2 per minute at 25 ^oC. The specific activity was expressed in terms of units per milligram of protein.

4.6.8.2. Superoxide dismutase assay

The assay of SOD was measured on the reduction of nitroblue tetrazolium (NBT) to water insoluble blue formazan, as described by Fedovich (1976). To the plasma (0.2 ml), 125 mM sodium carbonate (1 ml), 24 μM NBT (0.4 ml), and 0.1 mM EDTA (0.2 ml) were added. The reaction was initiated by adding 1 mM hydroxylamine hydrochloride (0.4 ml). Zero time absorbance was taken at 560 nm followed by recording the absorbance after 5 min at unit of enzyme required inhibiting the reduction of NBT by 50.0%. The specific activity was expressed in terms of units per milligram of protein.

4.6.8.3. Peroxidase assay

The peroxidase assay was carried out as per Nicholas (1962). To the liver homogenate (0.5 ml), 10 mM KI (1 ml), and 40 mM sodium acetate (1 ml) solution were added. The absorbance of potassium periodide was read at 353 nm, which indicates the amount of peroxidase. To the reaction mixture, 20 μl hydrogen peroxide (15 mM) was added, and the change in the absorbance in 5 min was recorded. Units of peroxidase activity was expressed as the amount of enzyme required to change the one OD per minute. The specific activity was expressed in terms of units/ milligram of protein.

4.6.8.4. GSH assay

The level of GSH was estimated by the method of Miron et al., (1979). The proteins were precipitated in the 12, 000X*g* supernatant by addition of TCA to a final concentration of 5% TCA. This was then centrifuged at 15 000Xg for 15 min to obtain the protein free supernatant. To 100 ml of this supernatant, 2 ml of 0.6 M 5, 5-dithio-bis (2-nitrobenzoic acid) dissolved in 0.2 M phosphate buffer (pH 8.0) was added. The absorbance was recorded at 412 nm. Reduced GSH was used as standard. The levels of GSH are expressed as μg/mg of protein.

4.6.8.5. Lipid peroxidation assay

TBA reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm (Buege and Aust, 1978). The TBARS was measured in terms of MDA and expressed as MDA equivalent. To the plasma and liver homogenate (0.5 ml), 1 ml of 0.15 M KCl was added and the peroxidation was initiated by adding 250 µl of 0.2 mM ferric chloride. The reaction mixture was incubated at 37^{o}C for 30 min. The reaction was stopped by adding 2 ml of an ice-cold mixture of 0.25 N HCl containing 15% trichloroacetic acid, 0.30% TBA, and 0.05% BHT and heated at 80^{o}C for 60 min. The samples were cooled and results were expressed as MDA equivalent, which was calculated by using an extinction coefficient of 1.56 X10^{5} M^{-1} cm^{-1}. One unit of lipid peroxidation was defined enzyme required to convert 1 mole of TBA in to TBARS. The specific activity was expressed in terms of units/milligram of protein.

4.6.8.6. Serum glutamic oxalacetic transaminase (SGPT)

This was estimated by monitoring pyruvate formed when α-ketoglutarate reacts with L-alanine, meadiated by SGPT. Pyruvate is made to react with 2, 4-Dinitrophenyl hydrazine to form hydrazone producing brown colour in alkaline medium which was monitored calorimetrically at 505 nm. The SGPT activity was calculated using standard graph with pyruvate as standard using diagnostic kit (Kumar diagnostic, Mysore, Karnataka, India)

4.6.8.7. Serum glutamic pyruvic transminase (SGOT)

Serum glutamine pyruvic transminase was estimated by monitoring oxaloacetate formed when α-ketoglutarate react with L-aspertate, meadiated by SGOT. Oxaloacetate is made to react with 2, 4-Dinitrophenyl hydrazine to form hydrazone producing brown colour in alkaline medium which was monitored calorimetrically at 505 nm. The SGOT activity was calculated using standard graph with pyruvate as standard using diagnostic kit (Kumar diagnostic, Mysore, Karnataka, India).

4.6.8.8. Serum alkaline phosphatase (SALP)

Serum ALP converts phenyl phosphate to inorganic phosphate and phenol. The phenol formed is made to react with 4-amino antipyrine in presence of oxidizing agent potassium ferricyanide to form a orange red complex and this was measured

spectrophotometrically at 505 nm. The SALP activity was calculated using standard graph with standard provided in diagnostic kit (Kumar diagnostic, Mysore, India)

4.6.8.9. Statistical analysis

All the experimental analyses were done in triplicate. Result values were expressed as mean ± SD in case of *in vitro* experiments. In case of *in vivo* analysis values were expressed as mean ± SD and one way analyses of variance (ANOVA) was used and the test was used for comparison of mean values. All tests were considered to be statistically significant and highly significant at $p < 0.05$ and 0.001 respectively.

4.7. Results

4.7.1. *In vitro* antioxidant properties of carotenoids

4.7.1.1. DPPH free radical scavenging activity

DPPH is a stable free radical containing an odd electron in its structure and usually is used for detecting radical scavenging activity in chemical analysis. The degree of decrease in the absorbance of DPPH indicates the free radical scavenging potentials. Free radical scavenging potential of acetone extract of *H. pluvialis* at different concentrations was analyzed by the DPPH method and the results are shown in Fig 91. The *H. pluvialis* extract exhibited dose dependent free radical scavenging ability at all tested concentrations. It exhibited 65, 74 and 86% of free radical activity at 5, 7 and 9 ppm level of carotenoids that is about 80-90% activity of respective level of BHA. The activity of the extract is attributed to their hydrogen donating ability (Shimada et al., 1992). The antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming a stable end product, which does not propagate further oxidation of lipids (Sherwin, 1978).

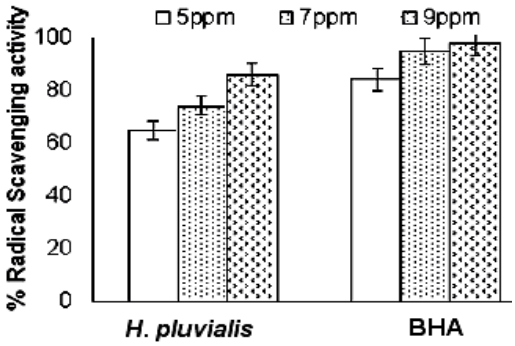


Fig. 91. Antioxidant activities of *H. pluvialis* extract using DPPH method. Data represents an average of 3 replicates. Bars indicates mean ± SD.

4.7.1.2. Antioxidant assay using β-carotene linoleate model system

The mechanism of bleaching of β-carotene is a free radical mediated phenomenon resulting from the hydro peroxides formed from linoleic acid. β-carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules, lose their double bonds by oxidation, the compound loses its chromophore and characteristic orange color, which can be monitored spectrophotometrically. The antioxidant activity of *H. pluvialis* extract as measured by the bleaching of β-carotene is shown in Fig 92. *H. pluvialis* exhibited 67, 78 and 89% of antioxidant property in β-carotene linoleate model system at 5, 7 and 9 ppm level of carotenoid, which is 85-95% of BHA.

4.7.1.3. Hydroxyl radical scavenging activity

Hydroxyl radical is supposed to be one of the fast initiators of the lipid peroxidation process, obstructing hydrogen atoms from unsaturated fatty acids (Kappus, 1991). Hydroxyl radical scavenging activity of *H. pluvialis* was estimated by generating the hydroxyl radical using Fe^{3+}/ascorbic acid system. The hydroxyl radicals formed by the oxidation reacts with dimethyl sulphoxide (DMSO) to yield formaldehyde that provides a convenient method to detect hydroxyl radicals by treatment with Nash reagent. The hydroxyl radical scavenging activity of the algal extract is shown in Fig 93. *H. pluvialis* exhibited 55, 68 and 78% of hydroxyl radical scavenging activity at 5, 7 and 9 ppm level of carotenoid, which is 85-90% of BHA. The *H. pluvialis* extract showed inhibitory effect on lipid peroxidation.

4.7.1.4. Changes of lipid peroxide level in rat tissues

As observed in model systems, *H. pluvialis* extract also exhibited inhibitory effect on lipid peroxidation in brain (85%), kidney (80%) and liver (79%) at 9 ppm level of carotenoid concentration (Figs. 94-96) and it was found to be dose dependent. The data shows that inhibitory activity of algal extract on lipid peroxidation in brain was similar to that in kidney and liver.

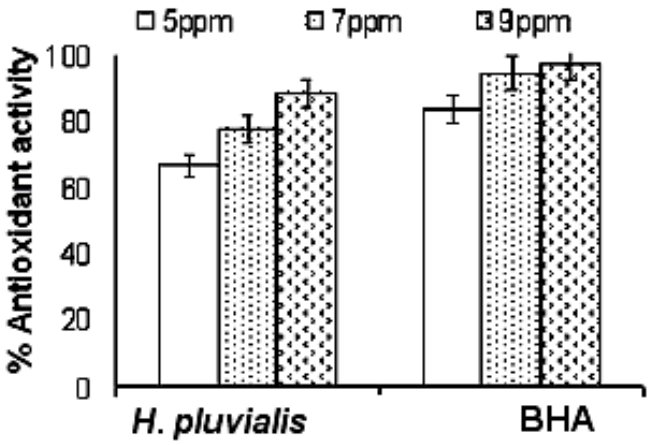


Fig. 92. Antioxidant activity of *H. pluvialis* extract using β-CLAMS method. Data represents an average of 3 replicates. Bars indicates mean ± SD.

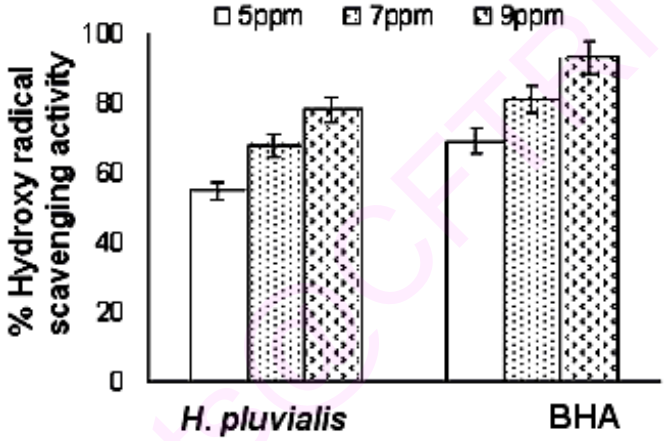


Fig. 93. Antioxidant activity of *H. pluvialis* extract using hydroxy radical scavenging. Data represents an average of 3 replicates. Bars indicates mean ± SD.

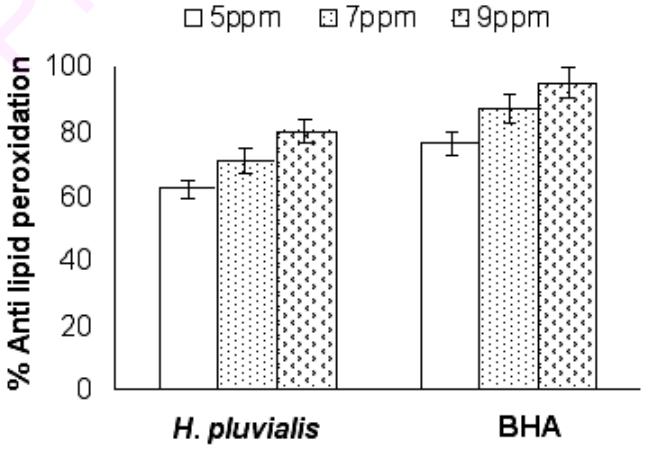


Fig. 94. Antilipid peroxidation of *H. pluvialis* extract on kidney homogenate. Data represents an average of three replicates. Bars indicate mean ± SD.

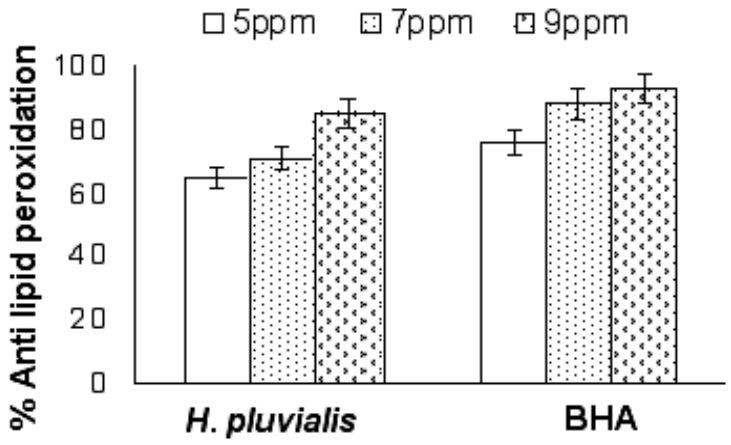


Fig. 95. Antilipid peroxidation of *H. pluvialis* extract on Brain homogenates. Data represents an average of three replicates. Bars indicate mean ± SD.

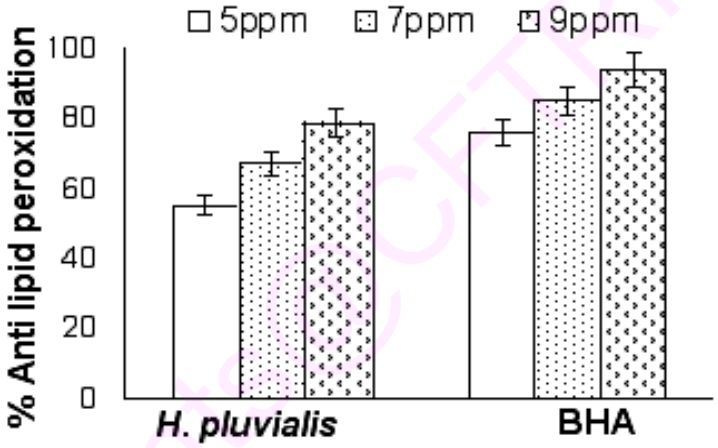


Fig. 96. Antilipid peroxidation of *H. pluvialis* extract on Liver. Data represents an average of three replicates. Bars indicate mean ± SD.

4.7.1.5. Inhibitory effect on lipid peroxidation in liposomes.

Lipid peroxidation is a free radical mediated propagation of oxidative damage to PUFA involving several types of free radicals and termination occurs through enzymatic means or by free radical scavenging by antioxidants. To evaluate the antioxidant activity of the algal extract, a liposome model system was used as reported by Tsuda et al., (1993). Malondialdehyde is the major product of lipid peroxidation that reacts with TBA to form a pink chromogen (diadduct), which can be detected spectrophotometrically at 532 nm. The antioxidative property of algal extract in the liposome system, induced by $FeCl_3$ plus ascorbic acid is shown in Fig 97. As like BHA, antioxidant property of the alga extract in liposome system was

significantly higher and which was found to be dose dependent. The extract exhibited 69, 74 and 87% inhibition of peroxidation of lecithin at 5, 7 and 9 ppm level of carotenoid. The result shows that the extracts used have a strong antioxidant action in liposome model system. The carotenoids present in green algae *Dunaliella salina* (Chidamabaramurthy et al, 2005), *Botryococus braunii* (Ranga Rao et al., 2006) and Seaweed *Kappaphycus alvarazzi* (Fayaz et al, 2005) may prevent the distructive effect of lipid peroxides *in vitro* by lowering their levels. Further, they have reported that the antioxidant property of the algal and the seaweed is due to the higher levels of carotenoids and phenolic compounds present in them. Similarly, the *H. pluvialis* extracts used in this study may play an important role in protecting the cells from lipid peroxides.

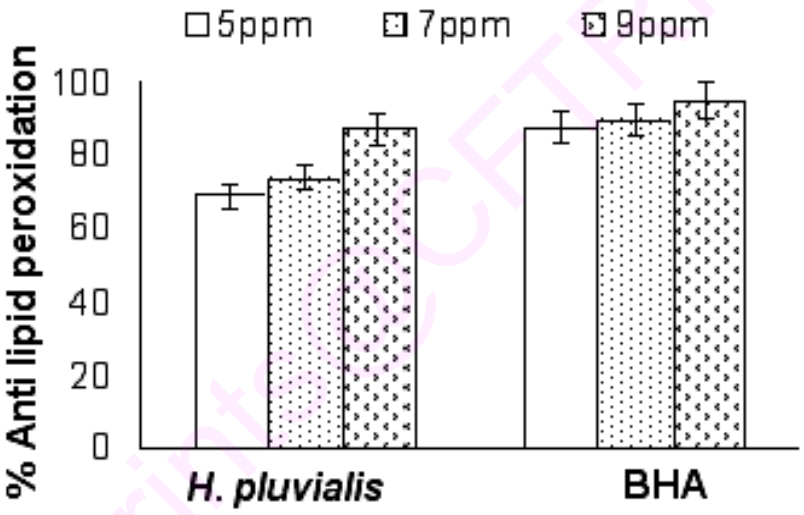


Fig. 97. Anti lipid peroxidation of *H. pluvialis* extract on egg liposome model. Data represents an average of three replicates. Bars indicate mean ± SD.

4.7.1.6. Inhibitory effect on LDL oxidation

Oxidative modification is known to play an important role in the pathogenesis of atherosclerosis and coronary heart diseases (Steinberg et al., 1981) and the dietary antioxidants that protect LDL from oxidation may therefore reduce atherogenesis (Pin-Der, 1998). The antioxidant activity of *H. pluvialis* extracts against human LDL oxidation is shown in Fig 98. The polyunsaturated fatty acids of human LDL were oxidized and the malondialdehyde formed has been estimated by using the TBA method. The average induction time for copper mediated LDL oxidation was around 20 min without addition of algal extracts. The algal extract protected LDL from

oxidation as measured by the prolongation of induction time of the formation of conjugated dienes. The algal extract exhibited 45, 64 and 75% protection at 5, 7 and 9 ppm level of carotenoid at the end of 2 h after induction of oxidation. Whereas, it was 51, 68 and 82% and 56, 73 and 84% protection at 5, 7 and 9 ppm levels of carotenoids used at the end of 4 h and 6 h respectively. The result indicates a dose dependent inhibition effect of *H. pluvialis* against LDL oxidation.

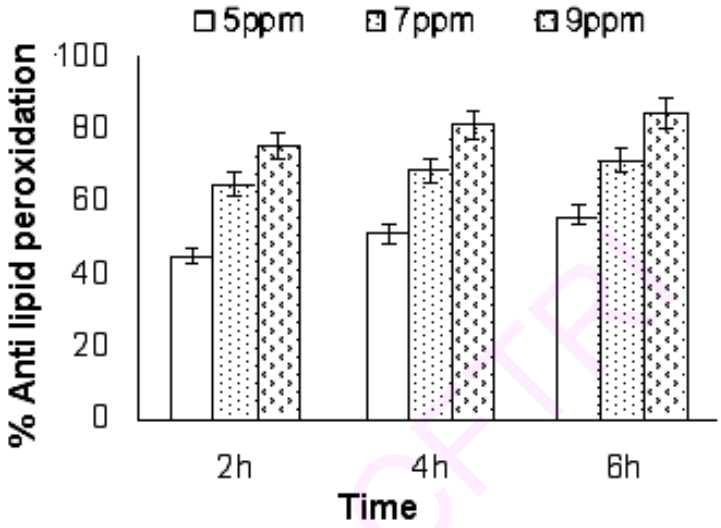


Fig. 98. Anti lipid peroxidation of *H. pluvialis* extract on human low density lipoprotein. Data represents an average of three replicates. Bars indicate mean ± SD.

4.7.1.7. Reducing power

The reducing power of *H. pluvialis* extract was found to be 0.52, 0.765 and 0.89 at 5, 7 and 9 ppm levels of carotenoids compared with standard vitamin C (Fig 99). The presence of reducing power indicates that the *H. pluvialis* extract has electron donors and can react with free radicals to convert them to more stable products and terminate radical chain reaction. Duh and Yen (1997) reported the reducing power of peanut hulls. Gordon (1990) reported that the antioxidant action of reductones is based on the breaking of the free radical chain by the donation of a hydrogen atom. The reducing power of *H. pluvialis* extract indicates that the marked antioxidant activity of algae is believed to be due to the presence of carotenoids. Particularly, high level of astaxanthin that may act as a strong antioxidant as reductones by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction.

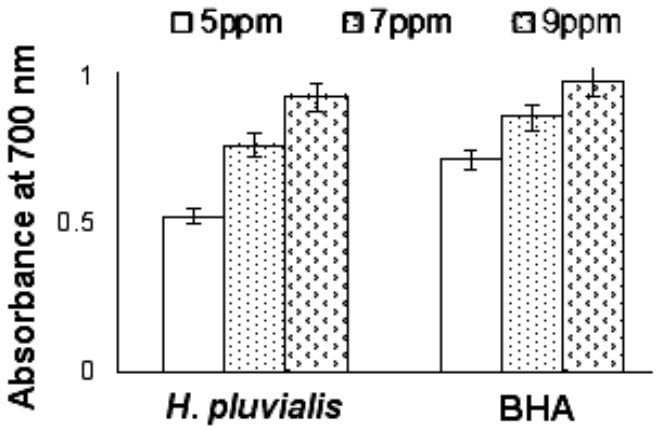


Fig. 99. Reducing power of *H. pluvialis* extract. Data represents an average of thee replicates. Bars indicate mean ± SD

4.8. Discussion

The role of antioxidants in health and disease has been realized beyond doubt and the search for different sources of antioxidants especially natural ones has acquired newer dimensions. The algae both micro and macro were also under exploration for bioactive molecules including antioxidants. Astaxanthin, astaxanthin esters, and total carotenoid from *Haematococcus pluvialis* were evaluated for anti ulcer properties in ethanol-induced gastric ulcer in rats by Kamath et al., (2008). Fayaz et al., (2005) reported antioxidant principles from red seaweed *Kappaphycus* and Aboul-Enein et al., (2003) reported antioxidant activity of microalgal extracts on lipid peroxidation. Chidambaramurthy et al., (2005) reported antioxidant principles from *Dunaliella salina* that produces β-carotene. Ranga Rao et al., (2006) reported antioxidant activity of *Botryococcus braunii* extracts which contained lutein as major carotenoid. The antioxidant activity of algal extracts was reported to be dependent on the chemical components of the extracts that mainly consisted of carotenoids, polyphenols, tocopherols and vitamin C etc. These substances can act as potent antioxidants in protecting lipid peroxidation, free radical scavenging and hydroxy radical scavenging activities by different modes of action (Aboul-Enein et al., 2003). Palozza and Krinsky, (1992) reported astaxanthin as highly potential antioxidant in protecting membranous phospholipids and other lipids against peroxidation.

Several studies have reported on the relation between phenolic content and antioxidant activity. Velioglu et al., (1998) reported a strong relationship between

total phenolic content and antioxidant activity in selected fruits, vegetables and grain products. However, there is a wide degree of variation between different phenolic compounds in their effectiveness as antioxidant (Robards et al., 1999). Active oxygen species regulate carotenoids biosynthesis in some microorganisms such as yeast, *Phaffia rhodozyma* (Schroeder and Johnson, 1995a), green algae, *H. pluvialis* (Kobayashi *et al.*, 1992) and *Dunaliella bardawil* (Shaish et al., 1993). The accumulated carotenoids might function as a protective agent against oxidative stress damage (Schroeder and Johnson, 1995). Carotenoids scavenge/quench several active oxygen species such as O_2, H_2O_2, peroxy radicals, and hydroxy radicals (OH) both *in vitro* and *in vivo* (Krinsky et al.,2001, Lim et al., 1992; Lawlor & Brien, 1995). Levy et al., (2000) reported the protective action of β-carotene against oxidation and oxidation mediated diseases in animals and also the carotenoid isomers in algae have shown to inhibit the LDL-oxidation in diabetes mellitus patients. Natural β-carotene was shown to have higher bioavailability compared to synthetic ones (Ben Amortz & Levy, 1996). Lutein constituted the major carotenoid in *B. braunii* extract whose bioavailability is higher than *β*-carotene and is considered to be an active agent in the prevention of chronic diseases such as cataracts, age related macular degeneration and atherosclerosis besides its use as feed additive in poultry farming as well as food dye (Krinsky et al., 2003). The present result shows that *H. pluvialis* extract is capable of preventing lipid peroxidation through scavenging free radicals and hydroxy radicals in living cells. Therefore, *H. pluvialis* extract can be used for various applications such as health supplements, pharmaceuticals and nutraceutical.

4.9. Antioxidant properties of astaxanthin & its esters in *in vivo* model

Among the groups the one which was treated with diester of astaxanthin concentration at 250 µg/kg has shown maximum activity i.e protection when compared to control and synthetic astaxanthin treated group. Treatment of toxin to astaxanthin and its esters have shown protection, which was estimated in terms of content of hepatic enzymes namely, catalase, peroxidase, superoxide dismutase and antilipid peroxidase. Treatments of rats with toxin at 2.0 g/kg body weight significantly reduced the levels of catalase, peroxidase and SOD by 31.65, 35.17 and 51.70%. On the other hand, lipid peroxidation increased by 1.7 folds as compared to normal due to the CCl_4 treatment. However, pretreatment of rats with 250 µg/kg b.w

of carotenoids preserved catalase, peroxidase and SOD activities, which are comparable with control values of the enzyme. Restoration of catalase was 42.44%. 49.40% and 58.06% higher when compared to toxin treated groups respectively at 250 µg/kg b.w. of astaxanthin and its esters, whereas it was 44.32% in 100 µg/kg b.w synthetic astaxanthin group. The similar trend was seen in case of peroxidase and SOD enzymes (Table 25). This shows the protection provided by feeding astaxanthin to the rats by restoration of the levels of these enzymes even after treatment of toxin. The lipid peroxidation was restored by 5.2 folds in case of 250 µg/kg diester of astaxanthin treated group.

Table 25. Effect of FASX, MASX, DASX and SASX* on hepatic enzymes in CCl_4 intoxicated and normal rats

Groups	Catalase (U/mg protein)	Peroxidase (U/mg protein)	SOD (U/mg protein)	% Anti lipid peroxidase
Normal	457.23 ± 6.98^{d}	10.12 ± 1.42^{c}	20.81 ± 1.75^{c}	22.45 ± 2.61^{c}
Control (CCl_4 treated)	312.51 ± 10.37^{e}	6.56 ± 1.74^{d}	10.05 ± 2.38^{e}	53.78 ± 3.80^{a}
FASX (100 µg/kg)	543.01 ± 9.61^{c}	15.98 ± 2.30^{b}	15.02 ± 1.73^{d}	26.46 ± 1.09^{b}
MASX (250 µg/kg)	623.84 ± 8.25^{b}	21.79 ± 3.06^{a}	26.13 ± 3.67^{b}	21.81 ± 2.74^{c}
DASX (250 µg/kg)	745.28 ± 6.05^{a}	23.65 ± 1.65^{a}	32.98 ± 4.01^{a}	10.33 ± 1.12^{d}
SASX (100 µg/kg)	561.31 ± 11.82^{b}	11.24 ± 2.38^{c}	16.81 ± 3.22^{d}	27.58 ± 2.91^{b}

*FASX refers to free astaxanthin, MASX refers to monoester of astaxanthin, DASX refers to diester of astaxanthin and SASX refers to standard astaxanthin. Values are mean ± SD. Values are not sharing a similar superscript within the same column are significantly different ($P<0.05$) as determined by ANOVA.

4.9.1. Hepatoprotective activity of astaxanthin and its esters

4.9.1.1. Isolation of astaxanthin & its esters by HPLC and LC-MS

H. pluvialis was grown in Bold basal medium and the biomass contained 2.35% (w/w) total carotenoid, 0.20% (w/w) chlorophyll respectively. Astaxanthin content was found to be 2.1% of biomass on dry weight basis (i.e. it constituted 88% of total carotenoid). The absorption spectra of the carotenoid extract from *Haematococcus* cells showed a major peak at 470-476 nm. Total carotenoid extract was done with

acetone: hexane (3:7) mobile phase using silica thin layer chromatography. Thin layer chromatography of total carotenoid showed FASX and EASX at relative front (RF=0.54, and 0.77). FASX and EASX were identified by the absorption spectra of FASX and EASX were checked at 476 nm by HPLC (Fig. 100A-C). These compounds were used for the experiments.

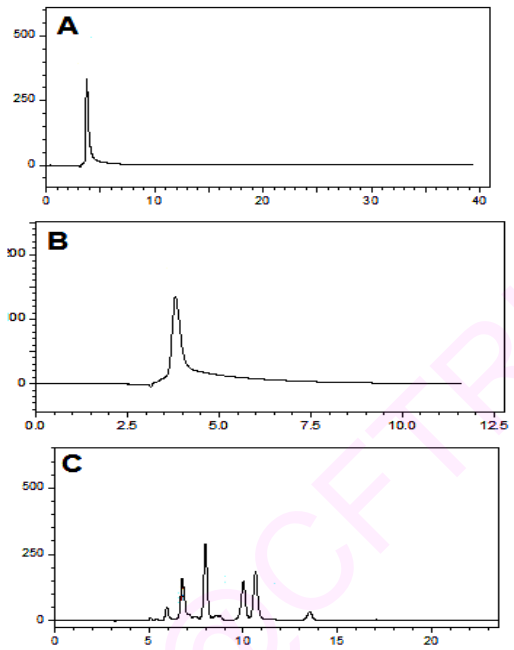


Fig. 100. HPLC profile of isolated SASX (A), FASX and EASX (B&C) from *H. pluvialis.* *FASX refers to free astaxanthin, EASX refers to astaxanthin esters, SASX refers to synthetic astaxanthin.

Administration of CCl_4 led to an increase in levels of serum enzymes by three fold as compared to control group. Treatment of rats with FASX, EASX and SASX at 250 µg/kg body weight markedly prevented the CCl_4 induced SGOT, SGPT; ALP levels. Fig. 101 & 102 show that the SGPT and SGOT activity increased in the CCl_4 toxin administered group, the concentrations of SGPT and SGOT were 153.39 ± 4.56 and 186.12 ± 5.19 units/ml respectively. These enzyme activities were significantly less for the test group animals, SGPT was 112.23 ± 5.94, 98.45 ± 6.54 and 122.28 ± 7.23 units/ml after treatment of FASX, EASX and SASX, whereas in SGOT was 105.98 ± 4.31, 86.67± 5.04, 114.01 ± 8.10 units/ml. The hepatoprotective activity of FASX, EASX and SASX on SALP were shown in **Fig. 103.** SALP activity was 94.87 ± 3.87 units/ml for CCl_4 treatment group and 72.45 ± 5.02 units/ml in normal group, whereas 63.21 ± 2.86, 50.38 ± 4.91, 68.10 ± 5.34 units/ml respectively, in the animals treated with FASX, EASX and SASX. Fig. 104 & 105 shows the total protein and albumin

levels in normal, CCl_4, FASX, EASX and SASX after treatment of CCl_4. The protein levels increased in EASX (9.42 ± 2.31) treated group when compared to control and CCl_4 groups. The albumin levels increased in FASX (7.12 ± 1.98) and EASX (8.54 ± 1.76) treated groups compared with CCl_4 group. Histology has shown in case of normal group, hepatocytes with normal architecture and portal triad, portal veins, hepatic artery and vein are visible (Fig. 106). However, CCl_4 treated group showed total loss of hepatic architecture, areas of necrosis. In the case of EASX treated group followed by CCl_4, the liver has retained the normal hepatic architecture with minor hemorrhage in case of 250 µg/kg treated group and the same was observed in the case of FASX and SASX treated groups.

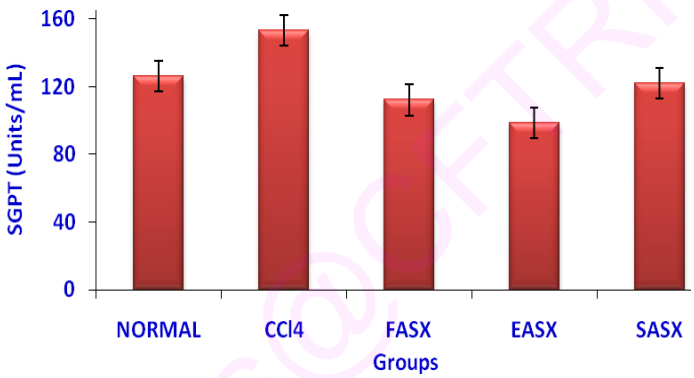


Fig. 101. Effect of astaxanthin & its esters on SGPT in CCl_4 intoxicated rats. CCl_4 were treated with vehicle (olive oil) of FASX, EASX and SASX at 250 µg/kg b.w. for 14 days. Animals of the normal group were treated with equal amounts of olive oil. Results were expressed as mean ± SD (n =5). *FASX refers to free astaxanthin, EASX refers to astaxanthin esters, SASX refers to synthetic astaxanthin.

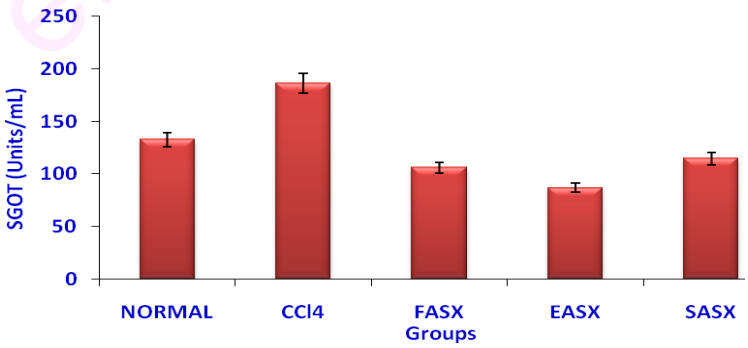


Fig. 102. Effect of astaxanthin & its esters on SGOT in CCl_4 intoxicated rats. CCl_4 were treated with vehicle (olive oil) of FASX, EASX and SASX at 250 µg/kg b.w. for 14 days. Animals of the normal group were treated with equal amounts of olive oil. Results were expressed as mean ± SD (n =5). *FASX refers to free astaxanthin, EASX refers to astaxanthin esters, SASX refers to synthetic astaxanthin.

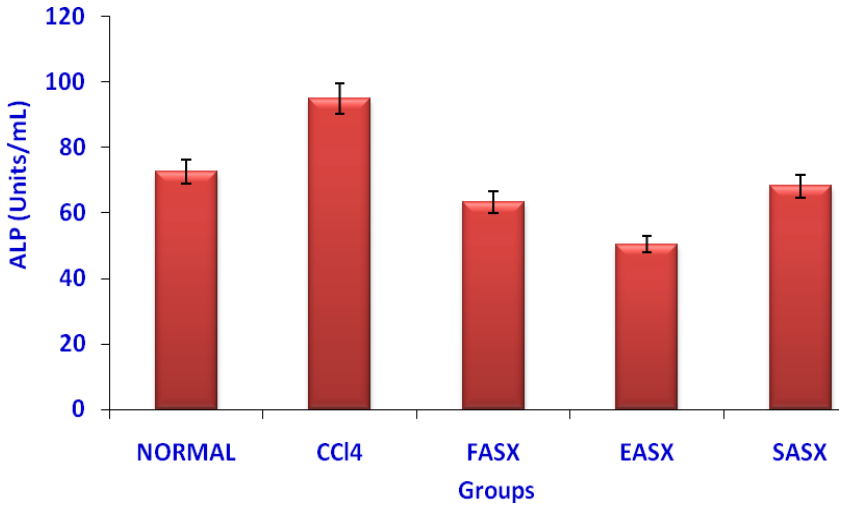


Fig. 103. Effect of astaxanthin & its esters on ALP levels in CCl_4 intoxicated rats. CCl_4 were treated with vehicle (olive oil) of FASX, EASX and SASX at 250 µg/kg b.w. for 14 days. Animals of the normal group were treated with equal amounts of olive oil. Results were expressed as mean ± SD (n =5). *FASX refers to free astaxanthin, EASX refers to astaxanthin esters, SASX refers to synthetic astaxanthin.

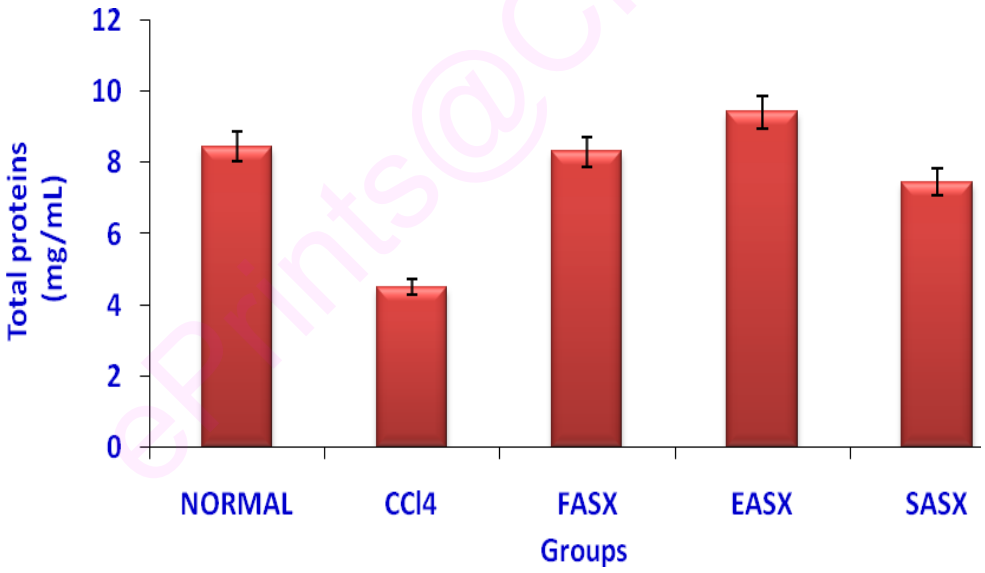


Fig. 104. Total protein content in normal, CCl_4 intoxicated rats, FASX, EASX and SASX. CCl_4 intoxicated rats were treated with vehicle (olive oil) and FASX, EASX and SASX at 250 µg/kg body weight for 14 days. Animals of the normal group were treated with equal amounts of olive oil. Results were expressed as mean ± SD (n =5). *FASX refers to free astaxanthin, EASX refers to astaxanthin esters, SASX refers to synthetic astaxanthin.

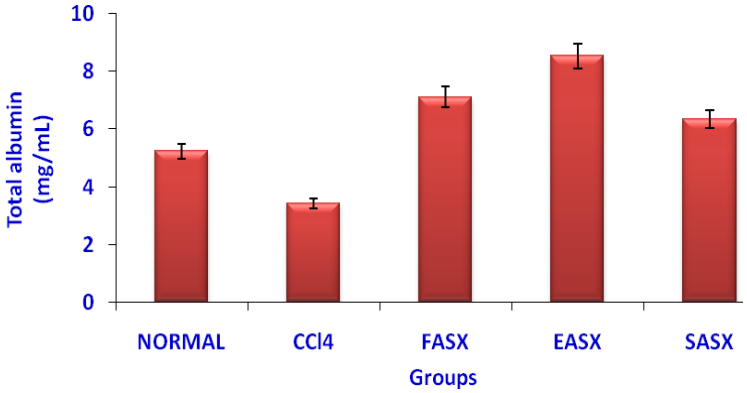


Fig. 105. Total albumin content in normal, CCl_4 intoxicated rats, FASX, EASX and SASX. CCl_4 intoxicated rats were treated with vehicle (olive oil) and FASX, EASX and SASX at 250 µg/kg body weight for 14 days. Animals of the normal group were treated with equal amounts of olive oil. Results were expressed as mean ± SD (n =5). *FASX refers to free astaxanthin, EASX refers to astaxanthin esters, SASX refers to synthetic astaxanthin.

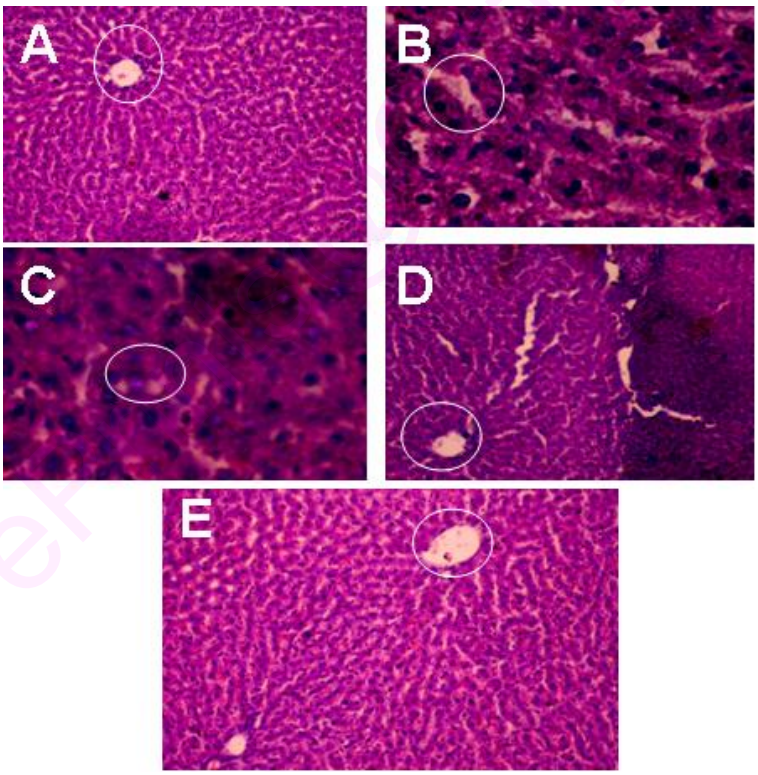


Fig 106. Histopathological observation of liver of different treated groups (10 X). Section through the liver of normal rats showing central vein and hepatocytes (A), section through the liver of CCl_4-treated rats showing central vein and hepatocytes (B) and section through the liver of FASX, EASX, SASX (250 µg/kg b.w) treated rats (C,D,E) showing the central vein (round marking) and hepatocytes. *FASX refers to free astaxanthin, EASX refers to astaxanthin esters, SASX refers to synthetic astaxanthin

4.9.2. Antibacterial properties of *H. pluvialis* extracts

The total phenolic content was estimated in hexane, chloroform, ethyl acetate, acetone and methanol extracts from *H. pluvialis*. The phenolic content 47.52, 131.98, 95.02, 68.03 and 85.23 (µg/mg) were found in hexane, chloroform, ethyl acetate, acetone and methanol extracts respectively. Antibacterial activity of *H. pluvialis* against selected bacteria is presented in Table 26. Among the different solvent extracts of *H. pluvialis,* chloroform extract showed highest antibacterial effect followed by ethyl acetate extract whereas hexane extract exhibited least activity against the bacteria tested. Highest zone of inhibition was observed in chloroform extract against *B. subtilus* (17 mm) followed by *E. coli* (14 mm). Acetone, ethyl acetate and methanol extracts showed highest zone of growth inhibition against *L. monocytogenes* (13 mm), *P. mirabilis* (13 mm) and *S. aureus* (13 mm) respectively.

Table 26. Antibacterial activity of *H. pluvialis* extracts

	Diameter of zone of inhibition (mm)				
Bacteria	**Hexane**	**Chloroform**	**Ethyl acetate**	**Acetone**	**Methanol**
B. subtilus,	11.01 ± 0.12^{d}	17.32 ± 0.18^{a}	11.23 ± 0.14^{d}	--	10.43 ± 0.12^{e}
E. aerogenes	--	14.54 ± 0.18^{b}	12.34 ± 0.26^{c}	10.05 ± 0.26^{e}	--
P. mirabilis	--	--	13.82 ± 0.18^{c}	--	--
M. *luteus*	--	12.09 ± 0.10^{c}	--	12.78 ± 0.24^{c}	12.81 ± 0.20^{c}
E. coli	--	15.87 ± 0.20^{b}	--	--	12.34 ± 0.13^{c}
S. aureus	10.23 ± 0.17^{e}	13.75 ± 0.31^{c}	12.61 ± 0.23^{c}	11.45 ± 0.17^{d}	13.52 ± 0.34^{c}
S. typhi	--	--	12.02 ± 0.11^{c}	--	--
P. aeruginosa	--	12.02 ± 0.21^{c}	--	--	--
K. pneumoniae	--	--	--	--	--
B. cereus	--	11.93 ± 0.14^{d}	--	--	12.27 ± 0.18^{c}
L. monocytogenes	10.66 ± 0.20^{e}	--	11.34 ± 0.18^{d}	13.96 ± 0.25^{c}	13.18 ± 0.31^{c}
S. fecalis	--	13.21 ± 0.28^{c}	12.98 ± 0.21^{d}	11.72 ± 0.10^{d}	--
Y. enterocolitica.	--	10.34 ± 0.17^{e}	10.54 ± 0.17^{e}	10.65 ± 0.21^{e}	11.32 ± 0.19^{d}

Values are mean ± SD. Values are not sharing a similar superscript with in the same column are significantly different ($P<0.05$) as determined by ANOVA.

4.9.2.1. MIC values of microalgal extracts

The MIC values of *H. pluvialis* extracts ranged from 150 ppm to 400 ppm (Table 27). Chloroform extract showed inhibitory activity against *B. subtilus* MIC of 150 ppm and also inhibited the growth of *M. luteus* and *E. coli* with 250 ppm. While ethylacetate extract inhibited the growth against *S. fecalis* and *P. mirabilis* at 200

ppm and 250 ppm respectively whereas methanol extract inhibited *S. aureus* and *L. monocytogenes* with MIC of 250 ppm. All the solvent extracts exhibited varied degree of antibacterial activity. The chloroform extracts of *H. pluvialis* showed higher activity when compared with other extracts.

Table 27. MIC values for *H. pluvialis* extracts against food borne pathogens.

	MIC (ppm)				
Bacteria	**Hexane**	**Chloroform**	**Ethyl acetate**	**Acetone**	**Methanol**
B. subtilus,	400	150	350	--	350
E. aerogenes	--	250	300	350	--
P. mirabilis	--	--	250	--	--
M. *luteus*	--	200	--	350	300
E. coli	--	200	--	--	300
S. aureus	350	250	300	400	250
S. typhi	--	--	300	--	--
P. aeruginosa	--	350	--	--	--
K. pneumoniae	--	--	--	--	--
B. cereus	--	350	300	--	300
L. monocytogenes	300	--	--	300	250
S. fecalis	--	250	200	350	--
Y. enterocolitica.	--	350	350	300	350

4.9.3. Discussion

The present study evaluated the antibacterial properties of micro algal crude extracts of *H. pluvialis* against important pathogenic bacteria. The chloroform, ethylacetate, acetone and methanol extracts of *H. pluvialis* extracts showed higher activity compared to hexane extracts. The *H. pluvailis* extracts of chloroform and ethylacetate extracts showed higher inhibitory activity against *B. subtilus, E. coli* and *E. aerogenes*, when compared with other extracts such as methanol, ethylacetate, acetone and hexane. Taken together the results indicated that the antimicrobial activity was related to the amounts of phenolic compounds contained in *H. pluvialis* organic extracts. Mundt et al., (2003) and Ozdemir et al., (2004) reported that antimicrobial activity of micro algae against some pathogenic organisms could be due to its fatty acids and hydroxyl unsaturated fatty acids, glycolipid and phenolic compounds. In the present investigation also the non-polar extracts of some algae exhibited greater antimicrobial activity than polar ones and their activity may also be attributed to the presence of fatty acids and lipid-soluble phenolic. At low concentration, phenols are reported to affect enzyme activity, especially of those

enzymes associated with energy production while at higher concentrations, they cause protein denaturation. In addition, effect of phenol and fatty acids on microbial growth could be the result of the ability of these compounds to alter microbial cell permeability, permitting the loss of macro-molecules from the interior and could be also interact with membrane proteins causing a deformation in their structure and functionality as well as affecting cellular activity as reported by Mundt et al., (2003). In the present investigation, linear inhibition of bacteria with increased concentration of extracts was noticed. Earlier report also indicated that antibacterial effects of phenolic compounds are concentration dependent (Prindle, 1977). Patterson et al., (1993) reported that the blue green alga extracts contained different antibacterial substances and reflect the variety of secondary metabolites. Kreitlow et al., (1999) reported 12 cyanobacterial strains for their antibiotic activities against 7 microorganisms, whereas antibacterial activity of fresh water micro-algae were reported by Jaya prakash Goud et al., (2007). The algal species in the present study were individually positive to one or more phytochemicals, which might have contributed to their antibacterial efficacy. The significant antibacterial activity *H. pluvialis* extracts against bacteria may be attributed to its chemical nature. The chloroform, methanol, ethylacetate extracts of *H. pluvialis* were found to have potential antibacterial activity against *Bacillus subtilus, Bacillus cereus, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus luteus, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Streptococcus fecalis* and *Yersinia enterocolitica* pathogens. Chloroform extract of *H. pluvialis* showed higher antibacterial activity when compared to other forms of extracts. The antibacterial effect of *H. pluvialis* against bacteria can be a preferred supplement to its known health benefits as antibacterial agents and usage in food system.

4.9.4. Estimation of nutritional quality of *H. pluvialis* cells

Based on the results of the above studies *H. pluvialis* were further subjected for evaluation of nutritional activity using albino rats. Treatment of *H. pluvialis* biomass at 2.5 g/kg and 5 g/kg b.w. has not shown any death or symptoms of toxicity. Animal of *H. pluvialis* treatment group were active similar to that of normal diet fed ones. Overall growth as measured by gain in body weight (once a week) shows that in

gained higher body weight compared control groups. Gain in body weight was maximum of 20% higher treated with 2.5 g/kg biomass, whereas in 5 g/kg *H. pluvialis* treated group was 14% higher when compared to control group (Table 28**).**

Table 28. Effect of *H. pluvialis* treatment on the body weight (grams) of experimental rats

Treatment	**Body weight**		
	initially	**1st week**	**2nd week**
Normal diet	221.43 ± 3.89^{c}	241.89 ± 4.76^{c}	252.29 ± 5.78^{c}
2.5 g/kg b.w *H. pluvialis* cells	225.82 ± 4.61^{b}	256.23 ± 6.81^{a}	271.34 ± 4.31^{a}
5.0 g/kg b.w. *H. pluvialis* cells	232.76 ± 7.52^{a}	248.23 ± 5.03^{b}	265.71 ± 3.97^{b}

Values are mean ± SD. Values are not sharing a similar superscript within the same column are significantly different ($P<0.05$) as determined by ANOVA.

Effect of algal treatment on the absolute and relative weight of vital organs is summarized in Table 30. Decrease in the weight of liver, lungs and testes were observed in both the groups, when compared to normal diet. Relative weight of kidney and thymus were significantly high in both 2.5 g/kg and 5.0 g/kg *H. pluvialis* treated group. Significant decrease in the relative weight of testis was observed in both the dose (Table 29)**.** Treatment of *H. pluvialis* biomass has not shown any adverse effect on the hematological profile of the experimental animals. Hemoglobin content of both 2.5 g/kg b.w. and 5 g/kg b.w. *H. pluvialis* treated animals were lowered, which was more in lower dose. White blood cells were in the normal range in all the group of animals. Its count decrease in case of 2.5 g/kg b.w. and it was not much altered in higher dose compared to normal group of animals. Serum protein was significantly lower in both the concentrations of *H. pluvialis* treated compared to the values of normal animals (Table 30).

Table 29. Effect of *H. pluvialis* treatment on the vital organs weight of experimental rats.

Organs	Normal	2.5 g/kg b.w of *H. pluvialis* cells	5.0 g/kg b.w. of *H. pluvialis* cells
Liver	7.01 ± 0.98^{a}	6.83 ± 1.21^{a}	6.83 ± 0.63^{a}
Heart	0.88 ± 0.13^{e}	0.84 ± 0.09^{e}	0.78 ± 0.02^{e}
Kidney	1.72 ± 0.06^{c}	1.81 ± 0.07^{c}	1.85 ± 0.13^{c}
Lungs	1.03 ± 0.12^{c}	1.05 ± 0.05^{d}	1.29 ± 0.16^{d}
Thymus	0.38 ± 0.09^{f}	0.41 ± 0.02^{f}	0.43 ± 0.05^{f}
Testis	2.56 ± 0.17^{b}	2.45 ± 0.14^{b}	2.39 ± 0.08^{b}
Spleen	0.71 ± 0.05^{e}	0.68 ± 0.06^{e}	0.66 ± 0.06^{e}

Values are mean ± SD. Values are not sharing a similar superscript within the same column are significantly different (P<0.05) as determined by ANOVA.

Table 30. Effect of *H. pluvialis* treatment on hematological profile of experimental animals

Hematology	Normal diet	2.5 g/kg b.w. of *H. pluvialis* cells	5 g/kg b.w. of *H. pluvialis* cells
WBC ($x10^3$/µl)	9.61 ± 1.02^{d}	9.34 ± 1.15^{d}	9.28 ± 0.95^{d}
RBC (x 10^6/ µl)	9.93 ± 0.98^{d}	9.95 ± 0.82^{d}	9.98 ± 1.01^{d}
HGB (g/dl)	14.96 ± 1.43^{c}	14.83 ± 1.23^{c}	14.71 ± 1.41^{c}
MCH (pg)	14.63 ± 1.35^{c}	15.68 ± 1.56^{c}	15.21 ± 1.92^{c}
MCHC (g/dl)	26.71 ± 1.98^{b}	24.56 ± 2.01^{b}	24.73 ± 2.18^{b}
MCV (fl)	54.58 ± 3.67^{a}	57.31 ± 4.07^{a}	55.72 ± 3.45^{a}
Total protein	6.27 ± 0.98^{e}	6.31 ± 0.76^{e}	6.22 ± 0.65^{e}

Values are mean ± SD. Values are not sharing a similar superscript within the same column are significantly different (P<0.05) as determined by ANOVA.

4.9.5. Discussion

Thus, the experiment clearly shows the protection offered to the animals fed with FASX and EASX of *H. pluvialis* is evident from restoration of the levels of serum enzymes even after treatment of the toxin. Both total protein and albumin were found to be decreased for the CCl_4 treated group of animals. However, these values were significantly higher for the FASX, EASX treated groups. This results indicates that carotenoids from *H. pluvialis* have a significant antihepatotoxic effect when compared with SASX, this finding may be attributed to the high biological

activity of EASX. The study involved supplementation of the diet with carotenoids to investigate the role of FASX and its esters in the *in vivo* model of CCl_4 induced toxicity. The effect of free radicals on the liver detoxification enzymes reduced the enzyme activity, mainly due to enzyme inactivation during the catalytic cycle. Carbon tetrachloride has been extensively studied as a liver toxicant, and its metabolites such as trichloromethyl radical (CCl_3) and trichloromethyl peroxyl radical (CCl_3O_2) are involved in the pathogenesis of liver and kidney damage. The massive generation of free radicals in the CCl_4 induced liver damage provokes a sharp increase of lipid peroxidation in liver. When free radical generation is massive, the cytotoxicity effect is not localized but can be propagated intracellularly, increasing the interaction of these radicals with phospholipids structure and inducing a peroxidation process that destroys organs (Castro et al., 1974). CCl_4 induces induces fatty liver and cell necrosis and plays a significant role in inducing triacylglycerol accumulation, depletion of GSH, increased lipid peroxidation, membrane damage, depression of protein synthesis and loss of enzyme activity (Ahmed et al., 2002). This is mainly due to metabolites of CCl_4, which provoke a sharp increase in the lipid peroxidation in liver and release of markers into plasma (Recknagel et al., 1980). Being cytoplasamic in location the damage marker enzymes like GOT, GPT and ALP are released in serum (Chenoweth and Hake, 1962; Yasuda et al., 1980). Scientific reports indicate that astaxanthin has positive effects on blood pressure as well as a cardio protective effect (Petri et al., 2007). Astaxanthin has been proven to suppress spontaneous liver carcinogenesis in male mice. The liver tumors was significantly decreased by astaxanthin treatment as compared with that in the control group (Hoyoku et al., 1999). Sanda et al., (2008) reported that the astaxanthin protects liver damage induced by methylnitros urea by inhibiting lipid and protein oxidation and stimulating the cellular antioxidant system. The damage to marker enzymes SGOT, SGPT and lactate dehydrogenase were evident in serum as reported earlier by Chenoweth and Hake (1962).

Carotenoids are also well known for their proantioxidant ability, in which they have shown membrane protection ability mainly by lipid peroxidation prevention and restoration of various antioxidant enzymes like superoxide dismutase, catalase, and peroxidases (Palozza and Krinsky, 1992). However, there are only a few reports available on direct protection ability of astaxanthin and carotenoids. *Cis* and *trans* β-

carotene from *Dunaliella* species have shown antioxidant activity in human neutrophils in comparison with synthetic all trans β-carotene (Liu et al., 2000). Park et al., (2010) showed that the dietary astaxanthin enhanced immune response and decreased a DNA oxidative damage biomarker and inflammation in young healthy females. Astaxanthin has shown various pharmacological activities, including anti-inflammatory (Kurashige et al., 1990; Ohgami et al., 2003) and antidiabetic activities (Uchiyama et al., 2002), as well as antioxidative effects (Kang et al., 2001; Aoi et al., 2003). Hussein et al., (2005a, 2005b) investigated an antihypertensive effect of astaxanthin in spontaneously hypertensive rats (SHR), which have been widely used as a model to study the mechanism, pathophysiology, and management of hypertension. Hussein et al., (2006) investigated the effect of astaxanthin on plasma levels of NO end products nitrite/nitrate (NO_2/NO_3, termed NOx) in spontaneously hypertensive rats. Lipid peroxidation in the serum and liver of astaxanthin-fed rats treated with carbon tetrachloride was significantly inhibited relative to rats fed on control diet (Kang et al., 2001).

Astaxanthin and its esters fed animals (250 µg/kg) have shown highly significant antioxidant activity in terms of restoration of hepatic enzymes after challenging animals with the toxin (carbon tetrachloride). The lipid peroxidation was restored by 5 folds in case of *Haematococcus* carotenoid treated group at the dose of 250 µg/kg b.w (Table 26). The free radicals reduce the activity of liver detoixification enzymes (catalase, SOD and peroxidase), mainly due to enzyme inactivation during the catalytic cycle. Under oxidative stress, *Haematococcus* containing astaxanthin and its esters along with other carotenoids and xanthophylls act as potent free radical scavengers, reducing the levels of hydrogen peroxide and superoxide anion and consequently lipid peroxidation and enzyme inactivation leading to restoration of the enzyme activity. Bioavailability of *trans* β-carotene is three times more than that of *cis*, while the same is found to be more with *cis* isomer of lycopene (Castenmiller and West, 1998). Tan and Chu (1991) fed carotenoid from palm oil to rats and measured hepatic cytochrome P_{450}- mediated metabolism of benzo pyrene. A study in humans revealed that maintenance of a carotenoid free diet for two weeks followed by feeding with β-carotene demonstrated a decrease in the serum lipid peroxide value, inducing *in vivo* antioxidant effect in humans (Mobarhan et al., 1990). The bioavailability of

other carotenoids like lutein, zeaxanthin, lycopene, and α-carotene has an effect on each other, which is favorable for biological response. It was also found that carotenoids in oils are absorbed and metabolized faster compared to other forms.

The present study indicates that the isolated astaxanthin & its esters molecules showed hepatoprotective activity compared with synthetic astaxanthin. Hence, accumulated astaxanthin & esters in *H. pluvialis* had a higher hepatoprotection and can be utilized as formulations and functional foods necessary for the purpose of hepatoprotection, which is having high demand in the market. Therefore, the astaxanthin & esters can be used for various applications such as health supplements, pharmaceuticals and nutraceuticals.

4.9.6. Bioavailability of astaxanthin from *H. pluvialis* using feeding trails

4.9.6.1. Effect of *H. pluvialis* biomass feeding to rats on their body and organ weight

After feeding of *H. pluvialis biomass* to rats there was no significant ($P < 0.05$) variation in body weight when compared to control group of rats (Table 31 & 32). No clinical signs of any toxicity, mortality were noticed during the experimental period.

Table 31. Relative weights (in grams) of different organs of the experimental rats after single dose of *H. pluvialis* as a source of astaxanthin.

	Liver	Kidney	Brain	Eye
Control	4.47 ± 0.61^{a}	1.11 ± 0.12^{a}	1.58 ± 0.08^{a}	0.23 ± 0.04^{a}
2h	4.90 ± 0.78^{a}	1.00 ± 0.11^{a}	1.45 ± 0.24^{a}	0.21 ± 0.03^{a}
4h	4.59 ± 0.63^{a}	0.95 ± 0.07^{a}	1.57 ± 0.10^{a}	0.22 ± 0.02^{a}
6h	4.31 ± 0.43^{a}	0.94 ± 0.14^{a}	1.59 ± 0.08^{a}	0.22 ± 0.03^{a}
9h	4.41 ± 0.78^{a}	1.03 ± 0.14^{a}	1.58 ± 0.09^{a}	0.23 ± 0.01^{a}

Rats were fed with a single dose of *H. pluvialis* biomass containing 200 μM equivalents of astaxnthin from the respective biomass, and were killed at 2, 4, 6, and 9 h after the dose. Values are mean ± SD (n=5). Values are not sharing a similar superscript within the same column in a group over 0-9 h are significantly different ($P<0.05$) as determined by ANOVA

Table 32. Body weights of rats randomized for different experimental groups.

	Body wt in grams
Control	129.2 ± 9.75^{a}
2h	129.8 ± 10.47^{a}
4h	126.6 ± 12.78^{b}
6h	129.0 ± 10.73^{a}
9h	124.60 ± 10.80^{c}

Values represent the mean ± SD of 5 analyses. That values are significant at $p < 0.05$ compared to untreated control.

4.9.6.2. Influence of feeding *H. pluvialis* biomass on plasma, liver and eye response of astaxanthin

Time course response of astaxanthin levels in plasma, liver and eyes of rat over 0-9 h after gavage of *H. pluvialis* biomass was recorded (Fig. 107**).** Astaxanthin was not detected in the plasma, liver and eye of zero-time group, but after gavage, its levels reached maximum in plasma (128.49 ± 2.58 pmol/ml), liver (131.23 ± 4.58 pmol/g) and eye (281.45 ± 5.12 pmol/g) at 2 h, 4 h and 6 h. The eye astaxanthin level was markedly higher at 6h and lower at 9h than those seen in plasma and liver. The AUC values of astaxanthin in plasma, liver and eyes were calculated and found that the plasma and liver showed no difference in their AUC values, whereas eye had higher AUC value. The mean astaxanthin AUC values of eyes were higher by 2.5 and 3 fold than that of plasma and liver.

4.9.6.3. Influence of feeding *H. pluvialis* biomass on antioxidant enzymes in plasma

Feeding of *H. pluvialis* (as source of astaxanthin) over 0-9 h to rats has influenced on activity of plasma hepatic antioxidant enzymes, catalase, superoxide dismutase and peroxidase (Table 33**).** Results showed that the maximum catalase, SOD, peroxidase activity (57%, 37% and 51%) was found at 2 h after intubation of *H. pluvialis* as a source of astaxanthin, when compared to control group. Catalase and peroxidase activities were 1.8 and 1.3 higher, when compared to SOD. The AUC values of catalase, SOD and peroxidase over 9 h were calculated for *H. pluvialis* fed groups. The AUC value of catalase activity is 1.1 fold higher in *H. pluvialis*.

4.9.6.4. Influence of feeding *H. pluvialis* biomass on antioxidant enzymes in liver

Intubation of *H. pluvialis* as source of astaxanthin over 9 h to rats have shown protection, which was estimated in terms of hepatic antioxidant enzymes, catalase, SOD and peroxidase (Table 34**).** The results shows that the maximum activity of catalase, SOD, peroxidase 34%, 39% and 54%, respectively was found at 4 h after intubation of *H. pluvialis*, when compared to control. The AUC values for catalase, SOD and peroxidase were calculated in *H. pluvialis* fed groups. The peroxidase activity increased 1.4 fold in *H. pluvialis*.

4.9.6.5. Influence of feeding *H. pluvialis* biomass on lipid peroxidation in plasma and liver

Changes of lipid per oxidation in plasma over 9 h intubation of *H. pluvialis* as source of astaxanthin are shown in Table 35. *H. pluvialis* biomass fed group, inhibition of lipid peroxidation was found to be 40% at 2 h, whereas in liver it was 41% at 4 h.

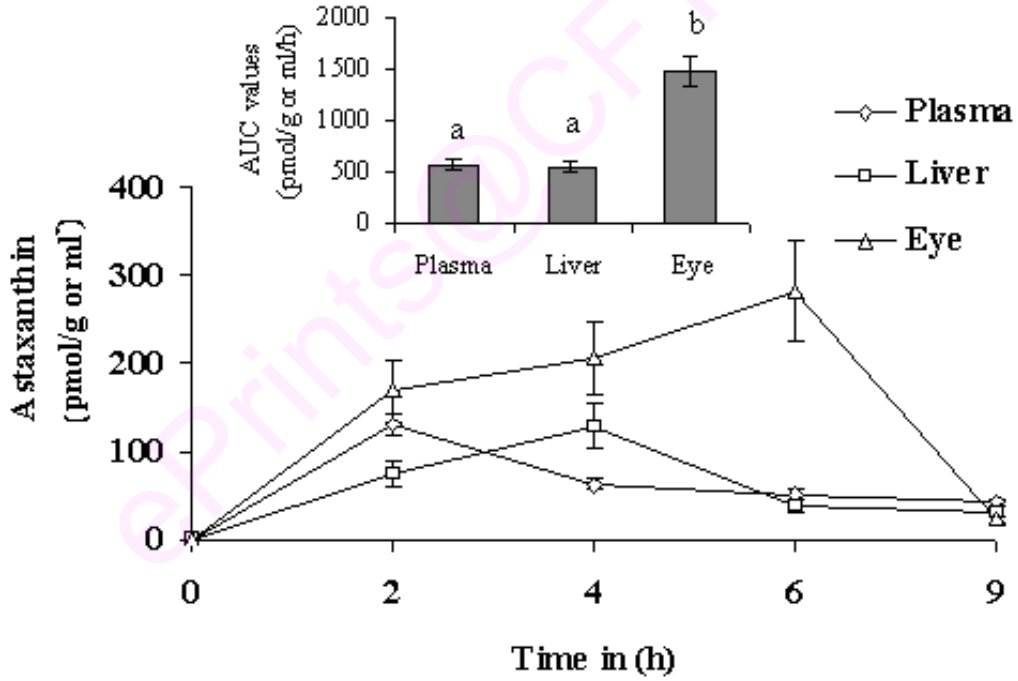


Fig. 107. Plasma, liver and eye response of astaxanthin after a single dose of *H. pluvialis* biomass dispersed in olive oil. The AUC values of astaxanthin in plasma, liver and eyes. No astaxanthin was detected in plasma, liver and eye at 0 h group. Values represent the mean ± SD of 5 analyses. That values are significant at $p < 0.05$ compared to untreated control.

Table 33. Activity of catalase, SOD and peroxidase in plasma of rats after a single dose of *H. pluvialis* biomass as source of astaxanthin.

Time in (h)	Catalase (U/mg protein)	SOD (U/mg protein)	Peroxidase (U/mg protein)
0	106.85 ± 3.59^{a}	7.57 ± 1.21^{c}	7.35 ± 1.55^{c}
2	250.10 ± 12.03^{b}	12.12 ± 3.25^{a}	15.05 ± 0.92^{a}
4	243.58 ± 8.03^{b}	11.14 ± 6.32^{a}	12.86 ± 0.61^{b}
6	199.23 ± 5.47^{c}	10.01 ± 0.54^{a}	10.51 ± 0.61^{b}
9	178.55 ± 15.42^{c}	9.09 ± 2.51^{b}	9.95 ± 1.14^{b}

Rats were fed with a single dose of biomass containing 200 µM equivalents of carotenoids from the respective biomass, and were killed at 2, 4, 6, and 9 h after the dose. Values are mean ± SD (n=5). Values are not sharing a similar superscript within the same column in a group over 0-9 h are significantly different ($P<0.05$) as determined by ANOVA

Table 34. Antioxidant enzymes in liver of rats after a single dose of *H. pluvialis* biomass as source of astaxanthin.

Time in (h)	Catalase (U/mg protein)	SOD (U/mg protein)	Peroxidase (U/mg protein)
0	251.52 ± 2.10^{c}	10.34 ± 0.54^{c}	7.44 ± 2.64^{c}
2	336.67 ± 4.51^{b}	13.80 ± 4.47^{b}	10.11±1.47c
4	383.08 ± 1.81^{a}	17.19 ± 3.87^{a}	16.28 ± 2.25^{a}
6	374.45 ± 4.34^{a}	16.29 ± 1.21^{a}	14.63 ± 3.99^{a}
9	366.44 ± 4.34^{a}	15.65 ± 0.85^{a}	11.32 ± 2.37^{b}

Rats were fed with a single dose of biomass containing 200 µM equivalents of carotenoids from the respective biomass, and were killed at 2, 4, 6, and 9 h after the dose. Values are mean ± SD (n=5). Values are not sharing a similar superscript within the same column in a group over 0-9 h are significantly different ($P<0.05$) as determined by ANOVA

Table 35. Antilipid peroxidation in after a single dose of *H. pluvialis* biomass as source of astaxanthin.

Time (h)	Plasma TBARS (nmol/mg protein)	Liver TBARS (nmol/mg protein)
0	1.34 ± 0.05^{b}	1.87 ± 0.08^{a}
2	0.57 ± 0.06^{d}	0.84 ± 0.06^{c}
4	0.59 ± 0.08^{d}	0.72 ± 0.05^{c}
6	0.78 ± 0.02^{c}	0.97 ± 0.03^{b}
9	0.82 ± 0.05^{c}	1.02 ± 0.04^{b}

Values are mean ± SD (n=5). Values are not sharing a similar superscript within the same column in a group over 0-9 h are significantly different (P<0.05) as determined by ANOVA.

4.9.6.6. Influence of feeding *H. pluvialis* biomass on triglycerides in plasma

Feeding of *H. pluvialis* as source of astaxanthin over 9 h to rats had significant influence on triglycerides in plasma. The maximum levels of triglycerides (31%) obtained at 2 h, while the lower levels (17%) were measured at 9h after intubation of *H. pluvialis*, when compared to control.

4.9.7. A repeated dose study

4.9.7.1. Influence of feeding *H. pluvialis* biomass on body and organ weight

Administrations of *H. pluvialis* did not show any significant (P < 0.05) variation in body weight when compared to control group of rats data presented in Table 36 & 37. No clinical signs of any toxicity, mortality were noticed during the experimental period

Table 36. Body weight of rats after a repeated dose of *H. pluvialis* biomass.

	Control	*H. pluvialis*
Initial	48.83 ± 2.13^{c}	49.33 ± 0.81^{c}
1st week	81.16 ± 3.46^{b}	83.28 ± 4.57^{b}
2nd week	104.50 ± 5.20^{a}	105.66 ± 3.11^{a}

Rats were fed a repeated dose of biomass containing 200 µM equivalent of carotenoids from the respective biomass, and were killed at 15th day after the dose. Values are mean ± SD (n=5). Values are sharing a similar within the same column in a group over 15 days are significantly different (P<0.05) as determined by ANOVA.

Table 37. Relative weights of organs after a repeated dose of *H. pluvialis* biomass as source of astaxanthin

	Liver (g)	Kidney (g)	Eye (g)	Intestine (g)	Brain (g)
Control	5.10 ± 0.34[a]	0.91 ± 0.09 [a]	0.23 ± 0.051 [a]	4.05 ± 0.70[b]	1.47 ± 0.07[a]
H. pluvialis	5.11 ± 0.45[a]	0.88 ± 0.075[a]	0.23 ± 0.040 [a]	5.36 ± 0.52[a]	1.43 ± 0.080[a]

Rats were fed a repeated dose of biomass containing 200 µM equivalent of carotenoids from the respective biomass, and were killed at 15th day after the dose. Values are mean ± SD (n=5). Values are sharing a similar within the same column in a group over 15 days are significantly different (P<0.05) as determined by ANOVA.

4.9.7.2. Influence of feeding *H. pluvialis* biomass on plasma, liver and eye response of astaxanthin in repeated dose study

Astaxanthin levels in plasma, liver and eyes of rat over 15 days after gavage of *H. pluvialis* biomass was recorded (Fig. 108). Astaxanthin was not detected in the plasma, liver and eye of control group, but after gavage, its levels reached maximum plasma (485.81 ± 34.58 nmol/ml), liver (896.51 ± 101.76 nmol/g) and eye (508.41± 71.32 nmol/g). The liver astaxanthin level was markedly higher than those seen in plasma and eyes. The AUC values of astaxanthin in plasma, liver and eyes were calculated**.** The mean astaxanthin AUC values of liver were higher by 1.8 and 1.7 fold than that of plasma and eyes.

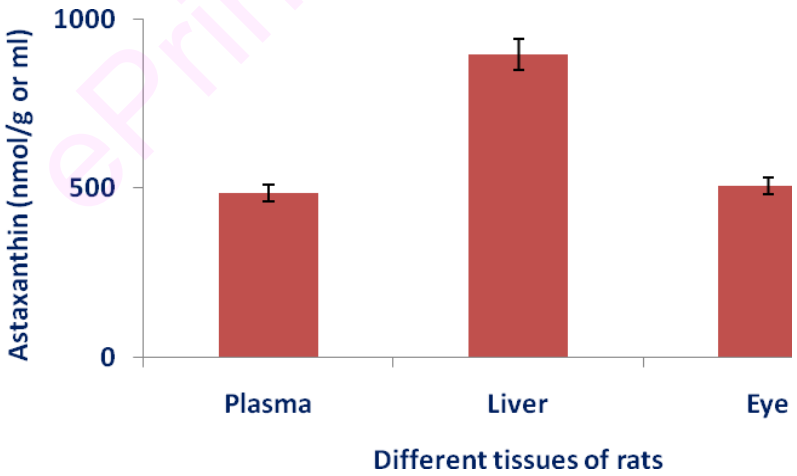


Fig. 108. Plasma, liver and eye response of astaxanthin after a repeated dose of *H. pluvialis* biomass dispersed in olive oil. Values represent the mean ± SD of 5 analyses. Values are significant at $p < 0.05$ compared to untreated control.

4.9.7.3. Influence of feeding *H. pluvialis* biomass as source of astaxanthin and its conversion into retinol in plasma, liver and eye response of rats

Retinol levels in plasma, liver and eyes of rat over 15 days after gavage of *H. pluvialis* biomass was recorded (Fig. 109). The plasma retinol levels was 102 pmol/ml, whereas in liver and eye were 27.69 and 79 pmol/g. The plasma retinol level was markedly higher than those seen in liver and eye. A 3.7 and 1.2 fold enhanced accumulation of retinol in plasma was observed in the experimental groups than that of liver and eye.

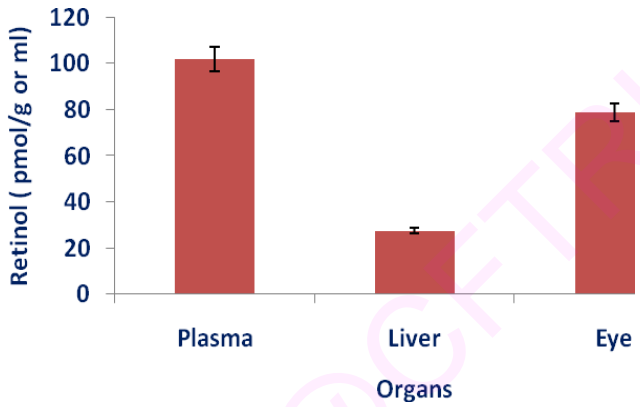


Fig. 109. Retinol levels in plasma, liver and eyes of rat tissues.

4.9.7.4. Influence of feeding *H. pluvialis* biomass on antioxidant enzymes in plasma and liver

Feeding of *H. pluvialis* over 15 days to rats had significant influence on activity of plasma hepatic antioxidant enzymes, catalase, superoxide dismutase (SOD) and peroxidase (Table 38). Results showed that maximum plasma catalase, SOD, peroxidase activity 52%, 58% and 71% higher in *H. pluvialis* fed group, when compared to control group. Intubation of *H. pluvialis* as source of astaxanthin over 15 days to rats have shown protection, which was estimated in terms of hepatic antioxidant enzymes, catalase, SOD and peroxidase (Table 38). The results shows that the activity of catalase, SOD, peroxidase 46%, 56% and 62%, respectively was found after intubation of *H. pluvialis*, when compared to control.

Table 38. Activity of catalase, SOD and peroxidase in plasma and liver of rats after a repeated dose of *H. pluvialis* biomass as source of astaxanthin.

	Catalase (U/mg protein)	**SOD (U/mg protein)**	**Peroxidase (U/mg protein)**
		Plasma	
Control	126.85 ± 4.78^{b}	8.54 ± 2.87^{b}	7.35 ± 1.55^{b}
H. pluvialis	267.29 ± 8.93^{a}	23.73 ± 3.25^{a}	25.91 ± 4.65^{a}
		Liver	
Control	208.11 ± 6.36^{b}	10.34 ± 0.54^{c}	7.44 ± 2.64^{d}
H. pluvialis	387.45 ± 4.89^{a}	24.36 ± 4.78^{a}	19.22 ± 2.31^{a}

Rats were fed a repeated dose of biomass containing 200 µM equivalent of carotenoids from the respective biomass, and were killed at 15th day after the dose. Values are mean ± SD (n=5). Values are not sharing a similar superscript within the same column in a group over 15 days are significantly different ($P<0.05$) as determined by ANOVA.

4.9.7.5. Influence of feeding *H. pluvialis* biomass on lipid peroxidation in plasma and liver

Changes of lipid peroxidation in plasma after intubation of *H. pluvialis* as source of astaxanthin over 15 days are shown in Table 39**.** The lipid peroxidation was found to be 60% in plasma after intubation of *H. pluvialis,* when compared to control. In the case of liver, the maximum lipid peroxidation was found to be 64% intubation of *H. pluvialis* biomass, when compared to control.

Table 39. Antilipid peroxidation in after a repeated dose of *H. pluvialis* biomass as source of astaxanthin.

	% of antilipid peroxidation	
	Plasma	Liver
Control	100.00^{a}	100.00^{a}
H. pluvialis	60.52 ± 2.61^{b}	64.62 ± 5.81^{b}

Rats were fed a repeated dose of biomass containing 200 µM equivalents of carotenoids from the respective biomass, and were killed at 15th day after the dose. Values are mean ± SD (n=5). Values are not sharing a similar superscript within the same column in a group over 15 days are significantly different ($P<0.05$) as determined by ANOVA.

4.9.8. Discussion

The present study was focused to elucidate the bioavailability of astaxanthin in *H. pluvialis* biomass by monitoring the postprandial plasma, liver and eye response in rats after administration of single and repeated dose study. The results indicate that the astaxanthin and its esters from *H. pluvialis* showed better bioavailability. The concentration of the carotenoids after a single dose and repeated dose of micro algal biomass reached to maximum level in plasma and liver, although their concentration levels varied among carotenoids significantly (Fig. 107 & 108). In single dose study, the eye astaxanthin level in rats from *H. pluvialis* biomass was 2.1 fold higher than plasma and liver at 6 h, whereas in repeated dose study, the peak concentration of liver astaxanthin levels was 1.8 fold higher than plasma and eye. However the carotenoid concentration varied significantly (Fig. 107 & 108). Presence of astaxanthin and its esters in *H. pluvialis* might be an added advantage to influence higher bioavailability of astaxanthin in rats. Lipophilic compounds such as astaxanthin are usually transformed metabolically before they are excreted and metabolites of astaxanthin have been detected in kidney, urine, liver, bile, gonads and skin (Page and Davies, 2002). The only study on humans to date confirmed the bioavailability of astaxanthin supplied in a single high dosage of 100 mg and its transport in the plasma by lipoproteins (Osterlie et al., 2000). Astaxanthin significantly influences immune function in several *in vitro* and *in vivo* assays using animal models. The bioavailability of *trans* β-carotene is reported to be 3 times more than that of *cis* (Castenmiller and West, 1998). Natural β-carotenes have shown to have higher bioavailability compared to synthetic ones (Ben Amortz and Levey, 1996). Lutein and zeaxanthin, two carotenoid pigments closely related to astaxanthin, are concentrated in the macula of the eye (Landrum et al., 1999). Biological properties of *Spirulina* are attributed to components including ω-3 or ω-6 fatty acids, β-carotene, α-tocopherol, phycocyanin and phenol compounds (Ross et al., 1990). The bioavailability of *Spirulina* carotenoids has been demonstrated in both the rat and chicken (Annapurna et al., 1991 and Chamorro et al., 2002).

Influence of the algal carotenoids on the activities of catalase, peroxidase and SOD was profound. The animals fed with micro algal biomass resulted in increase in activity of the liver and plasma antioxidant enzymes (Table 33, 34 & 38) and they

possibly act as potent free radical scavengers. Protection offered to the animals fed with astaxanthin carotenoids extract is evident from maintenance of the levels of the liver enzymes. Astaxanthin induce various enzymes in the cytochrome P_{450} system in rats and humans (Kistler et al., 2002), but this has so far not been confirmed in salmonid fishes (Page and Davies, 2002). Astaxanthin is much more effective than vitamin E at protecting mitochondria from rat liver cells against lipid peroxidation (Kurashige et al., 1990). The lipid peroxidation activity was slightly higher in astaxanthin at 2 h in plasma. This could possibly be attributed to the same mechanism being involved in these tissues. Similarly, in the case of liver, the maximum lipid peroxidation activity was found at 4 h. The antioxidant activity of algal extracts was reported to be dependent on the chemical components of the extracts that mainly consisted of carotenoids, polyphenols, tocopherols and vitamin C etc. Particularly, high level of carotenoids that may act as a strong antioxidant as reductones by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction. Our earlier studies have shown inhibition of lipid peroxidaiton by *B. braunii* extracts *in vitro* in brain, kidney, and liver tissues of rats (Ranga Rao et al., 2006). Fayaz et al., (2005) reported antioxidant principles from red seaweed *Kappaphycus* and Aboul-Enein et al., (2003) reported antioxidant activity of micro algal extracts on lipid peroxidation. Chidambaramurthy et al., (2005) reported antioxidant principles from *Dunaliella* that produces β-carotene. Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans (Park et al., 2010). Astaxanthin reduced triglycerides and increased HDL cholesterol in patients with mild hyperlipidemia (Yoshida et al., 2010). Cort et al., (2010) reported that astaxanthin protect in eye health which was found a protective effect in ocular hypertension in rats. Astaxanthin was found to protect against damage from ischemia through its intense antioxidant activity (Curek et al., 2010). In conclusion, it was evident that algal biomass is a potential source of carotenoids exhibiting bioavailability in experimental animals. The results also showed increased levels of antioxidant enzymes and prevention of lipid peroxidation through scavenging free radicals and hydroxyl radicals in living cells when microalgae biomass was fed to rats. This implies that *H. pluvialis* biomass can be used as source of carotenoids and also as nutritional or nutraceutical supplements.

4.9.9. Salient features

Carotenoid rich biomass of *H. pluvialis* significantly prevented antilipid peroxidation as seen in case of β-carotene linoleate model system, 1, 1-diphenyl-2-picryl hydrazyl method and hydroxyl radical scavenging model systems egg lecithin, brain, liver and kidney of rats measured in terms of thiobarbituric acid reactive species (TBARS). Hexane, chloroform, ethyl acetate, acetone and methanol extracts of *H. pluvialis* were tested against bacteria. The antibacterial activity was determined by agar-well diffusion assay and minimum inhibitory concentration. Chloroform extract of *H. pluvialis* recorded highest inhibition against *B. subtilus* (17 mm and MIC at 150 ppm). It was concluded that *H. pluvialis* extracts can be used as a bacteriostatic agents for suitable applications. In this study we have estimated the antioxidant and hepatoprotective activity of the astaxanthin & its esters of the algae against carbon tetrachloride induced radical toxicity as measured by various biochemical marker enzymes and witnessed by histopathological studies of rat liver. Among the groups, the astaxanthin diester group at 250 µg/kg showed increased antioxidant activities such as catalase 42.44%., peroxidase 49.40% and SOD 58.06%, when compared to control (CCl_4 treated group) and synthetic astaxanthin fed groups. The lipid peroxidation was restored by 5.2 folds in case diester of astaxanthin (250 µg/kg) treated group.

Haemtococcus pluvialis cells were further evaluated for nutritional quality using albino rats. Treatment of *Haematococcus pluvialis* biomass at the 2.5 g/kg and 5 g/kg b.w has not shown any death and toxic symptoms. *Haematococcus* treatment group was very active similar to that of normal diet fed group. Body weight gained 20% higher in *Haematococcus* treatment group when compared control. Treatment of *Haematococcus* biomass has not shown any adverse effect on the hematological profile of the experimental animals. Bioavailability of astaxanthin from *Haematococcus pluvialis* was studied using single dose and repeated dose administration to rats. Time course analyses of astaxanthin levels and antioxidant enzymes catalase, superoxide dismutase, peroxidase in plasma and liver of rats over 0-9 h after feeding of *H. pluvialis* biomass was done. The effect on hematological parameters and organ weight was not markedly different compared to normal diet fed animals. Single and repeated dose feeding of carotenoids to rats through

administration of *Haematococcus pluvialis* biomass showed peak levels of carotenoids in plasma 128.49 pmol/ml, liver 131.23 pmol/g, and eyes 281.45 pmol/g respectively. Astaxanthin from *H. pluvialis* showed better bioavailability. The antioxidant enzymes such as catalase (134.06%), peroxidase 104.76% were significantly high in plasma at 2h when compared to control. Whereas in liver at 4h the antioxidant activities of peroxidase 118.81%, TBARS 0.79 nmol/mg protein were evidently high offering protection from free radicals.

CHAPTER-IV

ANTICANCER PROPERTIES OF ASTAXANTHIN & ITS ESTERS IN *IN VITRO* AND *IN VIVO* MODELS

5.0. Background

Developing novel strategies to prevent skin cancer represents a desirable goal due to the rise in the incidence of skin cancer patients throughout the world (Greenlee et al., 2001, Gupta and Murkhtar, 2001). According to the World Cancer Report, skin cancer constitutes 30% of all newly diagnosed cancers in the world (Aziz et al., 2005). Skin carcinogenesis is a multi-step process comprising initiation, promotion and progression in which environmental toxins play a major role in tumorigenesis and their clinical appearance (Agarwal and Mukhtar, 1996).

Cancer is one of the most dreaded diseases of mankind. In spite of the advances made in basic scientific knowledge relating to cancer as well as clinical treatment of certain malignancies, the fact remains that death rate from some common forms of cancer such as lung, breast, and colon continue to rise (Sporn, 1996). Furthermore, most of the cytotoxic drugs used presently in cancer therapy are highly toxic to a wide spectrum of tissues such as gastro-intestinal tract, bone marrow, heart, lungs, kidney, and brain; latrogenic failure of these organs has been observed to be a frequent cause of death from cancer (Sporn and Suh, 2000). One of the most promising strategies for cancer control today is chemoprevention by natural substances from herbs, vegetables, fruits and spices (Gupta et al., 2004). Cancer chemoprevention either by specific natural or synthetic substances encompasses the objective of reversing, suppressing, or preventing carcinogenic progression to invasive cancer (Singh and Lippman, 1998). There is a growing interest seen in the pharmacological evaluation of various plant products to achieve chemoprevention (Gupta et al., 2004).

Skin is the largest organ in the human body and is exposed to a wide variety of environmental risk factors. UV-A (the wavelengths between 320 and 400 nm), a major high-energy component of sunlight, is capable of penetrating the derma, as opposed to UV-B (the wavelengths between 290 and 320 nm) (Bruls et al., 1984). It is suggested that exposure to UV-A causes skin damage by accelerating photosensitized oxidation involving free radicals. The superoxide anions and hydroxyls acts as reactive intermediates, whereas the singlet molecular oxygen acts as the direct oxidant. β-carotene and other carotenoids are well known to be potent anion quenchers and unique free

radical scavengers (Paiva and Russell, 1999). This pro-vitamin A carotenoid accumulates in the human skin through oral intake (Stahl et al., 1998) and there are several studies on the potential photoprotective effects of β-carotene supplementation against erythem induced by UV irradiation, in the human skin. However, the exact mechanism for astaxanthin in protecting skin tissue from UV-A light is still unclear.

Carotenoids are the most widespread group of naturally occurring pigments in nature. Astaxanthin is the major pigment in most aquatic animals. Although the conversions of β-carotene and other provitamin A carotenoids into retinal and retinoic acid are well documented in rat and humans (Goodman and Huang, 1965; Olson and Hayaishi, 1965; Goodman et al., 1966; Brubacher and Weiser, 1985; Ganguly and Sastry, 1985), little information is available on the metabolism of non-provitamin A carotenoids like astaxanthin. It is reported that although the carotenoids astaxanthin, canthaxanthin, and β-apo-89-carotenal induce xenobiotic-metabolizing enzymes in rat liver, other carotenoids like β-carotene, lycopene, and lutein do not (Astorg et al., 1994; Gradelet et al., 1996a, 1996b). The major dietary carotenoids present in human plasma are lycopene, β-carotene, β-cryptoxanthin, carotene, α, γ-carotene, phytofluence, phytoene, lutien and zeaxanthin. Carotenoid metabolites are reported to be involved in the chemoprevention of cancer (Khachik et al., 1995; King et al., 1997) and increase the gap junction communication (Hanush et al., 1995). The macular pigment has been postulated to improve acuity through the amelioration of the effects of chronic aberration (Reading and Weale, 1974) to preserve (Hammond et al., 1998) and protect (Snodderly, 1995) the central retina. The xanthophylls were isolated from macula and characterized using HPLC techniques (Bone et al., 2001). The dietary caroteoids 3`-epilutein, 3` dehydrolutein, (3R, 3`S) meso zeaxanthin, 3`-oxolutein and 3-methoxy zeaxanthin were also reported in human ocular tissues and serum (Khachik et al., 2002; Bernstein et al., 2001; Bhosale and Bernstein, 2005a, 2005b; Bhosale et al., 2007).

However, the pathways of biotransformation and biological functions of astxanthin metbaolites (oxidative breakdown products) and their isomers have not been understood. Khachick et al., (1999, 2002) for the first time reported oxidation products of lutein and zeaxanthin in human and non-primate models. Earlier reports suggest that

carotenoids with increased number of oxo- and hydroxyl functional groups show an enhanced antioxidant capacity (Hurst et al. 2004). Carotenoids could be oxidized to form cleavage products by various agents, which include oxidizing agents, azo compounds, free radical reactions, etc., (Stratton and Liebler, 1997). Conversions of β-carotene and other provitamin A carotenoids into retinal and retinoic acid are well documented in rats and humans (Ganguly and Sastry, 1985). Not much scientific information is available on the oxidative breakdown/metabolites of non-provitamin A carotenoids. Astaxanthin is highly reactive with free radicals and other reactive oxygen species. It is hypothesized that oxidized astaxanthin products may be involved in protecting from the ultraviolet rays and may act as better antioxidants than that parent compound itself. Epidemiological studies suggest that complete characterization of carotenoids and their metabolites in retina may help in understanding their functional properties (Khachik et al., 1999). In view this, it is vital to assess the formation of astaxanthin oxidation products and its cleavage/fragmentation pattern before understanding their biological function. Although considerable attention has been directed towards elucidating the functions of astaxanthin in plasma and liver, there is no detailed study on the identification and characterization of their full spectrum in human tissues. In the present investigation, the effect of astaxanthin & its esters on UV-DMBA induced skin carcinogenesis rats in *in vitro* and *in vivo* models were evaluated and also possible degradation/oxidation of astaxanthin & its esters metabolites were identified using HPLC and LC-MS. Further inhibitory effect of astaxanthin & its esters on human glioma cell lines (LN-229, HNGC2) and liver hepatocellular carcinoma cell line (HepG2) were evaluated using cell culture models.

5.1. Materials and Methods

5.1.1. *H. pluvialis*

The *H. pluvialis* culture conditions is given in details in Chapter-I methodology.

5.1.2. Effect of astaxanthin & its esters on skin tyrosinase activity

All the animal experiments were performed after due clearance from the Institutional Animal Ethics Committee (IAEC No. 116/08). Male Wistar rats [Out B - Wistar, IND-Cft (2C)] weighing 220-250 ± 5 g were housed individually in steel cages at room

temperature (28 ± 2 ^{o}C). A 12 h light dark/cycle was maintained and the rats received daily fresh pellet diet (Amrut feeds, Sangli, India) and had free access to tap water. Animals were exposed to UV radiation for 30 min/day using West Berlin, Universal-UV-Lampe, 254-366 nm, 200V ~ 50Hz for 8 weeks. Rats were shaved prior exposure of skin to UV. During the period of exposure the rats were group-housed in a stainless steel irradiation chamber, and the animals could move around freely in the chamber. Non-irradiated groups of animals were included as controls. The animals were sacrificed after ensuring skin lesions, which was observed after 56 days of UV treatment. Skin homogenates was prepared and examined for tyrosinase activity.

The tyrosinase activity, using L-Dopa as substrate, was measured according to the method of Kubo and Kinst-Hori (1998) with slight modifications. 0.1 ml of (1 mg/ml) L-Dopa solution was mixed with 0.7 ml of 0.1 M phosphate buffer (pH-6.0) and was incubated with 0.2 ml of skin homogenate (skin cancer induced rat) at 37 ^{o}C for 15 min. Dopachrome formation by measured fluorimeterically (Ex: 360 nm and Em: 720 nm). The increased tyrosinase activity was determined by increase in the absorbance at Ex: 360 nm and Em: 720 nm. The protective ability of astaxanthin and its esters on the tyrosinase activity was determined and expressed in mg protein per min.

5.1.3. Effect of UV on human buccal cells

Human buccal cells suspensions were prepared after washing with phosphate buffer saline (as described from our laboratory eariler). An aliquot 50 µl of buccal cells suspension were subjected to UV rays (360 nm) at 37 ^{o}C for 15-20 min. The protective ability of astaxanthin and its esters in the presence and absence of oxidants (10 µl) was observed under microscope (Olympus).

5.1.4. Red Blood Cells protection assay

Cellular damage/protection was observed under the micoscope by acridine orange ethidium bromide staining. Cellular changes are observed and recorded (Qin Yan Zhu et al., 2002). RBCs were obtained from healthy donors after taking their consent. Heparinized blood was centrifuged at 3000 x g for 10 min. After removal of plasma and buffy coat, RBCs were washed three times with phosphate buffer (pH-7.4) at room temperature and resuspended in PBS four times its volume for subsequent analysis. 100

µl of RBC were incubated with 50 µl of carotenoids fractions in presence of oxidants and the total volume was made upto 400 µl with PBS. This was incubated at 37 ^{o}C for 20 min and centrifuged. Hemoglobin released from cells in the supernatant, due to hemolysis was examined under the phase contrast microscope. Comparative effect of selected sources in protecting RBCs against oxidative damage was calculated.

5.1.5. DNA protection assay

The DNA-protective effect of carotenoid fractions was determined electrophoretically (Submarine electrophoresis system, Bangalore Genei, Bangalore, India) using calf thymus DNA (Rodriguez et al., 1998). Calf thymus DNA (1 mg in 15 ml) was subjected to oxidation by Fenton's reagent (30 mM H_2O_2, 50mM ascorbic acid and 80 mM $FeCl_3$). Relative difference in the migration between the native and oxidized DNA was ensured on 1% agarose gel electrophoresis after staining with ethidium bromide. Gels were documented (Herolab, Germany) and the intensity of the bands was determined (Easywin software). Protection to DNA was calculated based on the DNA band corresponding to that of native in the presence and absence of carotenoid fractions.

The DNA-protective effect of carotenoid fractions was determined spectro fluoremeterically using calf thymus DNA. Calf thymus DNA (1 mg in 15ml) was subjected to oxidation by Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid and 80 mM $FeCl_3$). Relative difference between the native and oxidized DNA was ensured based on the absorbance. Protection of DNA was calculated based on the relative absorbance of DNA in the presence and absence of carotenoid fractions.

5.2. UV-DMBA induced skin carcinogenesis in *in vivo* model

5.2.1. Experimental design

Healthy Albino Wistar rats (220 ± 5 g) used for the experiments were maintained under standard conditions of temperature, humidity and light and were provided with standard rodent pellet diet (M/s. Sai Durga feeds, Bangalore, India) and tap water ad libido. The study was approved by the Institutional Animal Ethical Committee (IAEC No.116/08), which follows the guidelines of CPCSEA (Committee for the purpose of Control and Supervision of Experiments on Animals, Reg. No. 49, 1999), Government of India, New Delhi, India.

All animals were divided into 13 groups (n=6 for each group), their body weights were recorded and their dorsal region were shaved prior to the start of the experiments. Total carotenoid and astaxanthin esters dissolved in groundnut oil were intubated in group by feeding 3, 4, 7, 10, 13 at 100 µg of astaxanthin & esters /kg b.w. and 6, 9, 12 at 200 µg astaxanthin & esters /kg b.w. respectively. For comparative analysis standard astaxanthin at a dose of 200 µg of astaxanthin /kg b.w. as positive control treated and only vehicle (groundnut oil) in control groups were kept. Group 2 ingested with groundnut oil and subjected to UV- DMBA treatment was considered as cancer group. Groups 5, 8, 11 and 14 served as sample controls for astaxanthin & esters at 100 and 200 µg/kg b.w. respectively. Healthy controls were also treated with groundnut oil. The samples/standard were intubated prior to the treatment for 14 days. From the 15^{th} day onwards UV-DMBA {UV radiation (West Berlin, Universal-UV-Lampe, 254-366 nm, 200V ~ 50Hz)-30 min/day and DMBA (100 µg/rat)} was given along with the intubation upto the development of skin lesions on the skin. A non-irradiated group of animals was included as control.

Tumors on the skin were recorded every week during the experimental period. Those having diameter >2 mm was considered as positive. After 45 days significant skin lesions were observed and animals were sacrificed. The rats were exsanguinated under mild ether anesthesia and blood was collected from heart into heparinized tubes and serum was separated immediately by centrifuging at 1000 x g for 15 min at 4 ^{o}C. Liver and skin were excised and washed with ice cold saline and immediately stored at -70 ^{o}C for further analysis. Serum, liver and skin were given for hematological and histopathological study. A 10% tissue homogenate (w/v) was prepared from part of the sample (liver/skin) in 0.15 M Tris-HCl (PH=7.4) and the homogenate was then centrifuged at 12,000 x g for 15 min. The supernatant thus obtained was taken for estimation of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), malondialdehyde (MDA), Serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), serum alkaline phosphatase (SALP), tyrosinase enzyme activity and protein content.

5.2.2. Tumor index

Tumors were determined in control, treated samples and UV-DMBA induced rats according to the method described by Corne et al., (1974).

5.2.3. Antioxidant enzymes SOD, catalase and GSH

For antioxidant enzymes SOD and catalase procedures are given in details in chapter-III. The level of GSH was estimated by the method of Miron *et al.*, (1979). The proteins were precipitated in the 12, 000 x *g* supernatant by addition of trichloroacetic acid (TCA) to a final concentration of 5% TCA. This was then centrifuged at 15 000 x *g* for 15 min to obtain the protein free supernatant. To 100 ml of this supernatant, 2 ml of 0.6 M 5, 5-dithio-bis (2-nitrobenzoic acid) dissolved in 0.2 M phosphate buffer (pH = 8) was added. The absorbance was recorded at 412 nm. Reduced GSH was used as standard. The levels of GSH are expressed as μg/mg of protein.

5.2.4. Lipid peroxidation activity

Lipid peroxidation activity procedure is given in details in Chapter-III methodology.

5.2.5. Protein content

Protein estimation procedure is given in details in Chapter-III methodology.

5.2.6. Extraction and analysis of astaxanthin and retinol in serum and liver of rats

Astaxanthin and retinol from serum and liver of UV-DMBA induced rats were extracted and analyzed by HPLC (Baskaran et al., 2003). In brief, to the serum (0.8 ml), 3 ml of dichloromethane: methanol (1:2, v/v) containing α-tocopherol (2 mM) was added. After mixing 1.5 ml of hexane was added, mixed well and centrifuged at 1000 x g for 15 min, and the resulting upper hexane/dichloromethane phase was collected. This extraction was repeated for the lower phase 2 more times using 1 ml of dichloromethane and 1.5 ml of hexane. The hexane extracts pooled and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 100 μl of mobile phase, and used for HPLC analysis for astaxanthin and retinol. Liver samples (1 g) were homogenized separately with 9 parts ice-cold isotonic saline with a Potter-Elvehjem homogenizer. Astaxanthin was extracted from 0.8 ml of the homogenate and analyzed by HPLC with the same procedure used for serum. Samples were handled on ice under dim yellow light to minimize isomerization and oxidation of carotenoids.

Astaxanthin and retinol levels in serum and liver were analyzed using HPLC (Shimadzu 10AS, Kyoto, Japan) reverse phase 25 cm × 4.6 mm, 5 μm, C_{18} column (Wakosil 11 5C 18RS) with an isocratic solvent system consisting of dichloromethane: acetonitrile: methanol (20:70:10, v/v/v) at a flow rate of 1.0 ml/min (Ranga Rao et al., 2010). Astaxanthin and retinol were monitored at 476 nm and 325 nm with UV- visible detector (Shimadzu, Kyoto, Japan). The peak identification and λ_{max} values of these components were confirmed by their retention times and characteristic spectra of standard chromatograms recorded with a Shimadzu model LC-10AVP series equipped with (SPD–10AVP) photodiode array detector. They were quantified from their peak areas in relation to the respective reference standards.

5.2.7. Hematological studies

EDTA anti-coagulated blood samples were used to obtain a complete blood count with a Hemavet Mascot Multispecies Hematology System Counter 1500R (Ravi Diagnostic Laboratory, Mysore, Karnataka, India). Lyphocytes (LYM), Mean cell hemoglobin count (MCHC), Platelet count (PLT), Mean cell volume (MCV), Packed cell volume (PCV), Red Blood Cells (RBC), Mean cell hemoglobin (MCH) and Hemoglobin (HGB) parameters were estimated in control and UV-DMBA treated groups.

5.2.8. Histopathological studies

Skin samples were fixed in 10% buffered formalin for 24 h. The processed tissues were embedded in paraffin blocks and sections made were stained with hematoxylin and eosin dye (Sibilia et al., 2003). The sections were analysed by observing under light microscope (Leitz, Germany) at 10 X magnification.

5.2.9. Toxicological studies

Activities of the enzymes SGOT, SGPT and SALP activity procedure is given in Chapter-III methodology.

5.3. Oxidative products and metabolites of astaxanthin in *in vivo* model

5.3.1. Animals

Animals details are given in 5.2.1. experimental design in Chapter-IV methodology.

5.3.2. Experimental design

All animals were divided into six groups (n=6 for each group), their body weights were recorded and their backs were shaved prior to the start of the experiments. Astaxanthin and its esters intubated to groups 4 and 5 at 200 µg/kg b.w. respectively. Vehicle (ground nut oil) control groups were maintained as group 1. Group 2 ingested with ground nut oil and subjected to UV- DMBA treatment was considered as cancer group. Groups 3 and 6 served as sample controls for astaxanthin and its esters at 200 µg/kg b.w. respectively. Healthy controls were also treated with groundnut oil. The samples/standard were intubated prior to the treatment for 14 days. From the 15^{th} day onwards UV-DMBA {UV radiation (West Berlin, Universal-UV-Lampe, 254-366 nm, 200V ~ 50Hz)-30 min/day and DMBA (100 µg/rat)} was given along with the intubation upto the development of skin lesions on the skin. A non-irradiated group of animals was included as control. Tumors on the skin were recorded every week during the experimental period. Those having diameter >2 mm was considered as positive. After 45 days, significant skin lesions were observed and animals were sacrificed. The rats were exsanguinated under mild ether anesthesia and blood was collected from heart into heparinized tubes and serum was separated immediately by centrifuging at 1000 x g for 15 min at 4 ^{o}C. Liver was excised and washed with ice cold saline and immediately stored at -70 ^{o}C for further analysis.

5.3.3. Isolation and extraction of astaxanthin from biological samples

Isolation and extraction of astaxanthin from serum and liver were given in Chapter-III methodology.

5.3.4. Analysis of astaxanthin metabolites by HPLC and LC-MS (APCI)

Astaxanthin and its metabolites were analyzed using HPLC (Shimadzu 10AS, Kyoto, Japan) and LC-MS, the procedure is given in chapter-III materials and methods.

5.4. Cytoprotective activity of astaxanthin & its esters on human glioma cell lines and liver hepatocellular carcinoma cell lines

5.4.1. Cell line and growth medium

Human glioma cell lines HNGC2 and LN-229 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1.5 gmL^{-1} sodium bicarbonate, 4 mm

glutamine and 5–10% fetal bovine serum (Gibco, USA). The human hepatoma cell line HepG2 was cultured in a minimal essential medium (MEM) containing 10% FBS. At confluence, HepG2 cells were sub cultured by trypsinization with 0.25% sterile trypsin at 1:5 split ratios approximately every 5-6 days. The cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 ^oC in an incubator.

5.4.2. Isolation of HNGC2 cells from human tumor tissue

The tumor tissue was washed in Dulbecco's modified Eagle's medium (DMEM) with 10x concentration of penicillin (200 U/ml) and streptomycin (100 U/ml). After three washes of 10 minutes each with 5 x and 1 x concentrations of penicillin and streptomycin, the tissue was finely dissected into 2- to 4-mm tissue fragments. Explant and adherent cultures were set up with these tissue fragments. For explant cultures, 10 to 12 explants were seeded randomly in eight 100-mm culture dishes (Falcon, San Jose, CA) and the plates were left to dry in the laminar hood for 10 to 15 minutes for the explants to adhere to plates. The explants were then incubated with 100 ml of fetal calf serum (FCS; Gibco BRL, Carlsbad, CA) at 37 ^oC in a 5% CO_2 atmosphere for 5 to 6 hours to promote better attachment of the explant to the dish. Later, the explants were fed with DMEM with 10% FCS and incubated at 37 ^oC in 5% CO_2 atmosphere and observed daily for outgrowth of cells. On the second day, cells started emerging from two to three explants from each dish. Growth from most of the explants dwindled in 4 to 5 days, but from one of the explants in the plate, cells grew vigorously and occupied the entire plate by the tenth day. The cells from this explant were detached using trypsin phosphate versine glucose (TPVG) and transferred to new culture dishes (Falcon). The cells from this primary culture could be propagated further and passaged continuously up to 25 to 30 generations without any change in cell morphology, phenotype, and growth characteristics, and were designated as HNGC-1. Around passage 28, a clone emerged from these HNGC-1 cells that was morphologically distinct, appeared refractile, displayed piling-up behavior, and was rapidly proliferating in culture. The clone was isolated from the HNGC-1 cells using cloning rings, expanded, and later cloned by limiting dilution. One clone was selected for study and expanded as a new cell line HNGC-2. Because HNGC-2 developed from HNGC-1 by spontaneous transformation of

only a small subpopulation of cells, the HNGC-1 could still be propagated independently as a cell line. Both cell lines HNGC-1 and HNGC-2 were cryo preserved at regular intervals and constituted a repertoire for further study during their continuous propagation. The characterization of cell lines was done between passages 12 and 20 for HNGC-1 and passages between 120 and 130 for HNGC-2 when otherwise stated.

5.4.3 Cell preparation

For cytotoxicity testing, the cells were utilized when they reached 60–80% confluent. The cells were diluted as needed and seeded as for LN-229, HNGC2 and HepG2 cells in 100 mL of media per well, sequentially plated in flat bottom 96-well plates (Becton Dickinson Labwane, USA). This number of cells was selected to avoid potential over confluence of the cells by the end of the four-day experiment while still providing enough cells for adequate formazan production. After plating, the 96-well plates were then incubated for 24 h to allow adherence of the cells prior to the administration of various samples for testing.

5.4.4. Addition of astaxanthin & its esters

The cells were incubated for 24 h at 37 ^{o}C and the details are mentioned above. The culture medium was replaced with 200 ml of solution containing fresh medium plus isolated microalgae metabolites, so that the final concentration variations of isolated microalgae metabolites were realized. To evaluate possible effect of blank on cell viability, cells were also incubated with blank, isolated bioactive molecules from microalgal culture. Control wells containing cells received only 200 ml of medium. After addition of all the test samples, the plates were returned to the CO_2 incubator. The study was conducted further up to a period of 72 h to allow both time-dependent and concentration-dependent drug-induced cytotoxicity. Furthermore, cells could be maintained in wells for this period without the need for refeeding. The antiproliferative effect of astaxanthin & its esters was analyzed by use of the MTT assay (Fan et al, 2003). The percentage cell viability was then determined. All experiments were performed in triplicate.

5.4.5 MTT assay

This assay was based on the measurement of the mitochondrial activity of viable cells by the reduction of the tetrazolium salt MTT (3-(4, 5-dimethyathiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) to form a blue water-insoluble product, formazan. After addition of various concentrations of isolated bioactive molecules from microalgal culture, cells were again incubated. After 24 h of incubation, MTT (5 mg/ml, 20 ml) was added to respective set of cells and the plates were incubated for an additional 4 h. After 4 h of incubation, the medium was removed and DMSO (200 ml, Sigma-Aldrich, USA) was added to dissolve the formazan crystals resulting from the reduction of the tetrazolium salt only by metabolically active cells. The absorbance of dissolved formazan was measured at 570 nm using a Bio-Rad microplate reader (Model 680). Since the absorbance directly correlated with the number of viable cells, the percent viability was calculated from the absorbance.

5.4.6. Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy was used to detect the apoptotic activity and uptake of bioactive molecules on cell lines. Before addition of various formulations, HNGC2, LN-229 and HepG2 cells were seeded at low density in 24 well plates (Becton Dickinson Labware, USA) on cover slips (ERIE scientific company, USA) and grown for 24 h to achieve semiconfluent cultures. When the cells were attached to the surface of the cover slips as a monolayer, they were incubated with different concentrations of microalgal metabolites and blank. The cells were further incubated for 24 h at 37 ^{o}C and 5% CO_2 in a humidified environment. After incubation, cells on the cover slip were washed with ice-cold phosphate buffered saline (PBS, Himedia, Mumbai, India) and fixed in 4% paraformaldehyde (Sigma-Aldrich, USA) for 10 min at room temperature. After two more rinses in PBS, cells were blocked in 5% BSA (ICN biomedicals, Germany) in PBS for 30 min at room temperature. Cells were washed three times in PBS in the dark and then incubated with DAPI (Molecular probes, USA) for 10 min. Cells were mounted with mounting medium containing DABCO (Sigma-Aldrich, USA). Confocal images were acquired using Zeiss LSM 510 confocal microscope (Germany).

5.4.7. Flow cytometric quantification of apoptosis of human glioma cell lines (HNGC2 and LN-229) and liver hepatocellular carcinoma cell line (HepG2)

Annexin-V-fluorescein isothiocyanate (FITC) (Pharmingen, Becton Dickinson, Franklin Lake, NJ) was known to have high affinity to phosphatidylserine, which was used to stain cells and to detect early apoptotic changes. Measurement of annexin-V binding was performed simultaneously with a dye exclusion test using propidium iodide (PI) (Sigma Chemical Co.) to discriminate between apoptosis and necrosis. Briefly, cells treated with extracts and untreated ones were trypsinized from six well dishes and then stained with annexin-V and PI. After the addition of 5µl of annexin-V-FITC and 10 µl of PI to the mixture, followed by incubation for 15 min in the dark, cells were analyzed on a flow cytometer (FACS, Beckton Dickinson, CA). Only fluoresceine-positive cells without PI staining were regarded as apoptotic cells.

5.4.8. DNA content assay by flow cytometry

Cultured cells with and without exposure to algae extracts were analyzed by flow cytometry at 24 h after the beginning of the treatment. Floating and trypsinized adherent cells were collected and adjusted to 1 X 10^6 cells, washed with PBS, and mixed in cold 70% ethanol for 4 h at 37 °C. Then, the cells were washed with PBS again, incubated in phosphate citrate buffer for 30 min, and treated with RNase A for 30 min at room temperature. Finally, the cells were stained with PI. After 1 h of incubation, cells were analyzed on a flow cytometer (FACS Calibur).

5.4.9. Statistical analysis

All experiments were done in triplicates and the data presented are the averages of mean of three independent experiments with standard deviation. The data were analyzed by one-way analysis of variance (ANOVA) using Microsoft Excel XP (Microsoft Corp., Redmond, WA), and the post-hoc mean separations were performed by Duncan's multiple-range test(DMRT) at $p < 0.05$ (25).

5.5. Results

5.5.1. Effect of SASX, FSAX, TC, MASX and DASX extracts on the skin tyrosinase inhibition

It was found that DASX and SASX had shown potent inhibitory effects on Dopa oxidase activity of mushroom tyrosinase and the inhibitory activities increased with increase of extract concentrations. The results showed that DASX and SASX had the highest tyrosinase inhibitory activity among the extracts, followed by FASX, MASX and TC extracts. On the basis of the half-inhibition concentration (IC_{50}) for the extracts, the DASX had the highest tyrosinase inhibitory ability as shown by the lowest value of IC_{50}, while TC extract showed the least ability (Fig. 110 & 111). The DASX is approximately 0.68, 2.35, 1.50 and 2.99 fold more effective than SASX, MASX, FSAX and TC respectively. From a comparison of the IC_{50} values, the tyrosinase inhibitory activity of DASX was found to be significantly more pronounced than other extracts. To obtain 50% tyrosinase inhibitory activity, the concentrations needed for SASX, FSAX, DASX, MASX and TC (µg/ml) were 3.10, 5.0, 3.20, 2.12 and 6.34 respectively. These results indicate that esterified astaxanthin was evident to have potent tyrosinase inhibitory activities.

5.5.2. Effect of SASX, FASX, TC, MASX and DASX extracts on human buccal cells

Protective ability of buccal cells by the carotenoids extracts further substantitates the cytoprotective ability. Fig. 112A indicates again the disruption and lysis of buccal cells upon treatment with oxidants as opposed to the control cells. Standard astaxanthin (75%), astaxanthin and its esters (80%), total extract (70%) protected these cells against the oxidant induced damage.

5.5.3. Effect of SASX, FSAX, TC, MASX and DASX extracts on Red blood cells (RBCs)

Red blood cells are very fragile. They are the first ones to get expose to the oxy radical *in vivo*, if the oxidants enters into the circulation. In the current study therefore, RBCs have been examined for their structural changes upon treatment with oxidants, possible protection exhibited by the total carotenoid extract, astaxanthin and its esters from *H. pluvialis*. *In vivo*, they are subjected to oxidation in presence of oxidative stress created

due to the generation of free radicals, leading to cellular damage and hence diseases. In the current study we examined the possible protection (70%) offered by esterified forms of astaxanthin from *H. pluvialis.* Oxidant induced damage of RBC's showed serration in the margin, which functionally cannot carry oxygen to the tissues and organs. As indicated in (Fig. 112B) control cells are round and red since it has hemoglobin intact. However upon oxidation, cells changed its morphology. Standard astaxanthin (70%), a standard antioxidant also showed good protection to RBC against oxidants.

5.5.4. Effect of SASX, FSAX, TC, MASX and DASX extracts on DNA damage

In cancer cell DNA is mutated due to attack by free radicals. Any agent that can prevent this oxidation of DNA, is therefore considered as anticarcinogen. In other words, we studied the DNA oxidation profile by agarose gel electrophoresis and evaluated the ability of extracts of total carotenoid extract, astaxanthin, monoester and diester in protecting the oxidant induced DNA damage. DNA fragmentation by Fenton's reagent was protected with the treatment of total carotenoid extract, astaxanthin, monoester and diester to oxidative stress. A dose-dependent protection was observed at 2-8 µg respectively (Fig. 112C). A significant (>60-70%) protection to native DNA during oxidation in the presence of these fractions was observed. These results indicate that esterified astaxanthin can quench free radicals and thereby may protect DNA against oxidative stress induced damage. The ability of astaxanthin to reduce the DNA damage depends on the type of reactive nitrogen oxygen species donor and the carotenoid concentration used.

5.6. UV-DMBA induced skin carcinogenesis in rat model

5.6.1. Hepatic studies: Body and organ weight and general observations

Body weight and relative organs weight at the termination of the experiment have been shown in Fig. 113. There is no significant difference in the weight gain profile of body weight, and the corresponding values of low and high doses were comparable to the control. The oral and topical administration of FASX, SASX, TC, MASX and DASX did not cause any apparent changes in clinical signs such as survivability, or any gross visible changes attributable to toxicity in the organs weight of rat.

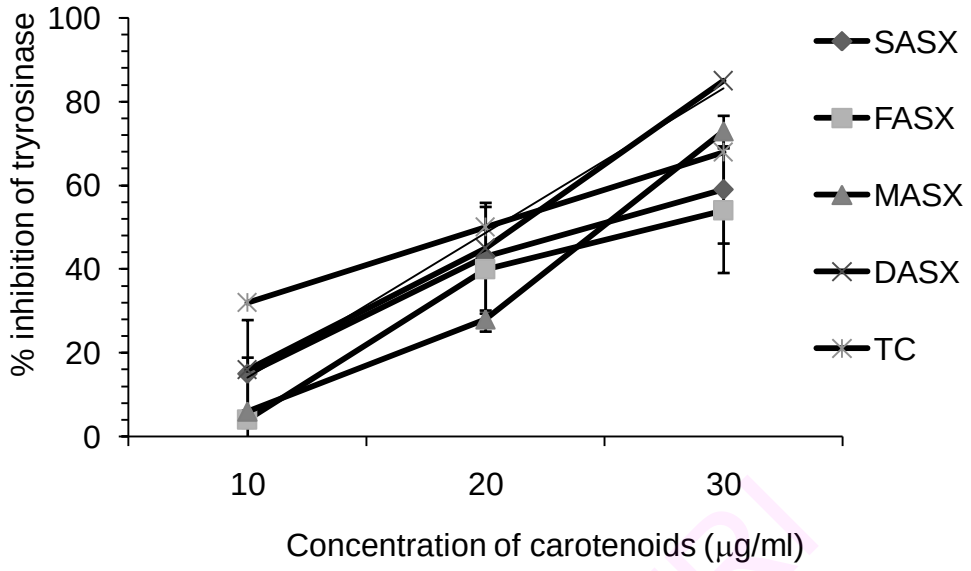


Fig. 110. Tyrosinase activity on UV induced skin carcinogenesis rats by FASX, SASX, TC, MASX and DASX from *H. pluvialis*. Values represent the mean ± SD of three analyses. FASX refers to free astaxanthin, SASX refers to Synthetic astaxanthin, TC refers to total carotenoid, MASX referes to monoester of astaxanthin, DASX refers to diester of astaxanthin.

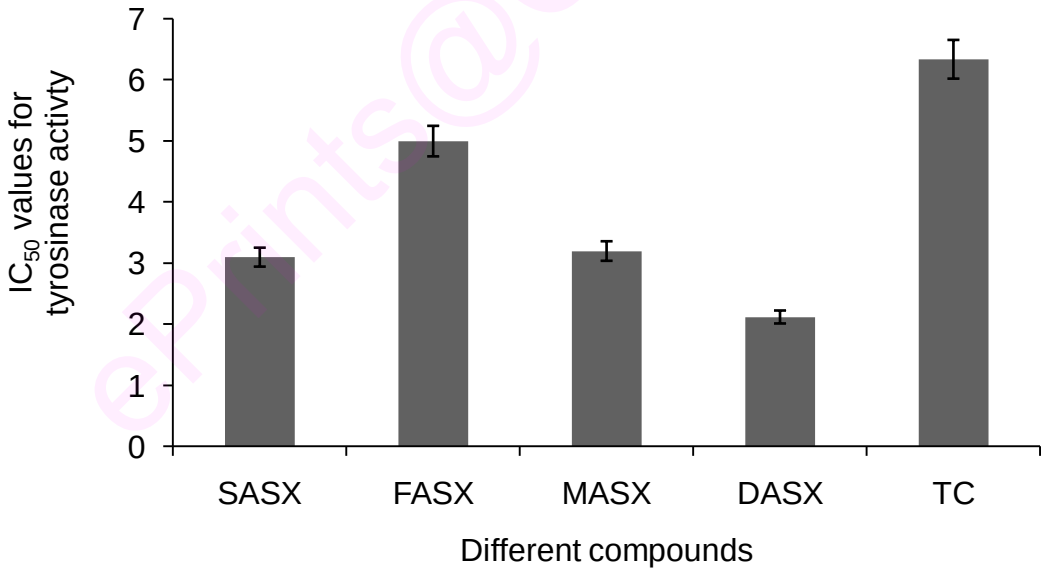


Fig. 111. IC_{50} values for tyrosinase (B) on UV induced skin carcinogenesis rats by FASX, SASX, TC, MASX and DASX from *H. pluvialis.* Values represent the mean ± SD of three analyses. FASX refers to free astaxanthin, SASX refers to synthetic astaxanthin, TC refers to total carotenoid, MASX referes to monoester of astaxanthin, DASX refers to diester of astaxanthin.

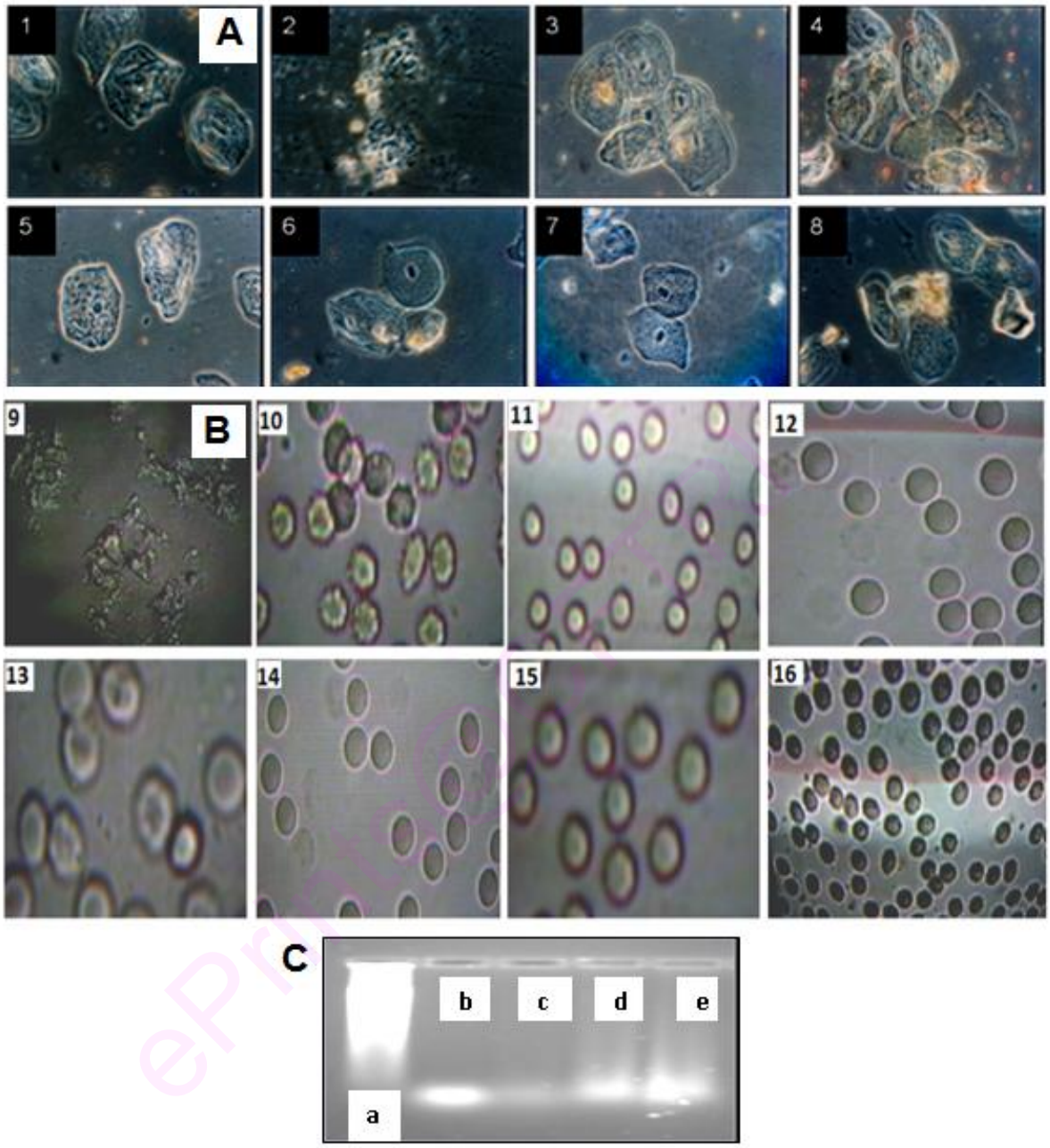


Fig. 112. Effect of SASX, FASX, TC, MASX and DASX on **(A).** Human buccal cells. 1. Control, 2. Oxidation induced cells, 3. Synthetic astxathin, 4. Free astaxanthin, 5. Monoester of astaxanthin, 6. Diester of astaxanthin, 7. Total carotenoid, 8. Synthetic β-carotene. **(B)**. Cytoprotective activity of Red blood Cells 9.Water blank, 10. Oxidative induced cells, 11. Synthetic astaxanthin, 12. Free astaxanthin, 13. Total carotenoid, 14. Monoester of astaxanthiin, 15. Diester of astaxanthin, 16. β-carotene. **(C).** DNA protection ability of astaxanthin and its esters from *H. pluvials.* One microgram of native calf thymus DNA in (lane-A), DNA treated with Fenton`s reagent (lane B), DNA pretreated with 4-8 µg of astaxanthin & it esters (lane C-E) were loaded on to the 1% agarose gel. Bands were visualized by staining with ethidium bromide and in the transilluminator increased mobility represents DNA. FASX refers to free astaxanthin, SASX refers to Synthetic astaxanthin, TC refers to total carotenoid, MASX referes to monoester of astaxanthin, DASX refers to diester of astaxanthin.

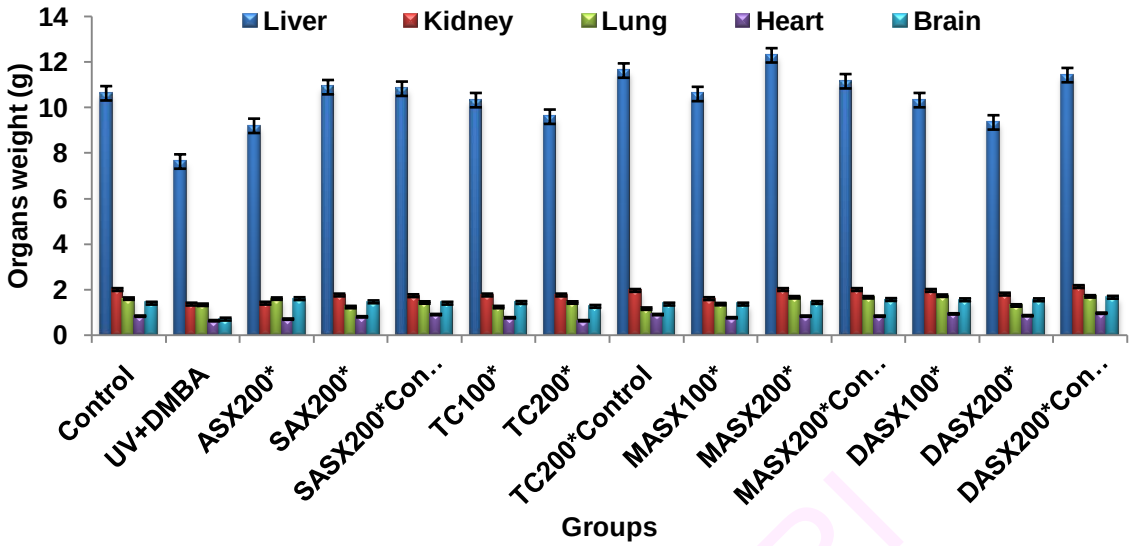


Fig. 113. Relative weights (in grams) of different organs of the experimental rats fed with astaxanthin and its esters compared with control group. FASX refers to free astaxanthin, SASX refers to synthetic astaxanthin, TC refers to total carotenoid, MASX referes to monoester of astaxanthin, DASX refers to diester of astaxanthin.

5.6.2. Effect of FASX, SASX, TC MASX and DASX on UV-DMBA induced rat skin carcinogenesis

The effect of FASX, SASX, TC, MASX and DASX on the DMBA–UV induced skin tumors in rats is shown in Fig. 114. When the data were analyzed for tumor incidence, oral administration of carotenoids significantly delayed the incidence of skin dermis, epidermis and papillomas in the carotenoid group. Compared with the non-carotenoid treated positive control group of rat, the time of appearance of the first tumor was delayed by 2 weeks in the carotenoid treated animals. When these data were assessed in the middle of the experiment at 4 weeks compared with 100% of rat with skin tumors in the non-carotenoid treated group, less than 30% of the carotenoid treated animals had tumors accounting for about 70% inhibition of the tumor incidence. Similarly, when the tumor data were evaluated for tumor multiplicity (cumulative number of tumors per group or number of tumors per rat), beginning with the first tumor appearance up to termination of the experiment, carotenoid showed a highly significant protection against DMBA-UV tumor promotion in rat at three weeks of tumor promotion. Examination of the liver in the non-carotenoid treated and carotenoid treated group showed that the former positive

control animals irradiated with DMBA-UV had enlarged liver, which was absent in the later group of rat receiving carotenoid. No toxic effect was observed in the experimental groups throughout the experiment.

5.6.3. Microscopic assessment of tumor index

FASX, SASX, TC, MASX and DASX administration DMBA and UV induced severe lesions including inflammatory patches, bleeding in mucosa and skin tumors with different size and degree in DMBA-UV induced rats as shown in Fig. 115 A&B. No such skin tumor lesions and bleeding were noticed in healthy control groups. Pre-treatment with astaxanthin esters showed 3-4 fold reduction in tumor index at 200 µg/kg b.w. as opposed to that of tumor-induced animals. Relatively only 40% protection was observed in total carotenoid treated group at similar dosage. Analogous to this, 61% mucin binding in astaxanthin ester treated group at 200 µg/kg b.w. revealed that protection against tumor may partly be via inhibiting mucosal damage that is generally caused by DMBA-UV.

5.6.4. Histopathological studies

DMBA-UV induced rats showed greater changes in the epidermis, dermis when compared to control group as shown in Fig. 116. Measurements of the epidermal thickness showed significant increases in the UV irritated compared to control group. UV irradiation increased in the epidermal thickness relative to the normal group after irradiation. Collagen fibers appeared to be lower in the UV-irradiated skin compared to the control group as observed by histochemical analysis for procollagen. The recovery of dermis and epidermis was observed in astaxanthin esters and total carotenoid treated groups when compared to DMBA-UV induced rats.

5.6.5. Hematological observations

Effect of astaxanthin and its esters on hematological studies of control and DMBA-induced groups data is presented in the Table 40. Following blood test, alterations were found in DMBA-UV treated animals. We observed that PLT, WBC and HGB were significantly increased in more advanced stages of the tumors in DMBA-UV treated animals. The PLT, WBC and HGB counts were also significantly higher in ASX, SASX, TC, MASX, DASX treated animals at 100 µg/kg b.w, whereas in treated with 200 µg/kg

b.w decreased PLT, WBC and HGB counts. The percentage of rats with hematological disturbances was significantly higher in the group of skin carcinogenesis with larger tumors than healthy controls. We also found a significant inverse relationship between PLT and HGB.

5.6.6. Astaxanthin and retinol levels in serum and liver

Vitamin A is essential for a number of physiological processes, such as regulation of cell differentiation, cell proliferation, vision and reproduction. The astaxanthin and retinol levels were measured in serum and liver of control and UV-DMBA induced skin carcinogenesis rats. The maximum astaxanthin (366 ng/ml) and retinol (72 ng/ml) were found in serum of DASX treated group, when compared to healthy control group (Fig. 117 A-B). Likewise in the liver, the astaxanthin and retinol content were found to be 22.5 ng/g and 440 ng/g respectively (Fig. 118 A-B).

5.6.7. Inhibitory effect of tyrosinase activity in serum, liver and skin

Tyrosinase is responsible for the pigment melanin biosynthesis in human skin. Melanogenesis begins with oxidation of tyrosine, which takes place within specialized organelles known as melanosomes, and is regulated by the rate-limiting enzyme tyrosinase (Cooksey et al, 1987). Skin tyrosinase has been widely used as the target enzyme for screening and characterizing potential tyrosinase inhibitors. Because the mode of inhibition depends on the structures of both the substrate and inhibitor, L-Dopa was used as the substrate in this study. Therefore, the activity studied in this investigation was concerned with O-diphenolase inhibitory activity of skin tyrosinase. Tyrosinase enzyme activity was measured in DMBA-UV treated rats (Table 41). The tyrosinase activity was increased in DMBA-UV treated rats. 73%, 74% and 78% inhibition of tyrosinase activity was observed in serum, liver and skin homogenates treated with DASX at 200 µg/kg b.w.

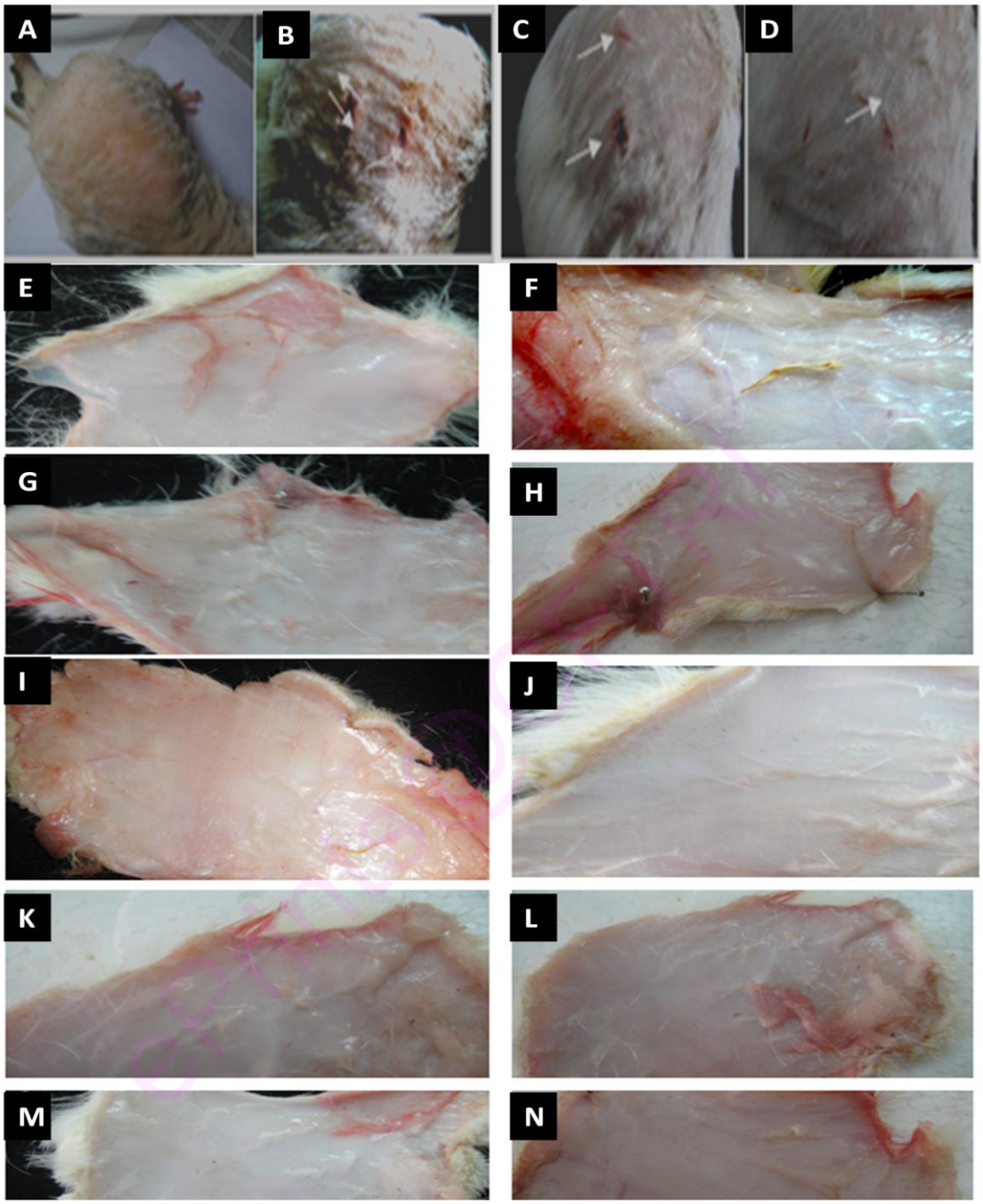


Fig. 114. Skin tumors observation (A). Healthy control, (B). UV-DMBA, (C). MASX200*, (D). DASX200*, and microscopic observations (E) Healthy control, (F). UV-DMBA, (G). ASX200*, (H). SASX200*, (I) TC100*, (J) TC200*, (K) MASX100*, (L) MASX200*, (M). DASX100*, (N). DASX200* of UV-DMBA induced skin carcinogenesis rats. *µg/kg b.w. FASX refers to free astaxanthin, SASX refers to synthetic astaxanthin, TC refers to total carotenoid, MASX referes to monoester of astaxanthin, DASX refers to diester of astaxanthin.

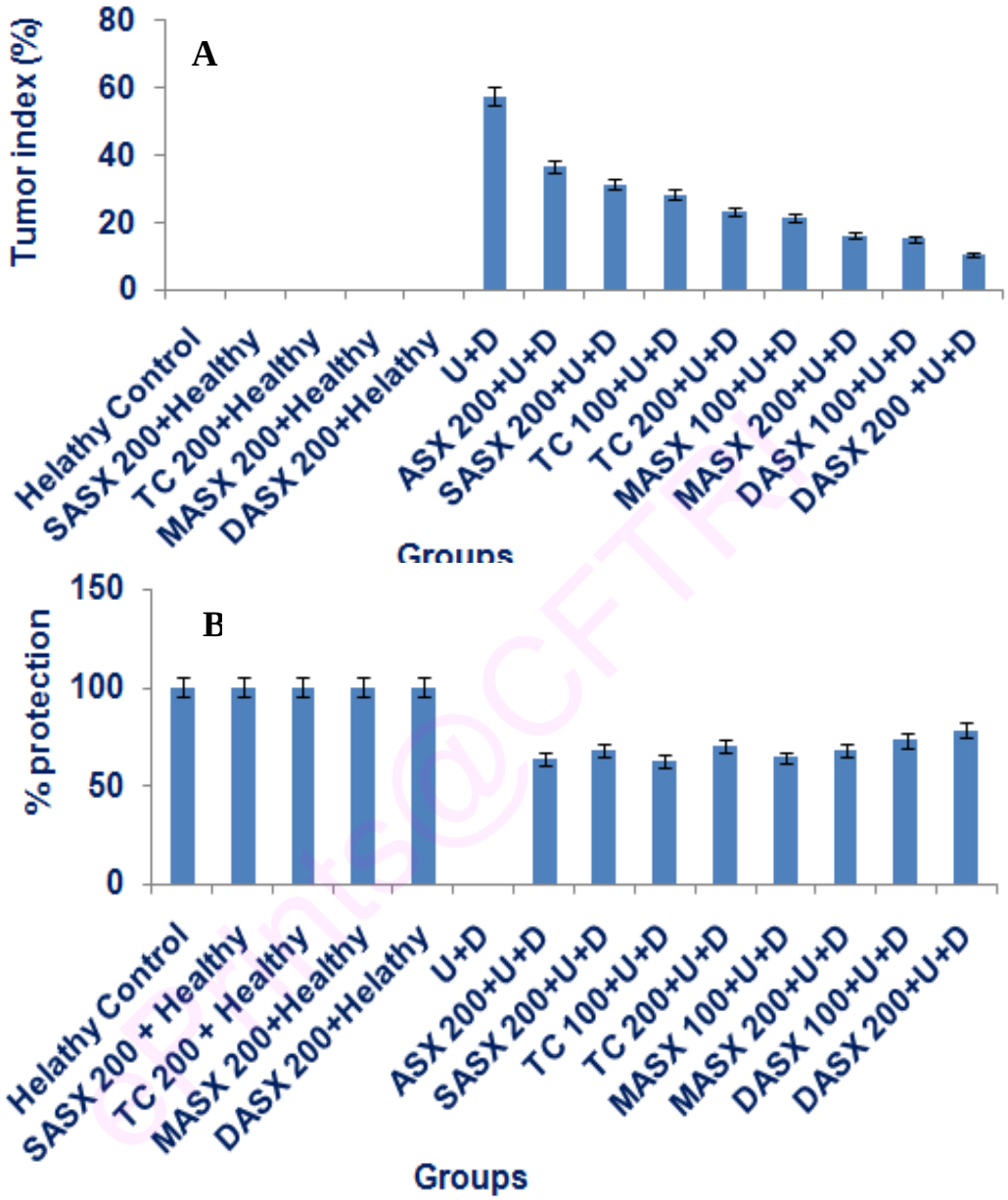


Fig. 115. Tumor protection (A) and Index (B) offered by astaxanthin esters against UV-DMBA induced skin carcinogenesis rats. Values represent the mean ± SD of 5 analyses. FASX refers to free astaxanthin, SASX refers to Synthetic astaxanthin, TC refers to total carotenoid, MASX referes to monoester of astaxanthin, DASX refers to diester of astaxanthin.

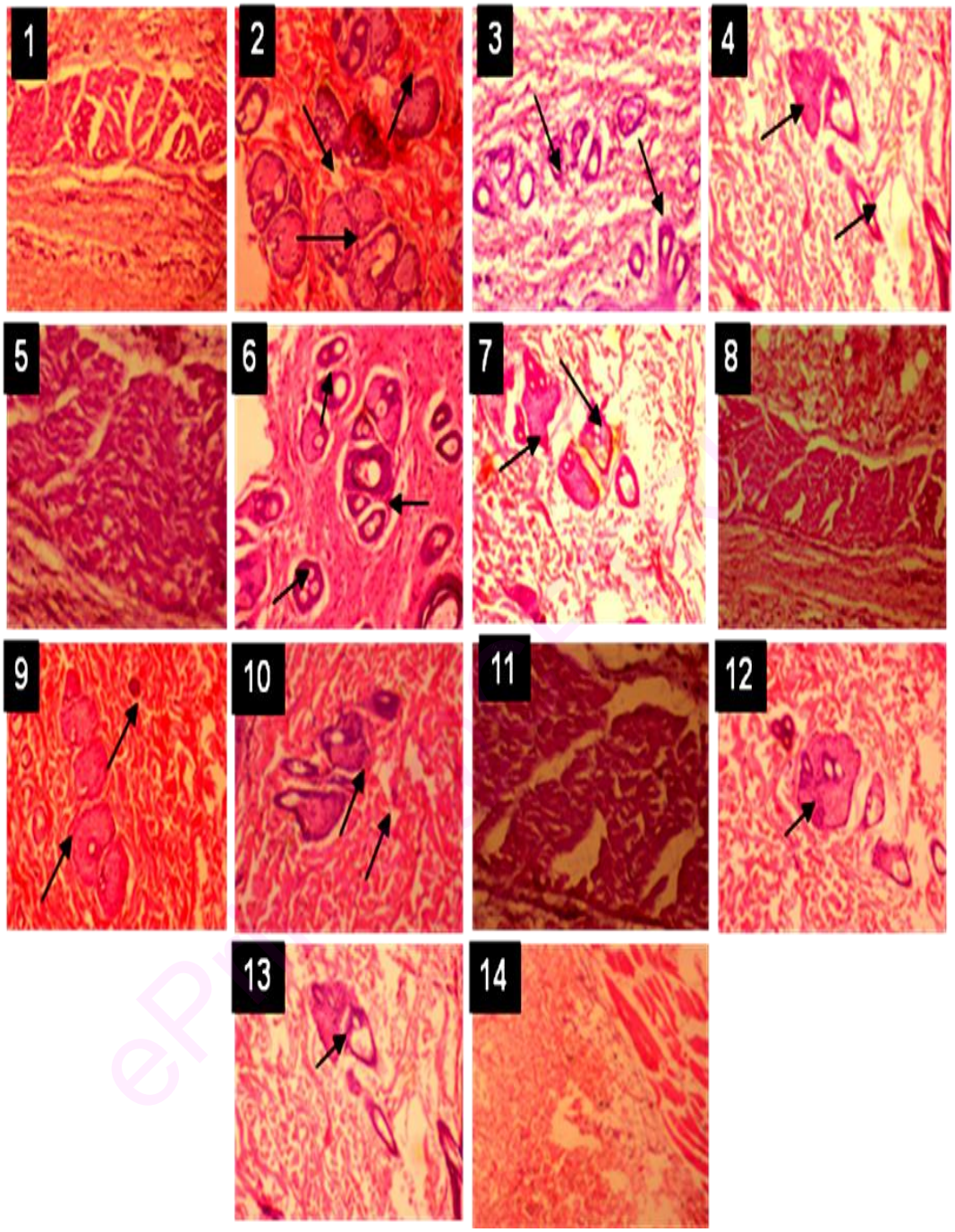


Fig. 116. Histopathological studies of UV-DMBA induced skin carcinogenesis rats. Observed dermis and epidermis in treated samples. 1. Healthy control, 2. UV+DMBA, 3.ASX200* 4. SASX200*, 5. SASX200* control, 6. TC100* 7. TC200*, 8. TC200* control, 9. MASX100*, 10. MASX200* 11. MASX200* control, 12. DASX100*, 13. DASX200* and 14. DASX200* control. FASX refers to free astaxanthin, SASX refers to Synthetic astaxanthin, TC refers to total carotenoid, MASX referes to monoester of astaxanthin, DASX refers to diester of astaxanthin.

Table 40. Effect of astaxanthin and its esters on hematological study in serum of UV-DMBA induced skin carcinogenesis rats

	Healthy control	UV+DMBA	ASX200*	SASX 200*	TC200*	MASX 200*	DASX 200*
WBC (x103/µL)	9.9 ± 0.28^{g}	16.2 ± 1.96^{e}	8.5 ± 1.84^{f}	9.9 ± 1.32^{g}	10.2 ± 2.45^{f}	10.4 ± 1.89^{f}	11.8 ± 2.13^{g}
RBC (x106/µL)	10.18 ± 0.43^{g}	9.23 ± 1.13^{g}	9.73 ± 1.92^{f}	9.52 ± 2.18^{g}	9.31 ± 1.16^{f}	9.58 ± 1.45^{g}	9.5 ± 1.21^{h}
HGB (g/dL)	14.9 ± 0.31^{f}	14.4 ± 1.85^{f}	14.8 ± 2.34^{e}	14.5 ± 2.65^{f}	14.8 ± 2.19^{e}	15.2 ± 2.37^{e}	14.3 ± 2.26^{f}
HCT (%)	55.5 ± 3.67^{c}	53.3 ± 4.51^{c}	56.6 ± 5.69^{c}	54.5 ± 4.87^{c}	55.8 ± 5.47^{c}	55.1 ± 5.81^{c}	52.3 ± 3.34^{c}
MCV (fL)	54.5 ± 4.19^{c}	57.4 ± 5.81^{c}	58.2 ± 4.81^{c}	57.2 ± 3.95^{c}	59.8 ± 3.45^{c}	57.5 ± 5.12^{c}	55.1 ± 4.06^{c}
MCH (pg)	14.6 ± 2.16^{f}	15.6 ± 2.13^{a}	15.2 ± 2.49^{a}	15.2 ± 2.36^{f}	15.9 ± 2.11^{e}	15.9 ± 1.95^{e}	15.1 ± 1.84^{f}
MCHC (g/dL)	26.8 ± 3.18^{d}	27.2 ± 1.25^{d}	26.1 ± 2.53^{d}	26.6 ± 3.14^{d}	26.5 ± 3.85^{d}	27.6 ± 2.68^{d}	27.3 ± 3.76^{d}
PLT (x103/µL)	904 ± 5.99^{a}	759 ± 6.31^{a}	993 ± 8.90^{a}	892 ± 7.63^{a}	833 ± 6.62^{a}	868 ± 7.73^{a}	792 ± 6.08^{a}
LYM (%)	76.6 ± 3.01^{b}	86.9 ± 5.09^{b}	71.8 ± 6.72^{b}	72.9 ± 5.81^{b}	81.3 ± 4.61^{b}	81.9 ± 3.39^{b}	76.1 ± 3.82^{b}
LYM# (x103/µL)	7.6 ± 1.23^{h}	13.9 ± 1.36^{f}	6.43 ± 0.98^{g}	7.2 ± 0.72^{h}	8.3 ± 1.04^{g}	9.1 ± 1.37^{g}	8.2 ± 0.69^{b}
Neutrophils	19.34 ± 1.78^{e}	10.45 ± 1.39^{g}	23.75 ± 1.85^{d}	21.48 ± 2.55^{e}	14.45 ± 2.08^{e}	11.23 ± 1.82^{a}	19.83 ± 2.31^{e}
Lymphocytes	76.11 ± 3.31^{b}	87.61 ± 4.28^{b}	72.82 ± 5.12^{b}	73.91 ± 4.68^{b}	81.68 ± 4.83^{b}	82.62 ± 5.76^{b}	76.61 ± 4.92^{b}
Eosinophils	2.12 ± 0.18^{i}	2.45 ± 0.32^{h}	2.31 ± 0.54^{h}	2.02 ± 0.32^{i}	2.31 ± 0.28^{h}	2.45 ± 0.73^{i}	2.23 ± 0.78^{j}
Monocytes	3.34 ± 0.05^{j}	1.78 ± 0.24^{i}	3.53 ± 0.31^{h}	4.15 ± 0.51^{j}	3.55 ± 0.23^{i}	5.61 ± 0.35^{h}	3.46 ± 0.63^{i}
Basophils	0	0	0	0	0	0	0

LYM-Lyphocytes, MCHC= Mean cell hemoglobin count, PLT= Platelet count, MCV=Mean cell volume, HCT or PCV= Packed cell volume, RBC= Red Blood Cells, MCH=Mean cell hemoglobin, HGB= Hemoglobin. FASX refers to free astaxanthin, SASX refers to Synthetic astaxanthin, TC refers to total carotenoid, MASX referes to monoester of astaxanthin, DASX refers to diester of astaxanthin.

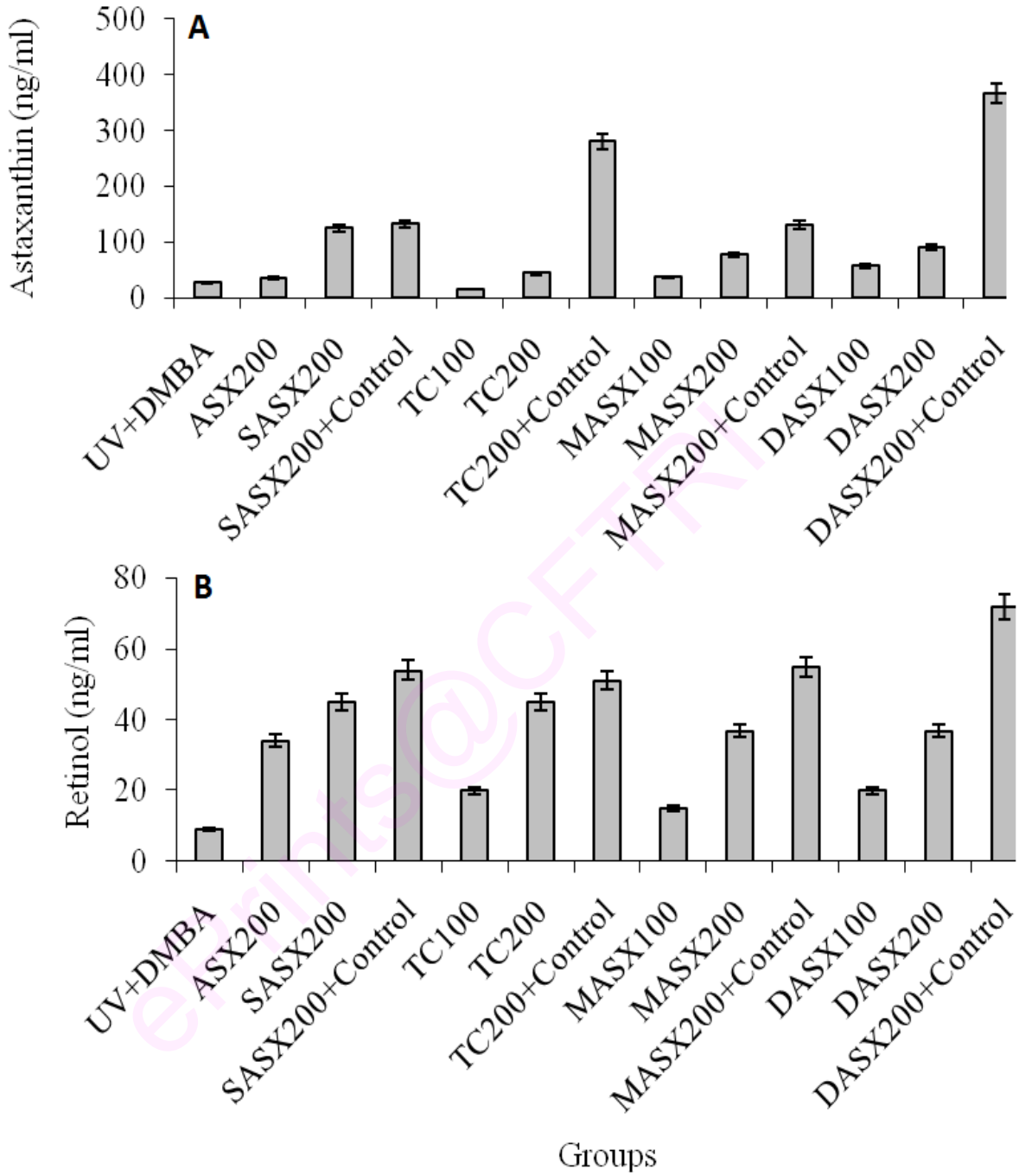


Fig. 117. Astaxanthin and retinol content in serum of UV-DMBA induced skin carcinogenesis rats. Values represent the mean ± SD of 5 analyses. Values are significant at $p < 0.05$ compared to untreated controls. FASX refers to free astaxanthin, SASX refers to Synthetic astaxanthin, TC refers to total carotenoid, MASX referes to monoester of astaxanthin, DASX refers to diester of astaxanthin.

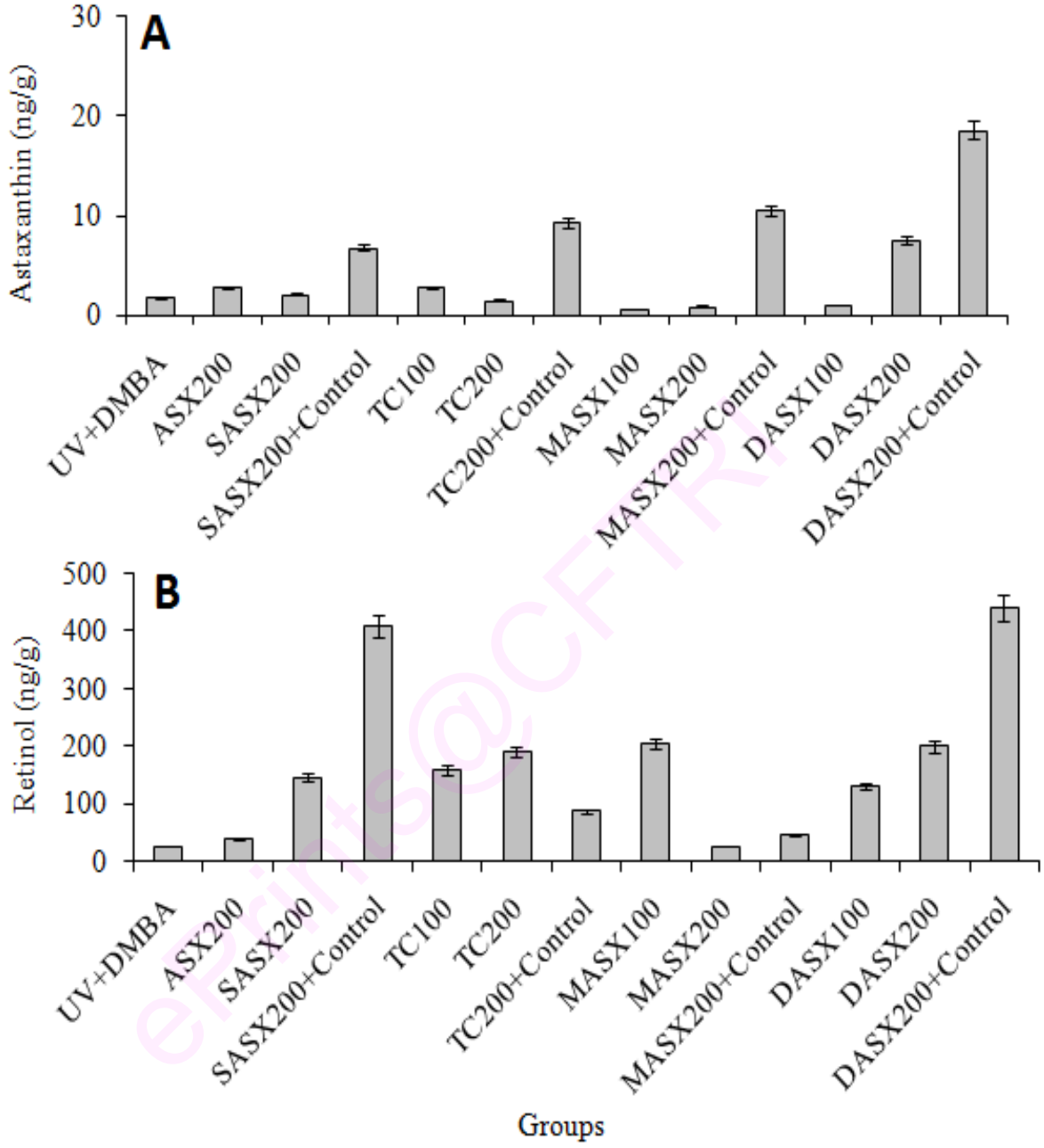


Fig. 118. Astaxanthin and retinol content in liver of UV-DMBA induced skin carcinogenesis rats. Values represent the mean ± SD of 5 analyses. Values are significant at $p < 0.05$ compared to untreated controls. FASX refers to free astaxanthin, SASX refers to synthetic astaxanthin, TC refers to total carotenoid, MASX referes to monoester of astaxanthin, DASX refers to diester of astaxanthin.

Table 41. Effect of astaxanthin & its esters on tyrosinase activity in serum, liver and skin homogenates of UV-DMBA induced skin carcinogenesis rats

	Serum (oxidation of Dopa in μmoles/mg protein)	Liver (oxidation of Dopa in μmoles/mg protein)	Skin (oxidation of Dopa in μmoles/mg protein)
Healthy control	0.19 ± 0.07^{h}	0.10 ± 0.04^{g}	1.65 ± 0.40^{i}
UV+DMBA	21.33 ± 3.07^{a}	9.90 ± 2.01^{a}	119.33 ± 9.04^{a}
ASX200*	13.21 ± 3.14^{b}	6.08 ± 0.23^{b}	72.27 ± 2.69^{c}
SASX200*	11.03 ± 1.72^{c}	5.57 ± 0.72^{b}	60.06 ± 3.39^{d}
SASX200* control	0.32 ± 0.07^{h}	0.07 ± 0.06^{g}	1.03 ± 0.14^{i}
TC100*	11.46 ± 2.14^{c}	6.07 ± 0.33^{b}	78.57 ± 9.44^{b}
TC200*	9.18 ± 0.65^{ef}	5.40 ± 0.73^{c}	52.62 ± 7.45^{e}
TC200*control	0.30 ± 0.16^{h}	0.06 ± 0.04^{g}	1.07 ± 0.85^{i}
MASX100*	10.28 ± 2.22^{cd}	5.48 ± 0.73^{c}	50.04 ± 8.31^{e}
MASX200*	8.24 ± 0.70^{f}	4.81 ± 0.91^{d}	39.27 ± 6.73^{f}
MASX200* control	0.19 ± 0.08^{h}	0.06 ± 0.05^{g}	1.25 ± 0.64^{i}
DASX100*	9.94 ± 1.84^{e}	4.32 ± 0.91^{e}	33.19 ± 0.49^{g}
DASX200*	7.60 ± 1.26^{g}	3.51 ± 0.99^{f}	26.06 ± 1.02^{h}
DASX200* control	0.24 ± 0.11^{h}	0.05 ± 0.03^{g}	1.169 ± 0.04^{i}

ASX refers to astaxanthin, SASX refers to standard astaxanthin, TC refers to total carotenoid, MASX refers to monoester of astaxanthin, DASX refers to diester of astaxanthin from *H. pluvialis*, *μg/kg/b.w. Values are expressed as mean ± SD (n=5). Values are not sharing a similar superscript within the same column are significantly different ($P<0.05$) as determined by ANOVA.

5.6.8. Changes in the antioxidant enzymes and lipid peroxidation levels in serum, liver and skin homogenates

Effect of astaxanthin and its esters on the antioxidant enzymes and lipid peroxidation levels were measured in serum, liver and skin homogenates of DMBA-UV induced rats. Table 42 indicates changes in the antioxidant enzymes and lipid peroxidation levels in serum of DMBA-UV induced rat models. SOD levels increased in serum (3 fold) and CAT (2.5 fold) and GSH decreased (1.6 fold) during DMBA-UV induced carcinogen's conditions and were normalized upon treatment with FASX, SASX, TC, MASX and DASX as in a dose dependent manner. An approximately 4 fold increase in TBARS levels depicts lipid peroxidation of DMBA-UV treated animals and was recovered up to 65% upon treatment with DASX at 200 μg/kg b.w.

Similarly, in the case of liver homogenates, the antioxidant enzymes and lipid peroxidation levels in liver homogenates of DMBA-UV induced rat models were

measured and the data is presented in Table 43. SOD levels increased in liver homogenate (2.7 fold) and CAT (2.2 fold) and GSH decreased (1.4 fold) during DMBA-UV treated rats and were normalized upon treatment with FASX, SASX, TC, MASX and DASX as in a dose dependent manner. An approximately 3.2 fold increase in TBARS levels depicts lipid peroxidation of DMBA-UV treated animals and was recovered up to 65% upon treatment with DASX at 200 µg/kg b.w.

In the case of skin homogenate, the antioxidant enzymes and lipid peroxidation levels were measured (Table 44). SOD levels increased by 2.7 fold and CAT by 2.3 fold and GSH decreased by 2 fold during DMBA-UV induced treated animals and were normalized upon treatment with FASX, SASX, TC, MASX and DASX as in a dose dependent manner. An approximately 2.6 fold increase in TBARS levels depicts lipid peroxidation of DMBA-UV treated animals and was recovered up to 60% upon treatment with DASX at 200 µg/kg b.w.

5.6.9. SGPT, SGOT and SALP levels in serum, liver and skin homogenates

SGPT, SGOT, and SALP levels were measured in serum, liver and skin homogenates of DMBA-UV induced rats, data is presented in Table 42-44**.** Toxicity studies with FASX, SASX, TC, MASX and DASX were carried out in rats for safety evaluation. There were no significant differences in total protein, TBARS levels, SGPT, SGOT and ALP between normal and DMBA-UV treated rats, indicating no adverse effect on the major organs. After the above treatment schedules, animals remained as healthy as control animals with normal food and water intake, body weight gain and behavior. The SGPT, SGOT and SALP activity were increased in serum, liver and skin homogenates of DMBA-UV treated animals. Increase of 1.8 fold SGPT, 1.7 fold SGOT and 1.8 fold SALP in serum were recorded, whereas in with DASX treated group these enzymes decreased at 1.4, 1.5 and 1.4 fold. Similarly, in the case of liver homogenate, increase in SGPT by 1.9 fold, SGOT by 1.6 fold and SALP by 2.1 fold were noticed, whereas in DASX treated group these enzymes showed decrease in 1.6, 1.4 and 1.7 fold. In skin homogenates, SGPT 1.7 fold, SGOT 2 fold and SALP 1.79 fold increase was noticed, whereas 1.59, 1.5 and 1.24 fold decreased in DASX treated group at 200µg/kg b.w.

Table 42. Effect of astaxanthin & it esters on antioxidant enzymes in serum of UV-DMBA induced skin carcinogenesis rats

	SOD (U/mg protein)	Catalase (nmol H_2O_2/ mg protein)	GSH (µg GSH / mg protein	TBARS (µmols/MDA /mg protein)	SGPT (U/mg protein)	SGOT (U/mg protein	SALP (U/mg protein)
Healthy control	11.33 ± 2.65^{m}	0.48 ± 0.009^{a}	2.82 ± 0.39^{a}	0.45 ± 0.11^{g}	108.89 ± 21.61^{m}	105.72 ± 3.28^{l}	227.56 ± 22.60^{l}
UV+DMBA	33.61 ± 3.53^{a}	0.21 ± 0.03^{e}	1.71 ± 0.24^{d}	4.56 ± 0.81^{a}	195.33 ± 16.45^{a}	184.66 ± 3.21^{a}	409.33 ± 19.10^{a}
ASX200*	23.18 ± 1.94^{c}	0.26 ± 0.02e	$2.30 \pm 0.16b^{c}$	3.11 ± 0.13^{c}	173.10 ± 11.77^{b}	153.79 ± 18.58^{d}	352.34 ± 26.67^{d}
SASX200*	19.04 ± 2.01^{f}	0.31 ± 0.03^{d}	2.28 ± 0.44^{c}	3.22 ± 0.12^{c}	166.26 ± 21.62^{c}	145.72 ± 10.42^{f}	$322.70 \pm 14{,}94^{f}$
SASX200* control	13.36 ± 3.46^{j}	0.35 ± 0.07^{c}	$2.34 \pm 0.17b^{c}$	0.46 ± 0.13^{g}	113.08 ± 16.32^{j}	111.03 ± 14.51^{j}	249.96 ± 11.91^{j}
TC100*	24.01 ± 6.58^{b}	0.23 ± 0.05^{e}	2.11 ± 0.2^{bc}	3.60 ± 0.09^{b}	168.28 ± 9.87^{c}	171.54 ± 12.19^{b}	370.94 ± 17.39^{b}
TC200*	19.89 ± 1.13^{e}	0.34 ± 0.03^{c}	2.17 ± 0.41^{bc}	3.08 ± 0.16^{c}	156.94 ± 31.64^{e}	152.69 ± 23.26^{e}	329.99 ± 18.72^{e}
TC200*control	17.18 ± 3.01^{g}	0.36 ± 0.03^{c}	2.17 ± 0.12^{bc}	0.47 ± 0.06^{g}	110.35 ± 12.56^{l}	106.16 ± 15.56^{k}	247.80 ± 15.42^{k}
MASX100*	24.01 ± 2.07^{b}	0.31 ± 0.07^{c}	2.33 ± 0.49^{bc}	2.34 ± 0.23^{d}	161.98 ± 13.55^{d}	159.03 ± 19.17^{c}	359.33 ± 12.90^{c}
MASX200*	13.81 ± 1.73^{i}	$0.41 \pm 0.08a^{b}$	2.74 ± 0.36^{a}	2.15 ± 0.21^{d}	$144.90 \pm 5.79g$	138.40 ± 29.01^{g}	307.89 ± 23.63^{g}
MASX200* control	12.77 ± 2.76^{k}	0.42 ± 0.10^{ab}	2.94 ± 1.84^{a}	0.46 ± 0.05^{g}	111.91 ± 12.55^{jk}	112.39 ± 73.18^{i}	257.51 ± 19.40^{i}
DASX100*	20.73 ± 0.58^{d}	0.29 ± 0.01^{cd}	2.52 ± 0.38^{b}	1.82 ± 0.10^{e}	148.35 ± 16.02^{f}	153.61 ± 13.05^{d}	323.69 ± 9.13^{f}
DASX200*	15.84 ± 1.81^{h}	0.36 ± 0.02^{b}	2.49 ± 0.14^{bc}	1.59 ± 0.23^{f}	138.63 ± 14.21^{h}	121.77 ± 8.17^{h}	287.16 ± 21.07^{h}
DASX200* control	11.58 ± 2.19^{l}	0.45 ± 0.10^{a}	2.73 ± 0.14^{a}	0.35 ± 0.10^{g}	114.61 ± 16.05^{i}	107.48 ± 13.50^{k}	226.26 ± 11.18^{l}

ASX refers to astaxanthin, SASX refers to standard astaxanthin, TC refers to total carotenoid, MASX refers to monoester of astaxanthin, DASX refers to diester of astaxanthin from *H. pluvialis*, *µg/kg/b.w. Values are expressed as mean ± SD. Values are not sharing a similar superscript within the same column are significantly different ($P<0.05$) as determined by ANOVA.

Table 43. Effect of astaxanthin & its esters on antioxidant enzymes in liver homogenates of UV-DMBA induced skin carcinogenesis rats

	SOD (U/mg protein)	Catalase (nmol H_20_2/ mg protein)	GSH (µg GSH / mg protein	TBARS (µmoles/MDA /mg protein)	SGPT (U/mg protein)	SGOT (U/mg protein)	SALP (U/mg protein)
Healthy control	2.81± 0.51^{gh}	1.57 ± 0.31^{a}	0.79 ± 0.05^{a}	0.28 ±0.05^{j}	39.86 ± 9.92^{k}	46.01±3.79i	215.69 ± 12.45^{k}
UV+DMBA	7.79 ±0.88^{a}	0.69 ± 0.20^{c}	0.47 ± 0.07^{e}	4.13 ± 0.21^{a}	77.82 ± 7.31^{a}	74.70 ± 14.76^{a}	457.33 ± 23.33^{a}
ASX200*	5.92 ± 0.20^{c}	1.00 ± 0.21^{b}	0.57 ± 0.06^{c}	2.91 ± 0.20^{b}	57.96 ± 5.27^{c}	67.13 ± 1.54^{b}	395. 89 ± 20.28^{b}
SASX200*	5.78 ± 0.38^{c}	1.05 ± 0.08^{b}	0.61 ± 0.08^{c}	2.62 ± 0.07^{d}	54.61 ± 8.28^{d}	59.54 ± 7.43^{d}	346.19 ± 19.56^{c}
SASX200* control	2.87 ± 0.93^{gh}	1.29 ± 0.35^{a}	0.68 ± 0.11^{b}	0.30 ± 0.04^{j}	44.16 ± 10.40^{h}	45.21 ± 7.96^{j}	233.90 ± 14.55^{j}
TC100*	6.41 ± 0.70^{b}	1.04 ± 0.25^{b}	0.57 ± 0.04^{c}	2.91 ± 0.22^{b}	61.69 ± 23.60^{b}	59.61 ± 4.05^{d}	341.13 ± 17.51^{d}
TC200*	5.85 ± 0.65^{c}	1.13 ± 0.09^{b}	0.58 ± 0.03^{c}	2.71 ± 0.06^{c}	50.87 v 11.36^{e}	57.15 ± 11.35e	318.18 ± 13.49^{e}
TC200*control	2.98 ± 1.00^{gh}	1.37 ± 0.06^{ab}	0.63 ± 0.07^{c}	0.44 ± 0.08^{i}	46.75 ± 3.47^{g}	50.15 ± 8.78^{h}	248.12 ± 16.98^{i}
MASX100*	5.80 ±0.65^{c}	1.14 ± 0.02^{b}	0.65 ± 0.06^{b}	2.64 ± 0.10^{d}	51.26 ±13.98^{e}	61.04 ± 5.03^{c}	324.49 ± 22.69^{e}
MASX200*	2.47 ± 0.90^{h}	1.32 ± 0.25^{a}	0.68 ± 0.07^{b}	2.30 ± 0.09^{e}	46.73 ±1.08^{g}	57.46 ± 4.41^{e}	293.78 ± 19.71^{g}
MASX200* control	4.77 ± 0.77^{d}	1.47 ± 0.29^{a}	0.71 ± 0.10^{a}	0.76 ± 0.30^{h}	42.59 ± 0.39^{i}	46.08 ± 8.43^{i}	245.78 ± 21.03^{i}
DASX100*	3.94 ± 0.77^{e}	1.23 ± 0.19^{a}	0.60 ± 0.10^{c}	1.99 ± 0.14^{f}	51.42 ± 14.81^{e}	56.14 ± 15.03^{f}	299.25 ± 13.79^{f}
DASX200*	3.56 ± 0.66^{f}	1.34 ± 0.19^{a}	0.68 ± 0.04b	1.79 ± 0.07^{g}	47.09 ± 16.74^{f}	51.15 ± 6.86^{g}	267.50 ± 15.25^{h}
DASX200* control	2.92 ± 0.37^{g}	1.52 ± 0.08^{a}	0.74 ± 0.11a	0.41 ± 0.12^{i}	41.55 ± 4.24^{j}	42. 71 ± 9.41^{k}	229.04 ± 18.48^{j}

ASX refers to astaxanthin, SASX refers to standard astaxanthin, TC refers to total carotenoid, MASX refers to monoester of astaxanthin, DASX refers to diester of astaxanthin from *H. pluvialis*, *µg/kg/b.w. Values are expressed as mean ± SD (n=5). Values are not sharing a similar superscript within the same column are significantly different (P<0.05) as determined by ANOVA.

Table 44. Effect of astaxanthin & its esters on antioxidant enzymes in skin homogenates of UV-DMBA induced skin carcinogenesis rats.

	SOD (U/mg protein)	Catalase (nmol H_20_2/ mg protein)	GSH (µg GSH / Mg protein	TBARS (µmoles/MDA /mg protein)	SGPT (U/mg protein)	SGOT (U/mg protein)	SALP (U/mg protein)
Healthy control	164.89 ± 17.35^{l}	0.16 ± 0.02^{a}	18.95 ± 2.54^{a}	0.33± 0.08^{h}	72.68 ± 15.06^{fg}	319.25 ± 25.44^{i}	329.31 ± 25.61^{j}
UV+DMBA	452.50 ± 24.70^{a}	0.07 ± 0.01^{d}	9.14 ±1.74^{k}	3.51 ± 0.15^{a}	126.17 ± 7.41^{a}	668.84 ± 32.80^{a}	591.93 ± 23.09^{a}
ASX200*	280.45 ± 7.50^{d}	0.11 ± 0.01^{c}	15.12 ± 4.96^{h}	3.14 ± 0.06^{b}	101.99 ± 23.97^{c}	549.87 ± 35.32^{b}	516.62 ± 34.21^{c}
SASX200*	243 .65 ± 75.89^{f}	0.12 ± 0.03^{c}	15.57 ± 7.68^{f}	2.93 ± 0.14^{c}	95.89 ± 15.60^{d}	421.40 ± 28.15^{g}	489.60 ± 21.90^{e}
SASX200* control	182.40 ± 20.09^{j}	0.14 ± 0.02^{b}	16.92 ± 3.21^{c}	0.32 ± 0.03^{h}	77.95 ± 24.35^{fg}	295.92 ± 15.61^{j}	390.18 ± 15.23^{h}
TC100*	309.40 ± 14.62^{b}	0.08 ± 0.01^{d}	13.14 ± 0.37^{j}	3.16 ± 0.10^{d}	115.05 ± 8.13^{b}	547.59 ± 18.82^{b}	528.50 ± 25.12^{b}
TC200*	233.71 ± 6.80^{g}	0.12 ± 0.02^{c}	14.47 ± 4.11^{i}	3.00 ± 0.13^{c}	99.65 ± 15.22^{c}	507.40 ± 29.02^{d}	499.80 ± 39.62^{d}
TC200*control	168.73 ± 16.73^{m}	0.14 ± 0.03^{b}	16.94 ± 3.04^{c}	0.30 ± 0.08^{h}	75.05 ± 27.48^{g}	316.12 ± 36.88^{i}	365.06 ± 34.49^{j}
MASX100*	292.02 ± 19.49^{c}	0.11 ± 0.04^{c}	14.50 ± 0.60^{i}	2.39 ± 0.03^{d}	93.09 ± 12.51^{d}	557.73 ± 14.91^{b}	499.00 ± 14.03^{d}
MASX200*	248.97 ± 21.58^{f}	0.13 ± 0.05^{c}	16.07 ± 1.03^{e}	2.31 ± 0.17^{d}	82.85 ± 12.97^{e}	484.05 ± 11.51^{c}	442.59 ± 12.10^{f}
MASX200* control	208.08 ± 30.27^{i}	0.15 ± 0.03^{a}	16.97 ± 4.81^{c}	0.68 ± 0.14^{g}	78.53 ± 16.11^{fg}	311.27 ± 16.29^{i}	378.39 ± 25.78^{i}
DASX100*	275.09 ± 26.21^{e}	0.12 ± 0.06^{c}	15.38 ± 3.77^{g}	2.16 ± 0.06^{e}	100.49 ± 29.97^{c}	536.-06 ± 18.92^{b}	484.47 ± 14.27^{e}
DASX200*	223.12 ± 10.36^{h}	0.13 ± 0.02^{c}	16.66 ± 4.31^{d}	1.90 ± 0.09^{f}	79.75 ± 3.09^{f}	447.20 ± 3.85^{f}	418.47 ± 18.25^{g}
DASX200* control	173.04 ± 13.11^{k}	0.16 ± 0.02^{a}	17.03 ± 0.68^{b}	0.31 ± 0.10^{h}	71.58 ± 14.42^{h}	331.53 ± 17.30^{h}	378.64 ± 20.95^{i}

ASX refers to astaxanthin, SASX refers to standard astaxanthin, TC refers to total carotenoid, MASX refers to monoester of astaxanthin, DASX refers to diester of astaxanthin from *H. pluvialis*, *µg/kg/b.w. Values are expressed as mean ± SD (n=5) Values are not sharing a similar superscript within the same column are significantly different (P<0.05) as determined by ANOVA

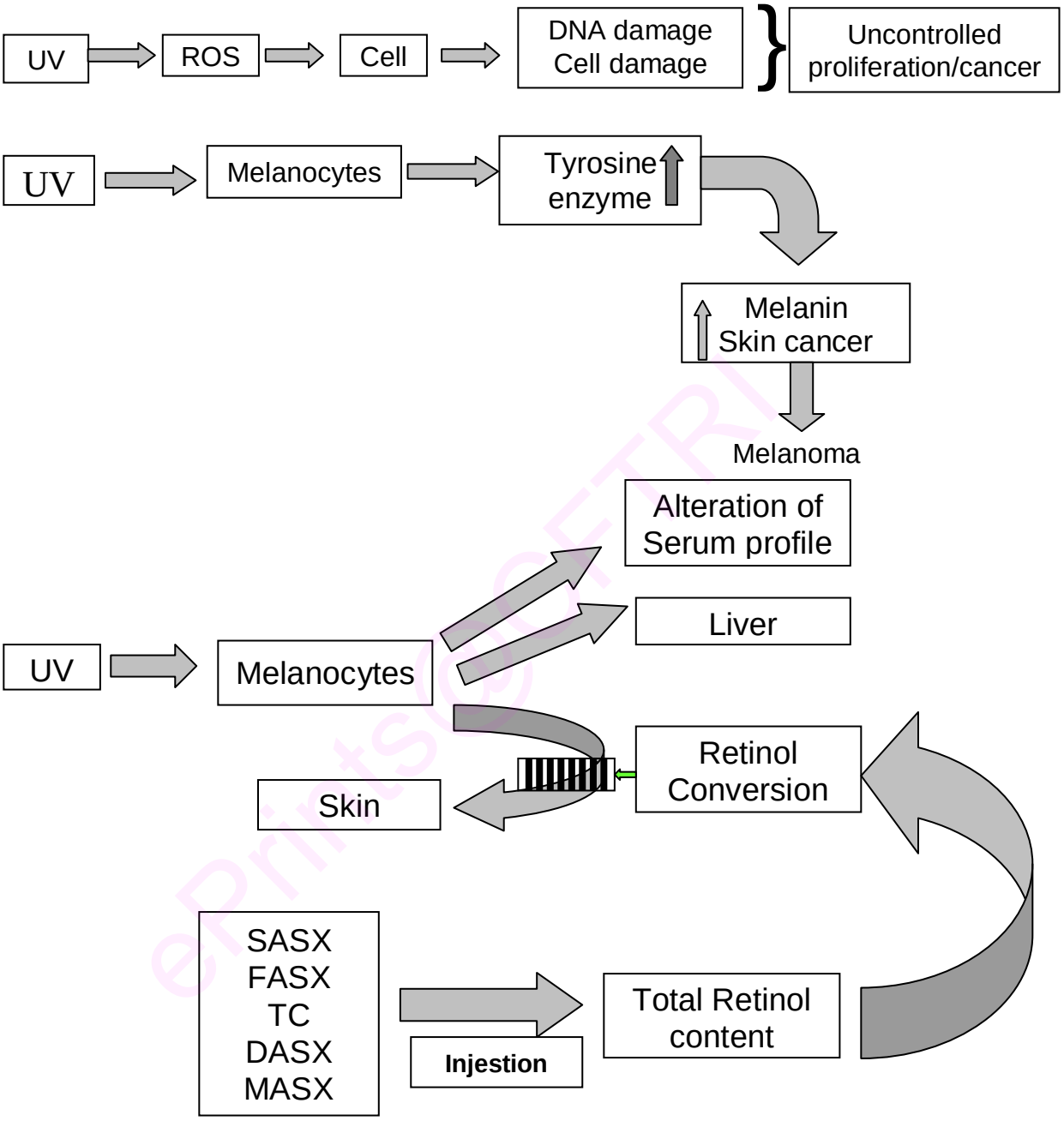


Fig. 119. Scheme for inhibition of skin carcinogenesis by SASX, FASX, TC, MASX and DASX from *H. pluvialis*

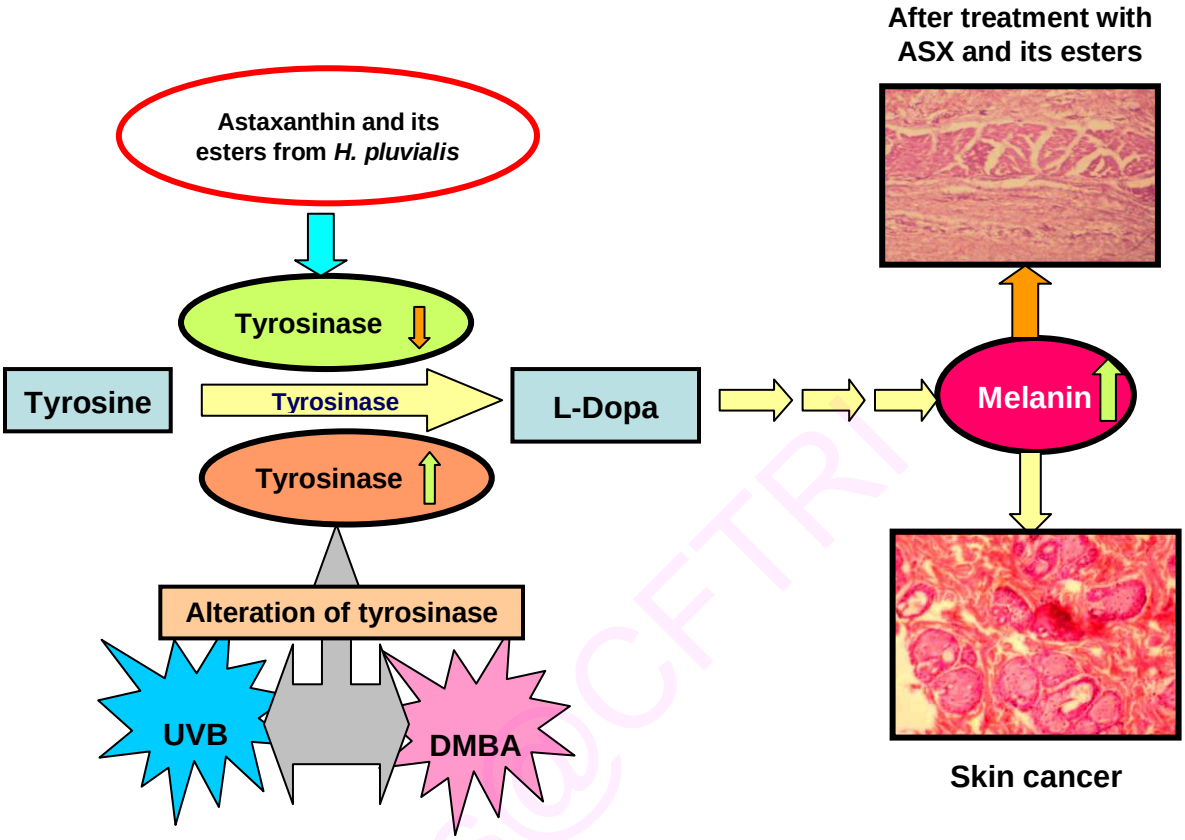


Fig. 120. Scheme for inhibition of tyrosinase activity in melanin production by astaxanthin and its esters treated animals

5.7. Possible degradation/oxidative products of astaxanthin & its esters in *in vivo* model

This study was aimed at optimizing the analytical conditions for enumeration of astaxanthin & its esters metabolites using HPLC and LC-MS *in vivo* model (plasma and liver) samples. The peak of astaxanthin & its ester metabolite fragments in the scanned range between 0 and 1000 m/z designated as A-Z, A_1 and B_1 (Table 45). The APCI detector parameters were adjusted suitably to give ion signal intensity for identification of peaks. The solvents in mobile phase such as acetonitrile (70%), methanol (10%) and dichloromethane (20%) seemed to have no effect on formation of positive ions. An integrated chromatographic peak area was measured by PDA at 450-476nm; Under the

conditions mentioned, a prominent peak at m/z 597 $[M+1]^+$ was detected for astaxanthin (Fig. 121A-B). This peak is more intense in astaxanthin fed samples. For convenience, in chain geometrical isomers of carotenoids, the terms all-E and Z, which refer to all *trans* and *cis* isomers of carotenoids, respectively, are used in this text instead of the old nomenclature.

5.7.1. Astaxanthin & its ester metabolites in *in vivo* model

Astaxanthin & esters metabolites were found in plasma and liver of UV-DMBA induced skin carcinogenesis fed with astaxanthin and the HPLC and LC-MS results were provided in Fig. 122C-D, 123 & 124. Astaxanthin ester metabolites were detected in plasma and liver after treatment with astaxanthin esters groups and compared with standards of astaxanthin and tocopherol (Fig. 122E-F, 125 & 126)

5.7.2. Biotransformation of astaxanthin in plasma and liver

In the case of plasma and liver, the characteristic fragmented ions obtained from astaxanthin as shown in (Fig. 123 and Table 45). The characteristic fragmented ions (unidentified metabolites) obtained from plasma were designated Q, R, S, T, U, V, W, X and Y. The peak that appeared in astaxanthin fed group was identified as 7,8 – Didehydroastaxanthin (595.62, $M+H^+$), 7,8,7,8,-Tetradehydroastaxanthin (593.61, $M+H^+$) 2′,3′-Anhydrolutein (551.45, $M+H^+$) corresponds to 2-hydroxy-5 ((1E,3E,5E,7E,9E,11E,13E,15E,17E)-18-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)-3,7,12,16-tetramethyloctadeca 1,3,5,7,9,11,13,15,17-nonaenyl)-4,4,6-trimethylcyclohexa-1,5-dienolate, 5 ((1E,3E,5E,7E,9E,11E,13E,15E,17E)-18-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl) 3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaenyl)-2,4,4,6-tetramethylcyclohexa-1,5 dienolate, and (1E,7E,9E,11E,13E)-5,9,14-trimethyl-2-tert-pentyl-16-(3-(2-(2,2,6-trimethyl-5-oxocyclohexyl)ethyl)oxiran-2-yl) hexadeca-1,7,9,11,13-pentaen-1-olate.

In the case of liver, the fragments resulting from astaxanthin were coded M, P, S, T, U, W, Y, Z, A_1 and B_1. (Fig. 127 and Table 45). The astaxanthin oxidative metabolites pattern in plasma was almost similar to that in liver. The HPLC profile of liver extract showed a well separated major peak which was identified as astaxanthin along with its

oxidative metabolites. Several unidentified metabolites were detected between 0-1000 m/z.

5.7.3. Biotransformation of astaxanthin esters in plasma and liver

No difference was found with respect to astaxanthin esters metabolites in plasma and liver of UV-DMBA induced skin carcinogenesis rats fed with astaxanthin esters. The HPLC results were given in (Fig. 122 E-F). Astaxanthin ester metabolites were detected in plasma and liver after incubation with astaxanthin esters. The peak that appeared after fed group was identified as astaxanthin esters ($C_{14:0}$, $C_{18:2}$ and $C_{18:3}$). The mass of 833.38, 857.22, 859.31 $[M+H]^+$ corresponded to astaxanthin esters such as astaxanthin hexadecenoate ($C_{16:1}$), hexadecanoate ($C_{16:0}$) and octadecenoate ($C_{18:1}$) esters were found in liver samples. The astaxanthin ester metabolites pattern in plasma was almost similar to that in liver. The HPLC profile of plasma extract showed a well separated major peak. Using APCI in positive ion mode, astaxanthin esters and several unidentified metabolites were detected between 0 and 1000 m/z. The characterstic fragmented ions obtained from plasma were designated as G, H, I, J, K , O, P, S, T, W, Y and Z (Fig. 125, Table 45), whereas in liver coded as A-I, L-P, S, U, W, Y, Z, A_1 and B_1 (Table 45 and Fig. 126).

5.8. Discussion

The present study indicate that astaxanthin & its esters is metabolized/oxidized in rat tissue to several components. The oxidation products appeared only after intake of astaxanthin & its esters (Fig. 123-126). Further these metabolites/ oxidation products were not detected in control samples. The available literature provides an indication of that astaxanthin & esters may play an active role in biological activities. Still critical evidence of the beneficial effects of astaxanthin & esters or their metabolites/oxidation products has not been elucidated in details. Among the carotenoids identified in plasma and liver of UV-DMBA skin carcinogenesis rats after intake of astaxanthin & its esters only 7,8–didehydroastaxanthin (595.62, $M+H^+$), 7,8,7,8,-Tetradehydroastaxanthin (593.61, $M+H^+$) 2′,3′-Anhydrolutein (551.45) were found in plasma and liver after intubation of astaxanthin. Samples were handled taking all safety measures to avoid the possibility of oxidation of astaxanthin and its esters. Further, we have found that

astaxanthin esters in plasma and liver (Fig. 125-126, Table 45) after intubation of astaxanthin and its esters. Heartmann et al., (2004) reported that dosing zeaxanthin to human volunteers resulted in considerable accumulation of all-*E*-3-dehydrolutein in plasma and postulated that because lutein concentration remains unaffected by zeaxanthin dosing, the increase in all –*E*-3-dehydrolutein might have been derived from zeaxanthin. Thurman et al., (2005) reported that long-term intake of lutein resulted in an accumulation of 3`-dehydrolutein in human plasma. Provitamin A carotenoids are cleaved at the central $C_{15}$-$C_{15}$` double bond by β-carotene dioxygenase into vitamin A (Goodman and Huang, 1965; Olson and Hayaishi, 1965). Wolz et al., (2008) reported that astaxanthin metabolites cleaved asymmetrically at the C_9 position in rat hepatocytes. Astaxanthin may be converted into several metabolites *in vivo*. In liver and plasma, oxidized molecules Q, R and U may be formed due to oxidative reactions. Based on the results given in Table 46, We assume that formation of astaxanthin & its esters oxidation products *in vivo* is possibly due to its oxidation/chemical reactions of astaxanthin & its esters, which splits the molecule into fragments as seen in peaks and the removal of methyl groups from (Fig. 124-127) Similar fragmented ions were also identified in liver samples, which indicate the occurrence of a chemical reaction in liver possibly. Earlier reports have suggested that light damages the retina by generation of free radicals (Khachik, 2002). Stratton et al., (1997) and Yamauchi et al., (1998) reported that lutein is involved in the chain-breaking peroxyl radical or quenching of the singlet oxygen. The formation of oxidized products indicates the formation of astaxanthin, which is very similar to epoxide, hydroxyl carotenoids and may have antioxidant potency. It has been reported that an increase in the number of hydroxyl groups in the carotenoid molecule amplifies antioxidant capacity. Interestingly, metabolites bearing molecular mass at *m/z* 603 (O), 601 (P), 369 (Y) were found in plasma and liver. Similar to earlier reports in mice and rat model (Asai et al., 2004, Sangeetha et al., 2010), which may be due to dehydrogenation and/or isomerization of carotenoids. Metabolites molecular mass at m/z 551 (U) were found in plasma and liver reported by Lakshminarayana et al., (2008). Lutein oxidation products *in vitro* (Photo oxidation) and *in vivo* in plasma and liver have

been reported (Lakshminarayana et al., 2008). Fucoxanthin and its metabolites investigated in retinol deficient rats in plasma and liver (Sangeetha et al., 2010). These metabolites may act as functional molecules *in vivo*, similar to lutein metabolites (Bhosale et al., 2007). Astaxanthin esters found in the liver were different from those in plasma. This is probably due to hydrolysis of astaxanhin & its esters to dehydroastaxanthin and other metabolites by gastro intestinal enzymes lipase, cholesterol esterase and carboxyl esterase and subsequently transported to liver (Asai et al., 2004). Metabolites found in plasma and liver could have resulted from either astaxanthin or by removal of water molecules due to enzymatic reactions involving oxygenases. Metabolites could be partly be due to enzymatic retoaldol cleavage (Strand et al., 1998). Biological transformation of carotenoids such as astaxanthin, lutein, zeaxanthin and canthaxanthin to precursors of vitamin A in fish has reported by (Matsuno, 1991; Goswami and Barua, 1981). Based on the present findings, the possible astaxanthin and its esters metabolic pathway in plasma and liver (Scheme. 1-3) of rats are proposed. From the LC-MS results, we speculate that these metabolites may be formed as a result of enzymatic reactions. This may be the first report on astaxanthin & its esters metabolites in plasma and liver of rat model.

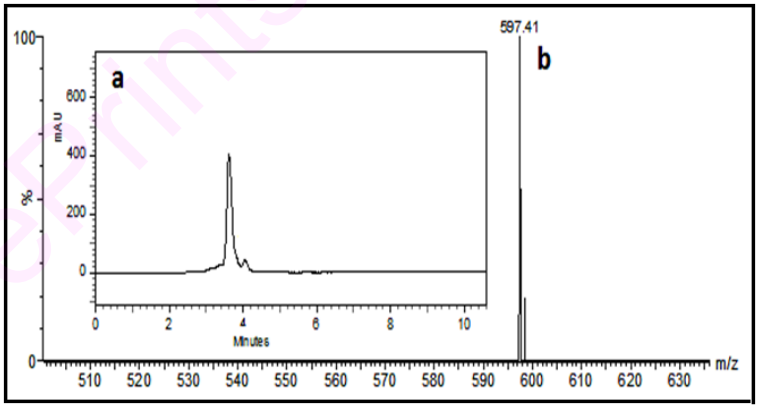


Fig. 121. A typical HPLC profile and spectrum of (a) standard astaxanthin and (b) its mass spectrum. HPLC and LC-MS conditions adopted were outlined under Materials and methods.

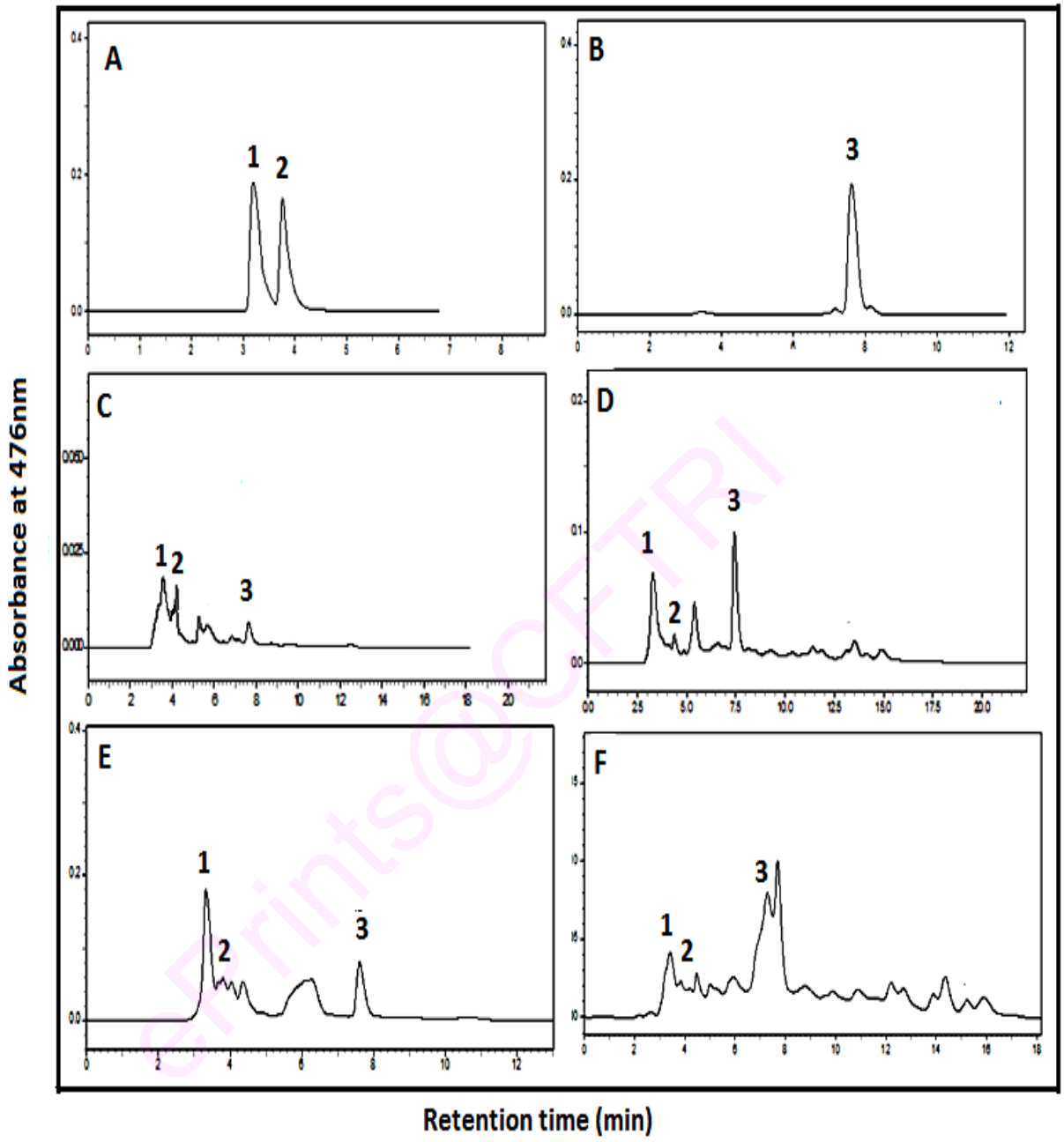


Fig. 122. HPLC profile of astaxanthin & its metabolites from plasma and liver of UV-DMBA induced skin carcinogenesis rats incubated with astaxanthin. Standard astaxanthin & lutein (A), Standard tocopherol(B), Plasma astaxanthin (C), Liver astaxanthin (D), Plasma astaxanthin esters (E) and Liver astaxanthin (F).

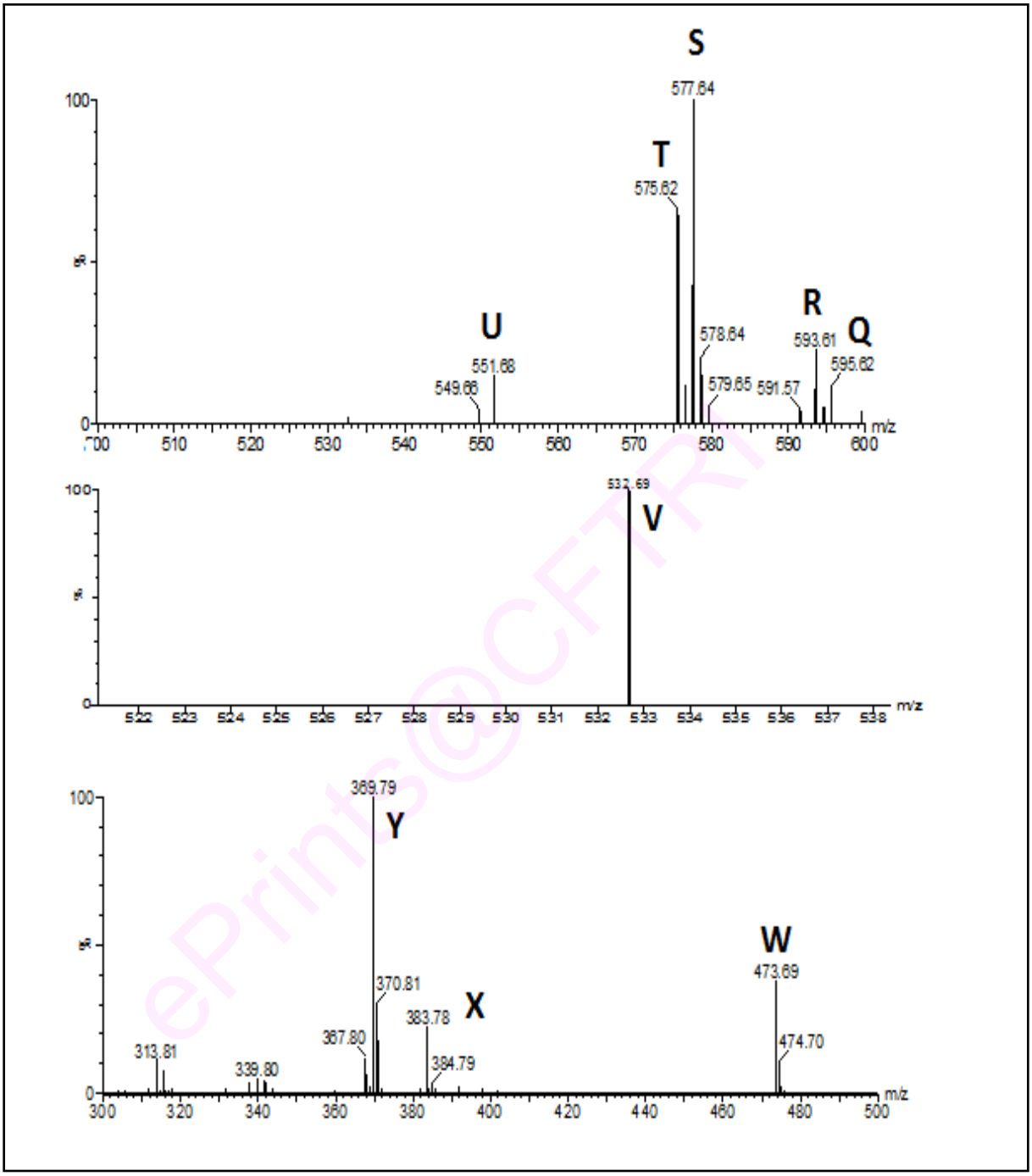


Fig. 123. APCI-MS profiles of astaxanthin metabolites detected in plasma of UV-DMBA induced skin carcinogenesis rats fed with astaxanthin.

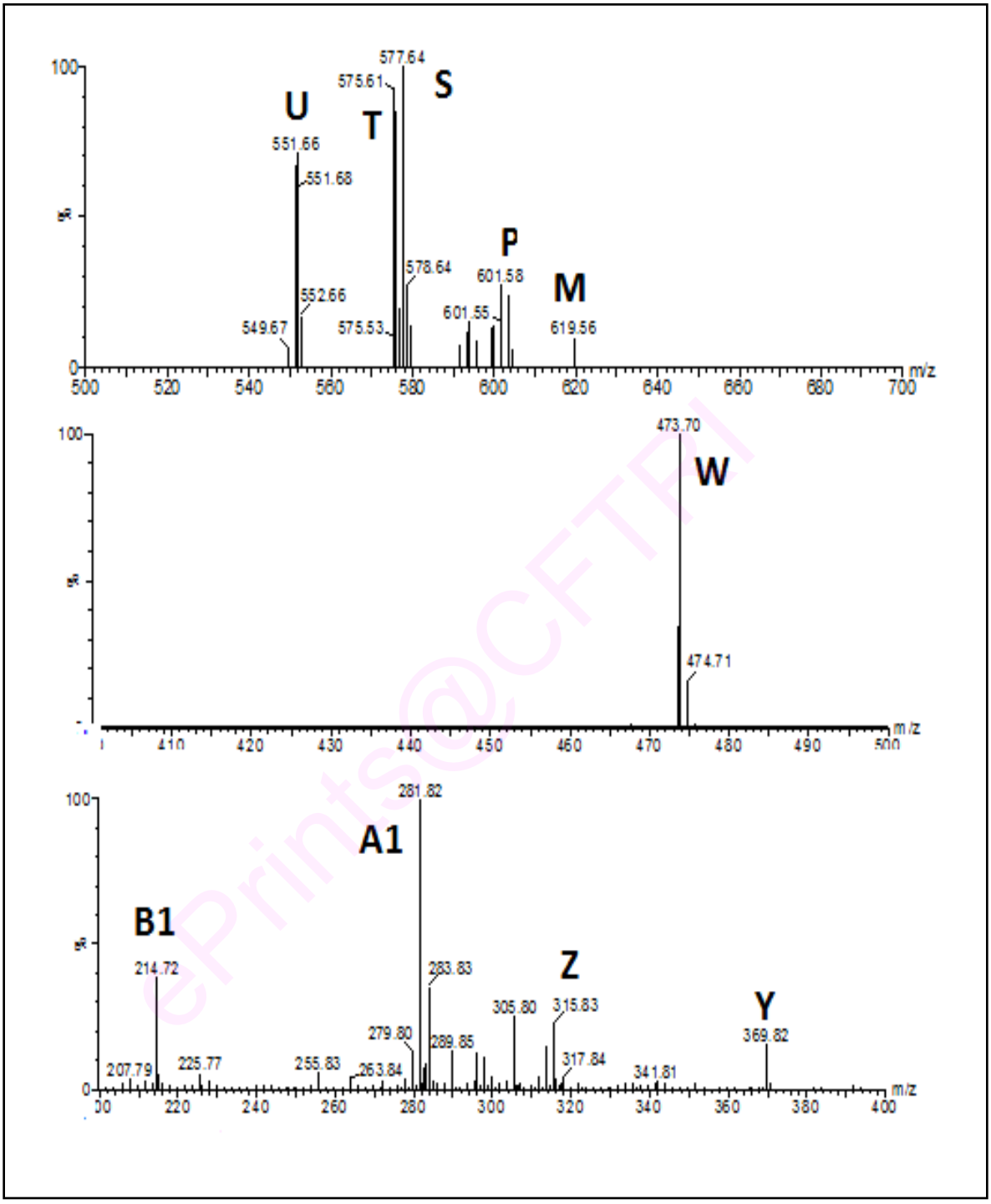


Fig. 124. APCI-MS profiles of astaxanthin metabolites detected in liver of UV-DMBA induced skin carcinogenesis rats fed with astaxanthin

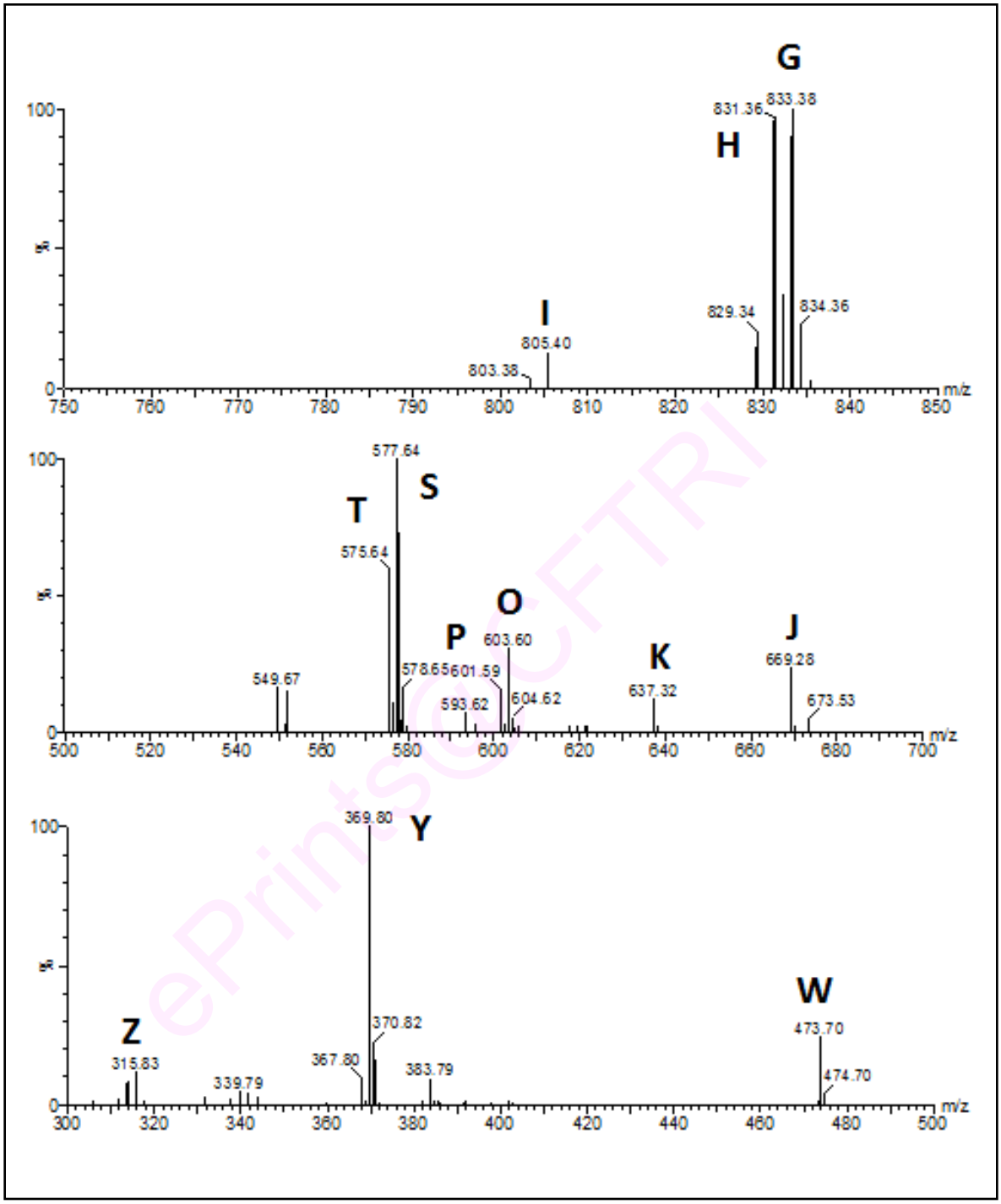


Fig. 125. APCI-MS profiles of astaxanthin esters metabolites detected in plasma of UV-DMBA induced skin carcinogenesis rats fed with astaxanthin esters.

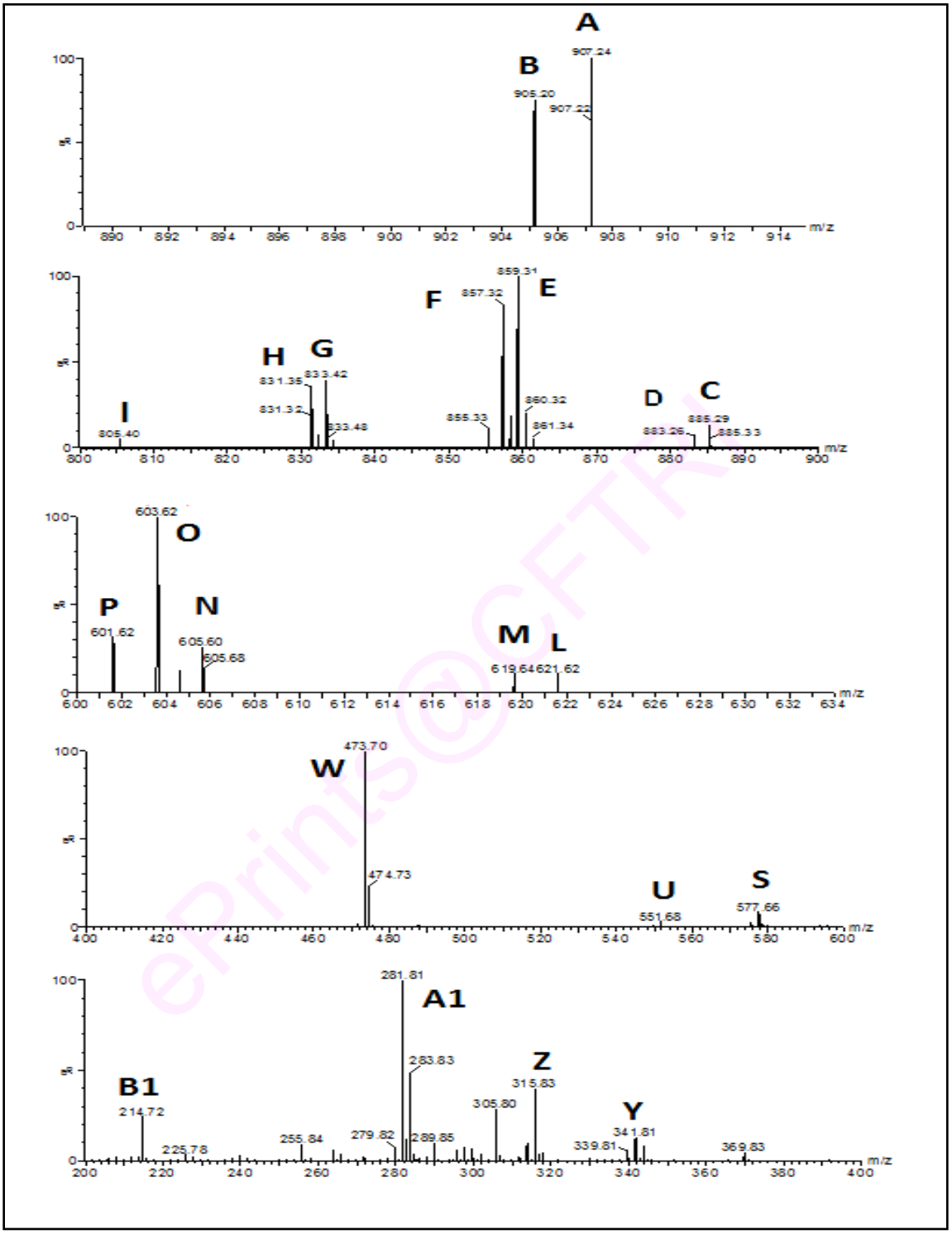
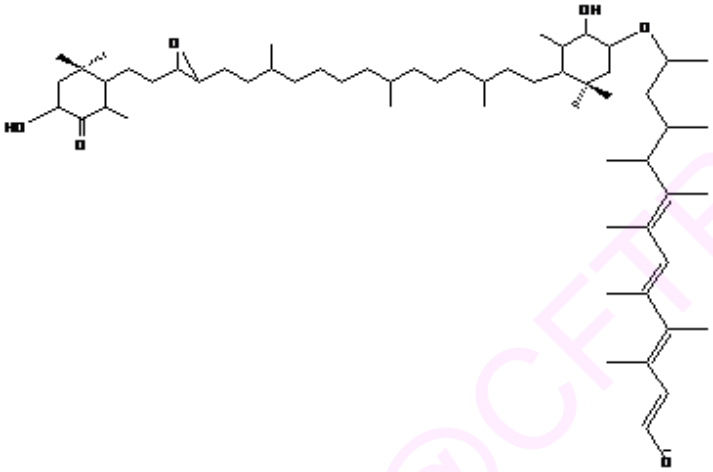
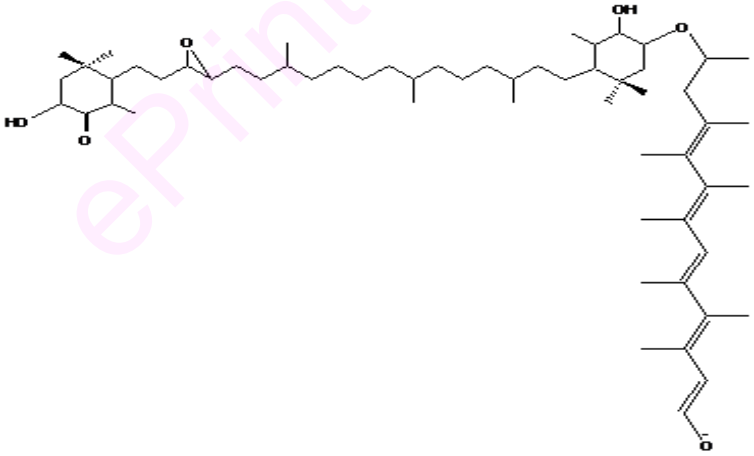
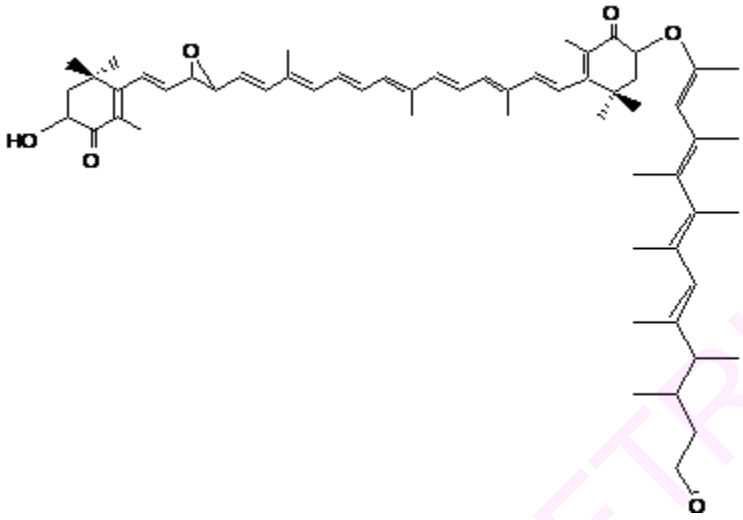
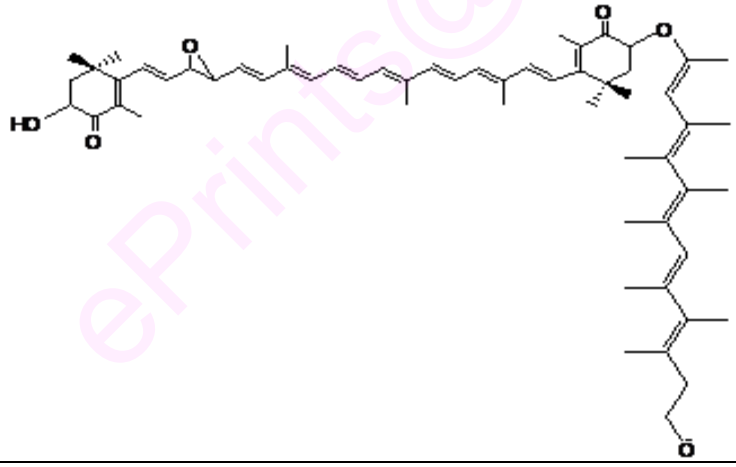
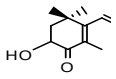
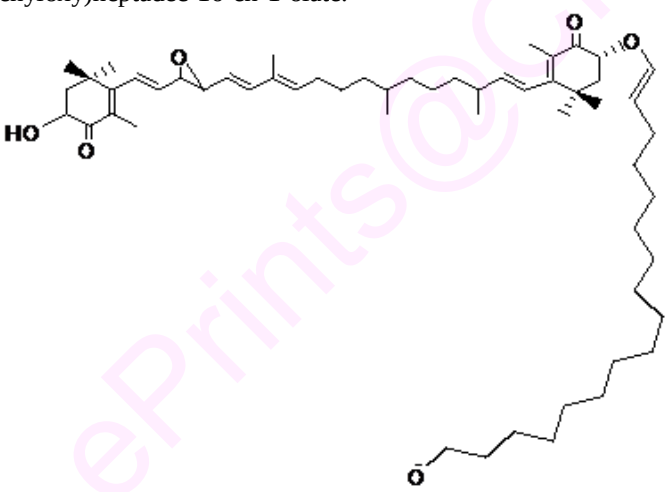


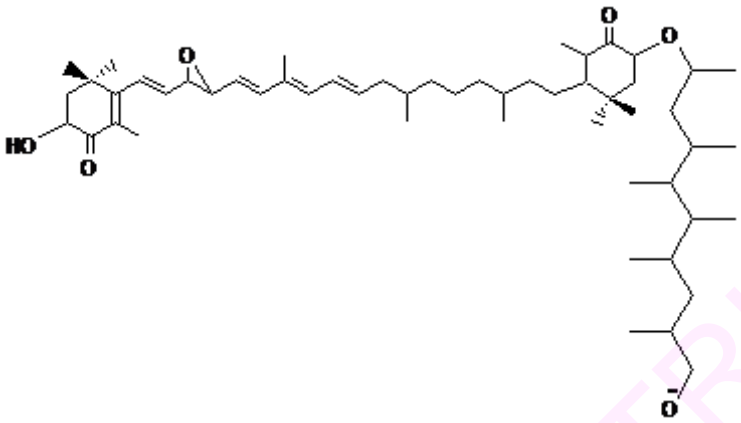
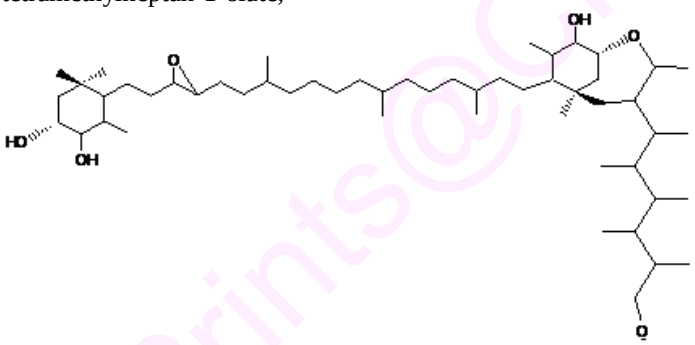
Fig. 126. APCI-MS profiles of astaxanthin esters metabolites detected in liver of UV-DMBA induced skin carcinogenesis rats fed with astaxanthin esters.

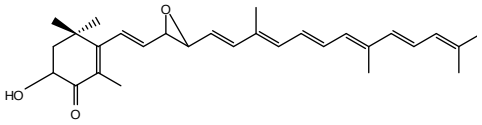
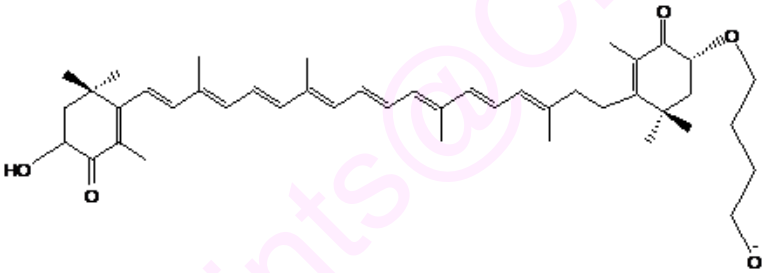
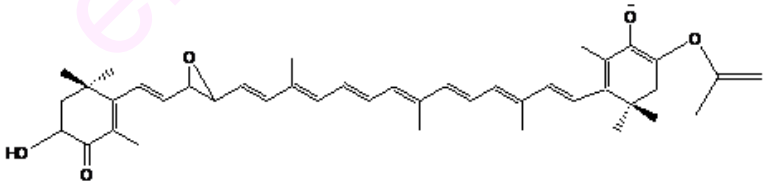
Table 45. Mass spectral fragmented ions derived from astaxanthin & its esters *in vivo*, elucidated by APCI+ ion mode, showing their chemical structure, molecular mass, and molecular formula.

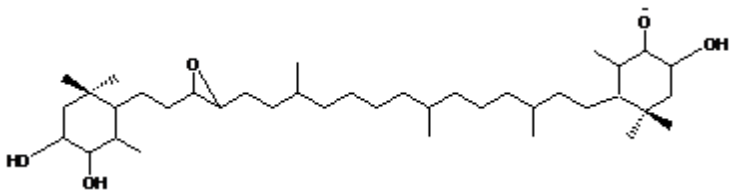
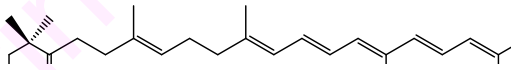
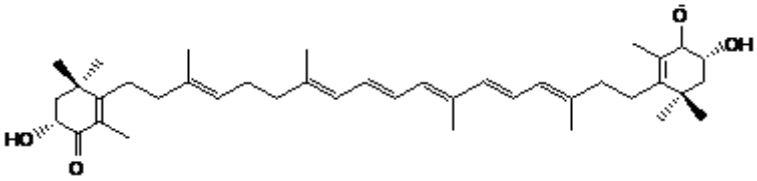
Alphabet	Structure and name of the compound	Mass	Formula
A	(1E,3E,5E,7E)-12-(2-hydroxy-4-(14-(3-(2-(4-hydroxy-2,2,6-trimethyl-5-oxocyclohexyl)ethyl)oxiran-2-yl)-3,7,12-trimethyltetradecyl)-3,5,5-trimethylcyclohexyloxy)-3,4,5,7,8,9,10-heptamethyltrideca-1,3,5,7-tetraen-1-olate. 	907.78	$C_{59}H_{103}O_6^-$
B	(1E,3E,5E,7E,9E)-12-(2-hydroxy-4-(14-(3-(2-(4-hydroxy-2,2,6-trimethyl-5-oxocyclohexyl)ethyl)oxiran-2-yl)-3,7,12-trimethyltetradecyl)-3,5,5-trimethylcyclohexyloxy)-3,4,5,7,8,9,10-heptamethyltrideca-1,3,5,7,9-pentaen-1-olate 	905.76	$C_{59}H_{101}O_6^-$

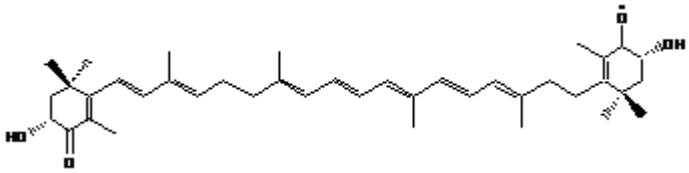
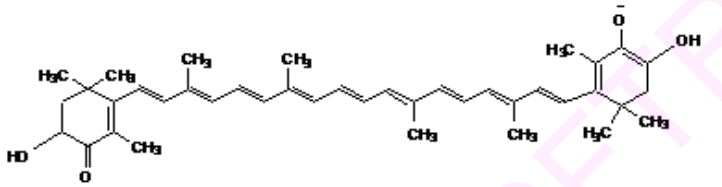
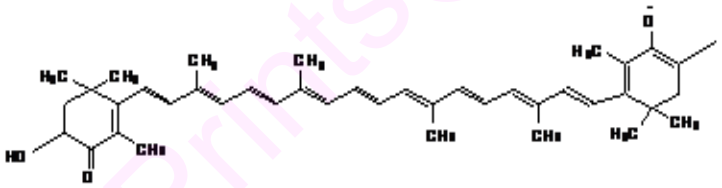
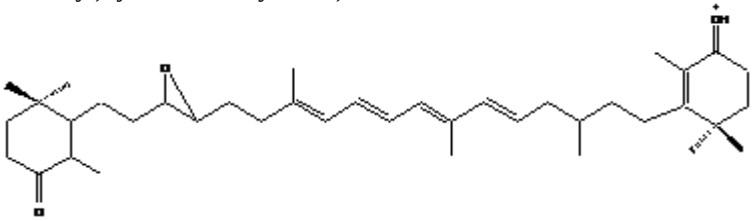
C	5E,7E,9E,11E)-12-(4-((1E,3E,5E,7E,9E,11E,13E)-14-(3-((E)-2-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)vinyl)oxiran-2-yl)-3,7,12-trimethyltetradeca-1,3,5,7,9,11,13-heptaenyl)-3,5,5-trimethyl-2-oxocyclohex-3-enyloxy)-3,4,5,7,8,9,10-heptamethyltrideca-5,7,9,11-tetraen-1-olate, 	885.60	$C_{59}H_{81}O_6^-$
D	(3E,5E,7E,9E,11E)-12-(4-((1E,3E,5E,7E,9E,11E,13E)-14-(3-((E)-2-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)vinyl)oxiran-2-yl)-3,7,12-trimethyltetradeca-1,3,5,7,9,11,13-heptaenyl)-3,5,5-trimethyl-2-oxocyclohex-3-enyloxy)-3,4,5,7,8,9,10-heptamethyltrideca-3,5,7,9,11-pentaen-1-olate, 	883.59	$C_{59}H_{79}O_6^-$

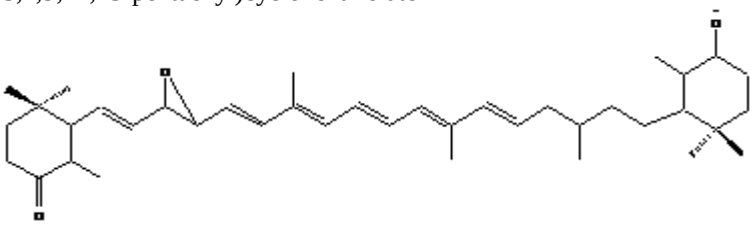
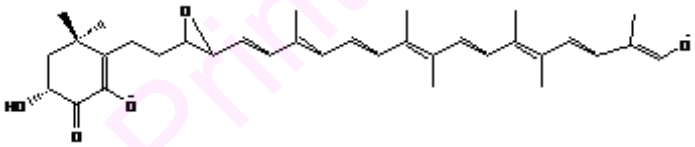
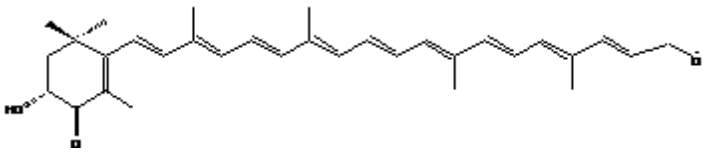
E	17-((1R)-4-((1E,11E,13E)-14-(3-((E)-2-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)vinyl)oxiran-2-yl)-3,7,12-trimethyltetradeca-1,11,13-trienyl)-3,5,5-trimethyl-2-oxocyclohex-3-enyloxy)heptadecan-1-olate 	859.68	$C_{56}H_{91}O_6$-
F	(E)-17-((1R)-4-((1E,11E,13E)-14-(3-((E)-2-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)vinyl)oxiran-2-yl)-3,7,12-trimethyltetradeca-1,11,13-trienyl)-3,5,5-trimethyl-2-oxocyclohex-3-enyloxy)heptadec-16-en-1-olate. 	857.67 Miao et al., 2006 Holtin et al., 2009	$C_{56}H_{89}O_6^-$

G	9-(4-((9E,11E,13E)-14-(3-((E)-2-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)vinyl)oxiran-2-yl)-3,7,12-trimethyltetradeca-9,11,13-trienyl)-3,5,5-trimethyl-2-oxocyclohexyloxy)-2,4,5,6,7-pentamethyldecan-1-olate 	833.67	$C_{54}H_{89}O_6^-$
H	6-((6S)-7-(14-((2R)-3-(2-(4,5-dihydroxy-2,2,6-trimethylcyclohexyl)ethyl)oxiran-2-yl)-3,7,12-trimethyltetradecyl)-9-hydroxy-3,6,8-trimethyl-2-oxabicyclo[4.3.1]decan-4-yl)-2,3,4,5-tetramethylheptan-1-olate, 	831.74	$C_{53}H_{99}O_6^-$

I	(6E,8E)-9-(4-((1E,3E,5E,7E,9E,11E,13E)-14-(3-((E)-2-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)vinyl)oxiran-2-yl)-3,7,12-trimethyltetradeca-1,3,5,7,9,11,13-heptaenyl)-3,5,5-trimethyl-2-oxocyclohex-3-enyloxy)-2,4,5,6,7-pentamethylnona-6,8-dien-1-olate 	805.54	$C_{53}H_{73}O_6$-
J	4-((1R)-4-((3E,5E,7E,9E,11E,13E,15E,17E)-18-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)-3,7,12,16-tetramethyloctadeca-3,5,7,9,11,13,15,17-octaenyl)-3,5,5-trimethyl-2-oxocyclohex-3-enyloxy)butan-1-olate 	669.45	$C_{44}H_{61}O_5^-$
K	5-((1E,3E,5E,7E,9E,11E,13E)-14-(3-((E)-2-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)vinyl)oxiran-2-yl)-3,7,12-trimethyltetradeca-1,3,5,7,9,11,13-heptaenyl)-4,4,6-trimethyl-2-(prop-1-en-2-yloxy)cyclohexa-1,5-dienolate 	637.39	$C_{42}H_{53}O_5^-$

L	3-(14-(3-(2-(4,5-dihydroxy-2,2,6-trimethylcyclohexyl)ethyl)oxiran-2-yl)-3,7,12-trimethyltetradecyl)-6-hydroxy-2,4,4-trimethylcyclohexanolate 	621.55	$C_{39}H_{73}O_5^-$
M	1-(2-hydroxy-4-((7E,9E,11E,13E)-14-(3-((E)-2-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)vinyl)oxiran-2-yl)-12-methyltetradeca-7,9,11,13-tetraenyl)-3,5,5-trimethylcyclohex-1-enyloxy)ethenolate	619.40	$C_{39}H_{55}O_6$-
N	(6R)-6-hydroxy-3-((3E,5E,7E,9E,11E,15E)-18-((R)-4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)-3,7,12,16-tetramethyloctadeca-3,5,7,9,11,15-hexaenyl)-2,4,4-trimethylcyclohexanolate 	605.46	$C_{40}H_{61}O_4$-
O	(6R)-6-hydroxy-3-((3E,5E,7E,9E,11E,15E)-18-((R)-4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)-3,7,12,16-tetramethyloctadeca-3,5,7,9,11,15-hexaenyl)-2,4,4-trimethylcyclohex-2-enolate 	603.44 Sangeetha et al., 2010 et al., 2008	$C_{40}H_{59}O_4^-$

P	(6R)-6-hydroxy-3-((3E,5E,7E,9E,11E,15E,17E)-18-((R)-4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)-3,7,12,16-tetramethyloctadeca-3,5,7,9,11,15,17-heptaenyl)-2,4,4-trimethylcyclohex-2-enolate. 	601.43 Schweiggert et al., 2005 Lakshminarayana et al., 2008	$C_{40}H_{57}O_4^-$
Q	2-hydroxy-5-((1E,3E,5E,7E,9E,11E,13E,15E,17E)-18-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)-3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaenyl)-4,4,6-trimethylcyclohexa-1,5-dienolate. 	595.38 Sangeetha et al., 2010	$C_{40}H_{51}O_4^-$ Reported
R	5-((1E,3E,5E,7E,9E,11E,13E,15E,17E)-18-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)-3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaenyl)-2,4,4,6-tetramethylcyclohexa-1,5-dienolate. 	593.40	$C_{41}H_{53}O_3^-$
S	(2,4,4-trimethyl-3-((5E,7E,9E,11E)-3,7,12-trimethyl-14-(3-(2-(2,2,6-trimethyl-5 oxocyclohexyl)ethyl)oxiran-2-yl)tetradeca-5,7,9,11-tetraenyl)cyclohex-2-enylidene)oxonium 	577.46	$C_{39}H_{61}O_3^+$

T	2,4,4-trimethyl-3-((5E,7E,9E,11E,13E)-3,7,12-trimethyl-14-(3-((E)-2-(2,2,6-trimethyl-5 oxocyclohexyl)vinyl)oxiran-2 yl)tetradeca-5,7,9,11,13-pentaenyl)cyclohexanolate 	575.45	$C_{39}H_{59}O_3$- reported
U	(1E,7E,9E,11E,13E)-5,9,14-trimethyl-2-tert-pentyl-16-(3-(2-(2,2,6-trimethyl-5-oxocyclohexyl)ethyl)oxiran-2-yl)hexadeca-1,7,9,11,13-pentaen-1-olate	551.45 Sangeetha et al., 2010 Lakashminarayana et al., 2008 Bhosale et al., 2007	$C_{37}H_{59}O_3$-
U	(5R)-2-(2-(3-((1E,3E,5E,7E,9E,11E,13E,15E)-3,7,8,11,12,15-hexamethyl-16-oxidohexadeca-1,3,5,7,9,11,13,15-octaenyl)oxiran-2-yl)ethyl)-5-hydroxy-3,3-dimethyl-6-oxocyclohex-1-enolate 	532.71	$C_{34}H_{44}O_{52}^-$
W	(2E,4E,6E,8E,10E,12E,14E,16E,18E)-19-((R)-4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)-4,8,13,17-tetramethylnonadeca-2,4,6,8,10,12,14,16,18-nonaen-1-olate 	473.31	$C_{32}H_{41}O_3^-$

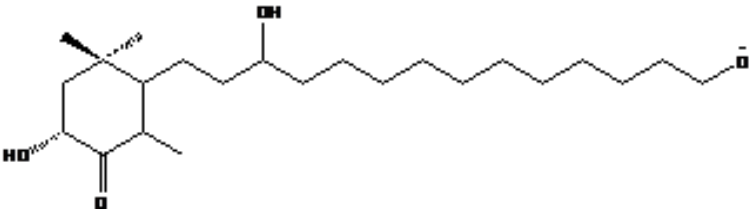
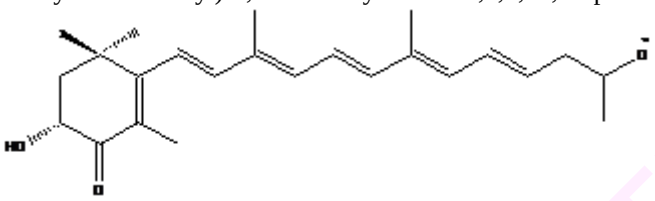
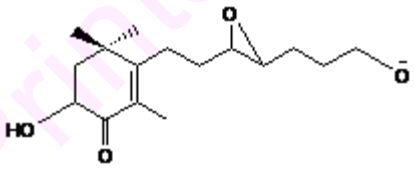
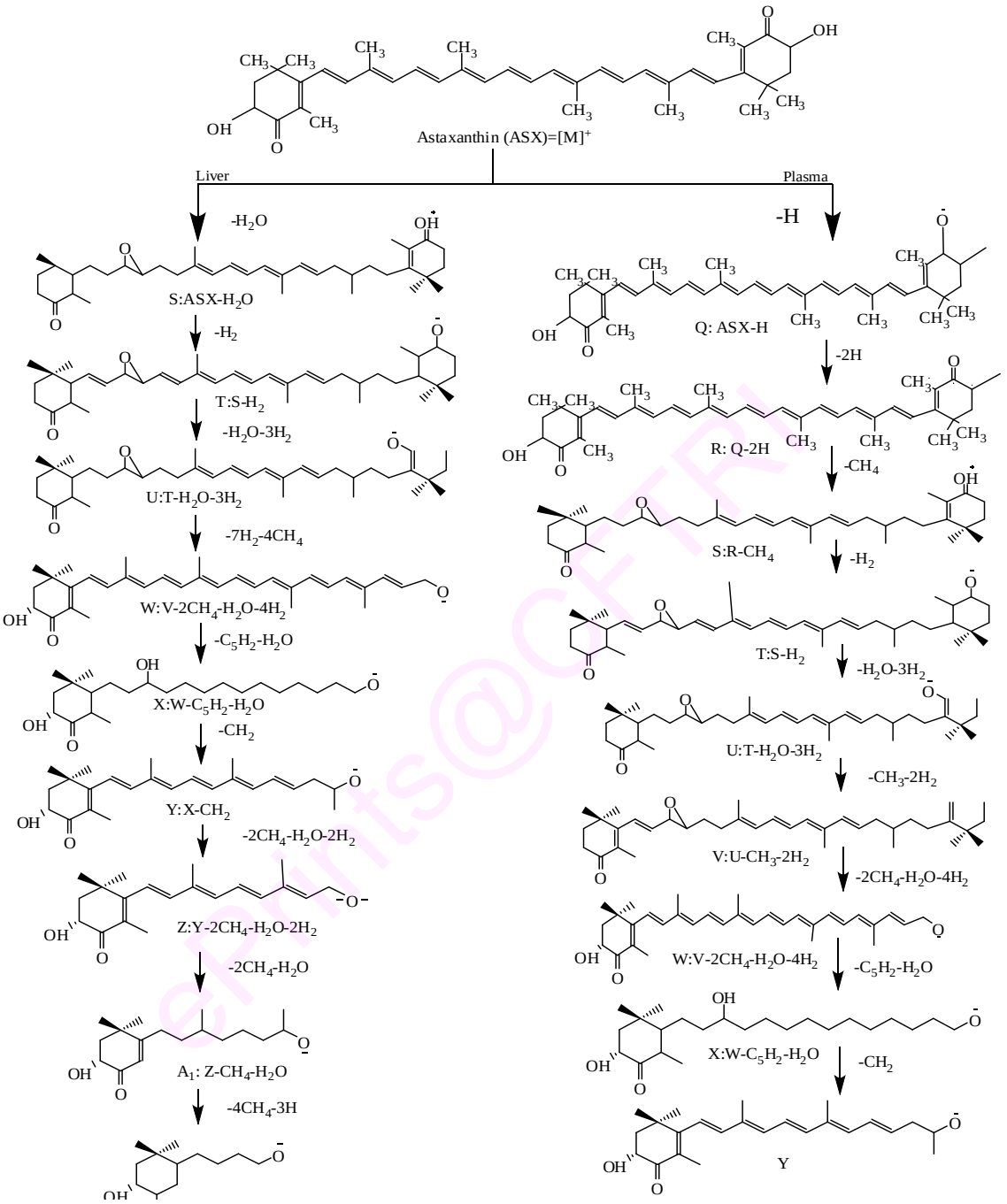
X	12-hydroxy-14-((4R)-4-hydroxy-2,2,6-trimethyl-5-oxocyclohexyl)tetradecan-1-olate 	383.59 Lakshminarayana et al., 2008	$C_{23}H_{43}O_4^-$
Y	(4E,6E,8E,10E,12E)-13-((R)-4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)-7,11-dimethyltrideca-4,6,8,10,12-pentaen-2-olate 	369.52 Sangeetha et al., 2010	$C_{24}H_{33}O_3^-$
Z	(1E,3E,5E,7E)-8-(3-((4R)-4-hydroxy-3,6,6-trimethylcyclohex-1-enyl)oxiran-2-yl)nona-1,3,5,7-tetraen-1-olate	315.20	$C_{20}H_{27}O_3$-
A_1	3-(3-(2-(4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-enyl)ethyl)oxiran-2-yl)propan-1-olate 	281.18	$C_{16}H_{25}O_4^-$
B_1	6-hydroxy-2,4,4-trimethyl-3-(3-oxidopropyl)cyclohexanolate	214.16	$C_{12}H_{22}O_{32}$-

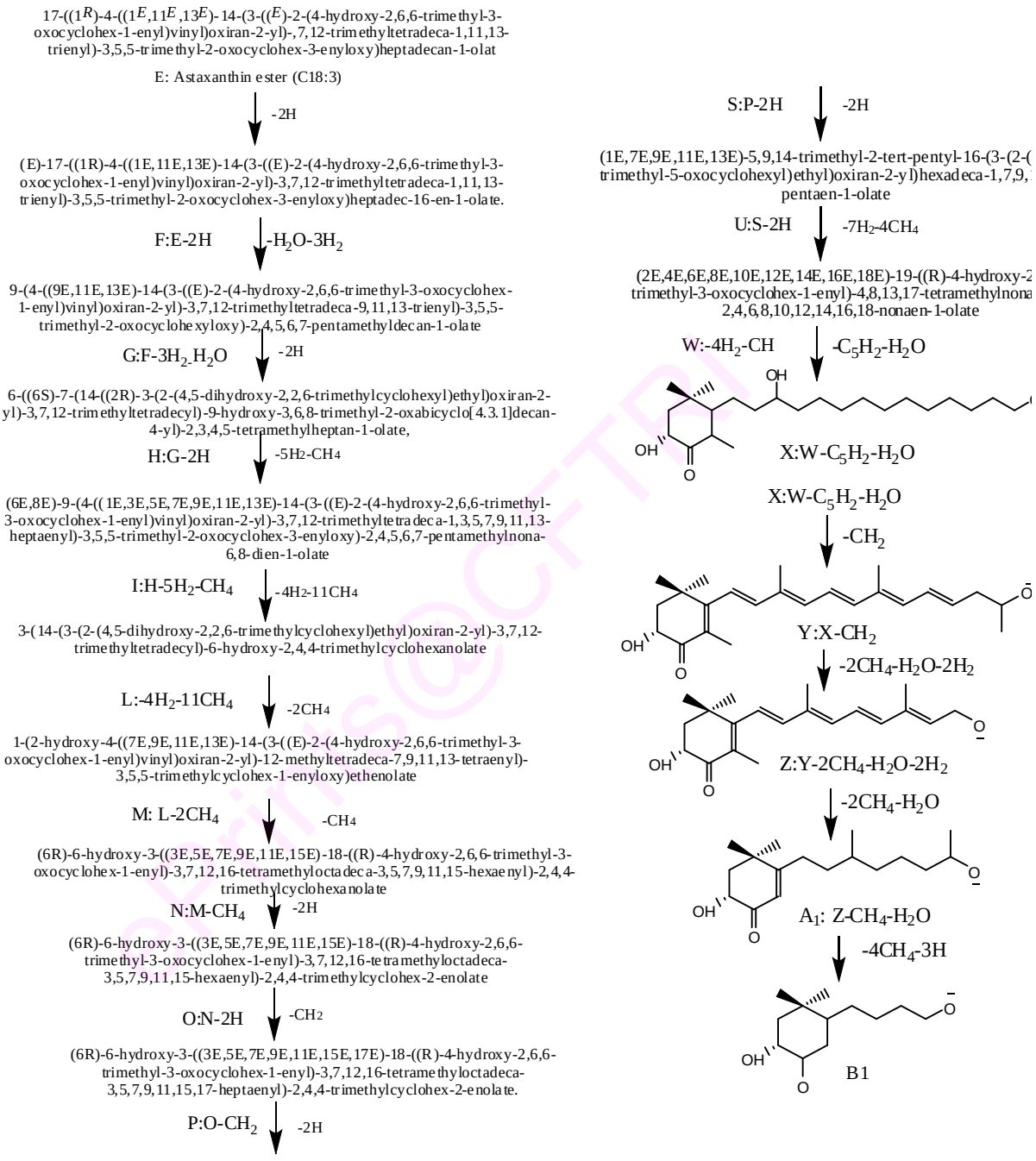
Table 46. Comparison of the astaxanthin & its esters fragmented ions identified in *vivo* experiments.

Biological samples	**Identified fragmented ions**																											
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	A_1	B_1
Astaxanthin																												
Plasma	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-
Liver	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-
Astaxanthin esters																												
Plasma	-	-	-	-	-	-	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+		+	+	-	-
Liver	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	+		+	-	+	+	+	+	+	+

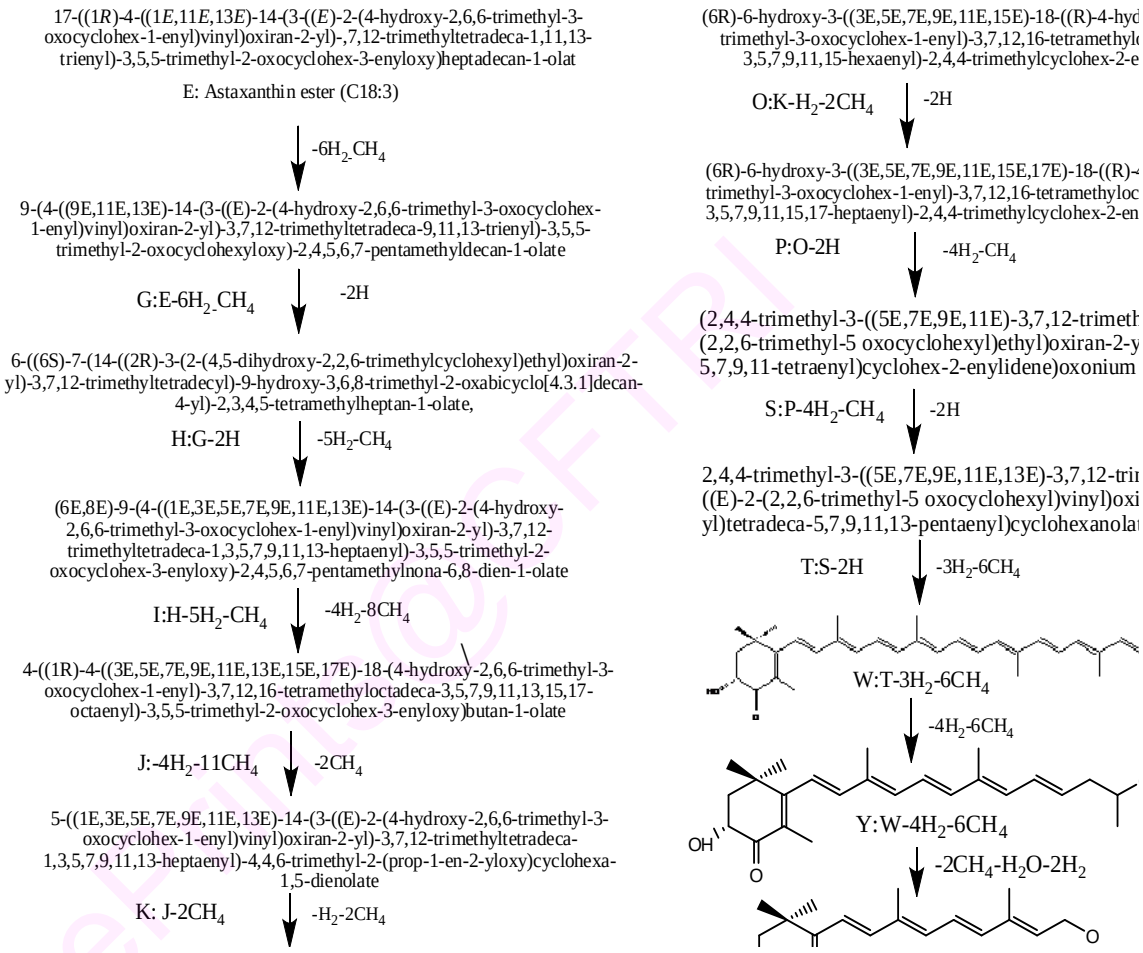
+, presence of molecule, -, absence of molecule



Scheme 1. Possible pathway of astaxanthin biotransformation by oxidation/metabolism in *in vivo.*



Scheme 2. Possible pathway of astaxanthin esters biotransformation by oxidation/metabolism in *in vivo* (liver).



Scheme 3 Possible pathway of astaxanthin esters biotransformation by oxidation/metabolism in *in vivo* (plasma).

5.9. Anticancer properties of astaxanthin and its esters using cell culture models

5.9.1. Anticancer properties of astaxanthin from *H. pluvialis*

The cytotoxic effect of astaxanthin and its esters on human glioma cell lines and liver hepatocellular carcinoma cell lines were evaluated. The *in vitro* cytotoxicity of astaxanthin and its esters were investigated on human glioma cell lines (LN-229 and HNGC2) and liver hepatocellular carcinoma cell line (HepG2) by using MTT assay.

5.9.2. Antiproliferative properties of astaxanthin from *H. pluvialis* on human glioma cell lines (HNGC2 and LN-229) and liver hepatocellular carcinoma cell line (HepG2).

The antiproliferative effect of astaxanthin on human glioma cell lines (LN-229, HNGC2) and liver hepatocellular carcinoma cell line (HepG2) were studied. The *in vitro* cytotoxicity of astaxanthin and only culture media for negative control, was investigated in human glioma cell lines (LN-229 and HNGC2) and HepG2 liver cell line by using an MTT assay. The results after incubation of astaxanthin at 24 h, 48 h, and 72 h were shown in Fig. 127-129. The wells that received only media were regarded as a negative control with a cell viability of 100% Fig. 130. shows the percent viability of LN-229, HNGC2 and HepG2 cell lines after exposing to different concentration of astaxanthin for 24 h, 48h and 72 h respectively. In the case of LN-229 cells, at the end of the 24h, 48h, 72h the cytotoxicity was 99, 92 and 68% was observed at 15 µmol/ml, whereas in 30 µmol/ml incubation it was 87, 67 and 42%. At the end of the 72 h, the maximum activity was found to be 42% at 30 µmol/ml. The cell viability decreased in all the tested concentration. The 50% inhibition was observed at 25.86 µmol/ml. At the end of the 24 h, astaxanthin had significant inhibition of the cell growth and enhanced cytotoxicity was 89% and 73% at 15-30 µmol/ml, whereas at the end of the 48 h and 72 h it was 83 and 36% and 72 and 22% were observed in HNGC2. At the end of the 72 h, the maximum cytotoxicity was found to be 22% at 30 µmol/ml. The 50% cytotoxicity effect was found at 20 µmol/ml. Similarly in HepG2 cells, after incubation of astaxanthin at 24 h, 48 h and 72 h the cytotoxicity was found to be 81, 78, 73% at 15 µmol/ml, whereas it was 58, 52, and 31% at 30 µmol/ml. The highest activity was 31% observed at 30 µmol/ml after 72h

incubation. At the end of the 24 h incubation, the 50% inhibition was found to be 25.86 µmol/ml. At the highest concentration 30 µmol/ml, the cell viability decreased in all the cells.

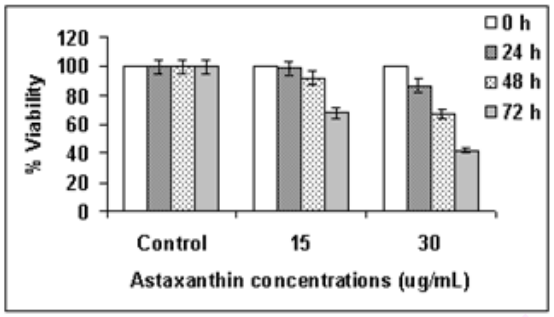


Fig. 127. Viability of LN-229 cell lines after 24 h, 48 h and 72 h exposure to different astaxanthin.

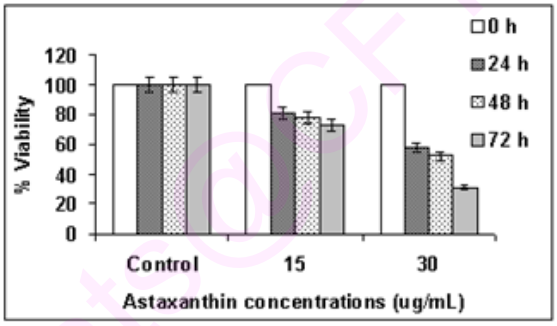


Fig. 128. Viability of HNGC2 cell lines after 24 h, 48 h and 72 h exposure to different astaxanthin.

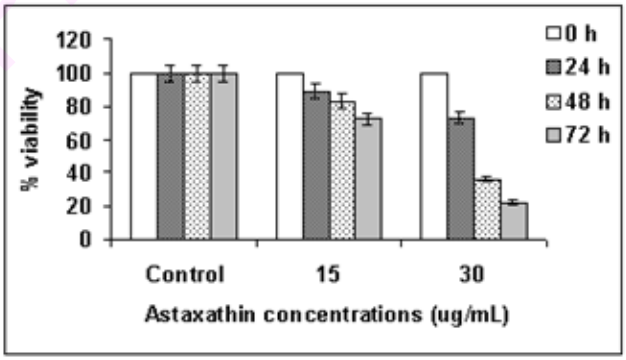


Fig. 129. Viability of HepG2 cell lines after 24 h, 48 h and 72 h exposure to different astaxanthin.

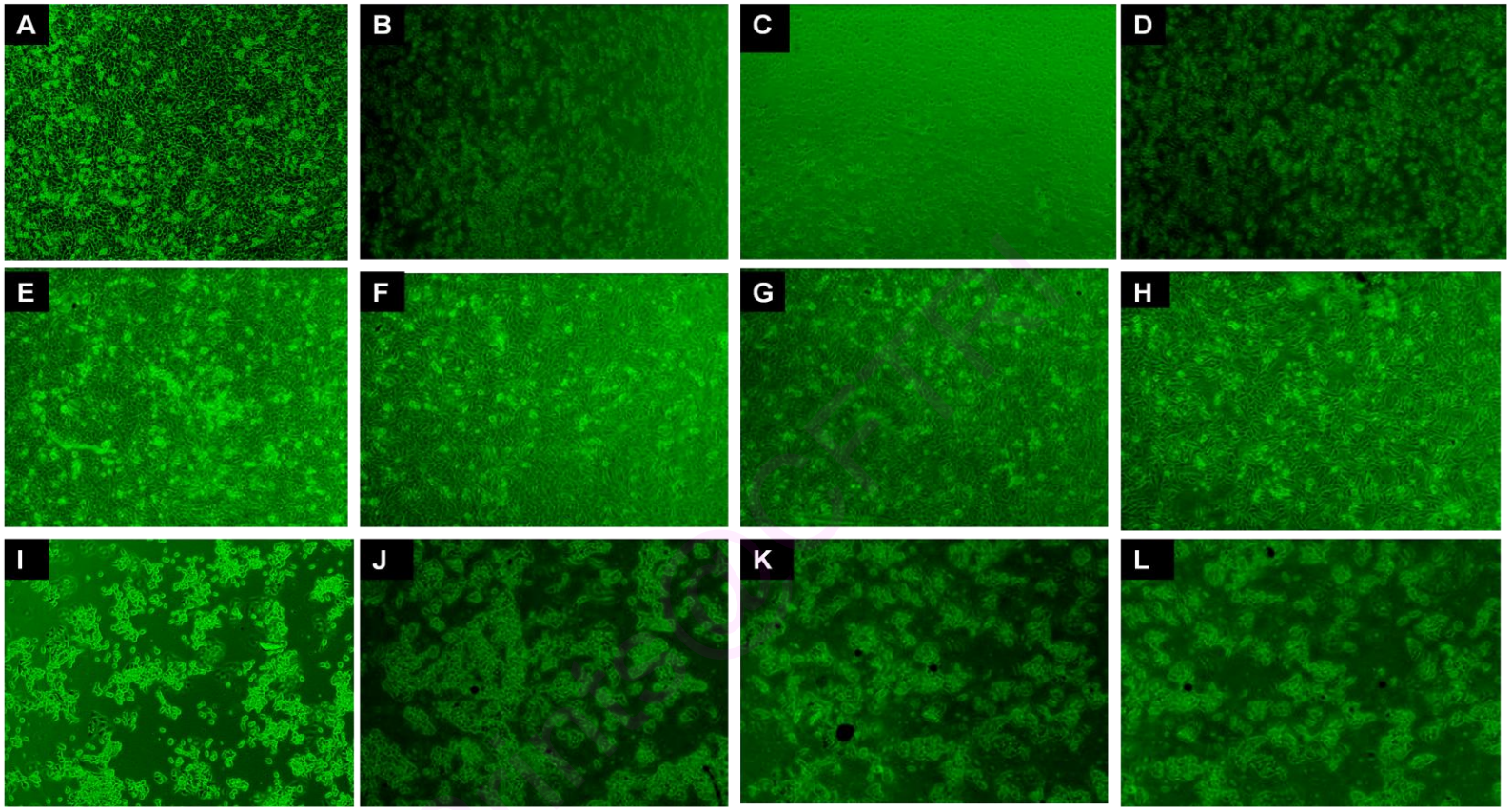


Fig. 130. Morphology of LN-229, $HNGC_2$ human glioma cells and $HepG_2$ liver hepatic cells treated with astaxanthin and visualized under inverted microscope. A. Control LN-229 cells, (B-D) incubation of astaxanthin in LN-229 cells at 24 h, 48 h, and 72 h, (E). Control $HNGC_2$ cells, (F-H) Incubation of astaxanthin in HNGC2 cells at 24 h, 48 h and 72 h, (I) Control $HepG_2$ cells, (J-L). Incubation of astaxanthin in $HepG_2$ cells at 24 h, 48 h and 72 h.

5.9.3. Antiproliferative properties of monoester of astaxanthin from *H. pluvialis* on human glioma cell lines (LN-229, HNGC2) and liver hepatocellular carcinoma cell line (HepG2).

The antiproliferative effect of monoester of astaxanthin on human glioma cell lines (LN-229, HNGC2) and liver hepatocellular carcinoma cell line (HepG2) were studied. The *in vitro* cytotoxicity of monoester of astaxanthin and only culture media for negative control, was investigated in human glioma cell lines (HNGC2, LN-229) and HepG2 liver cell line by using an MTT assay. The antiproliferative effect of monoester of astaxanthin on human glioma cell lines (LN-229, HNGC2) and liver hepatocellular carcinoma cell line (HepG2) were studied. The *in vitro* cytotoxicity of astaxanthin and only culture media for negative control, was investigated in human glioma cell lines (LN-229 and HNGC2) and HepG2 liver cell line by using an MTT assay. The results after incubation of astaxanthin at 24 h, 48 h, and 72 h were shown in Fig. 131A-C. The wells that received only media were regarded as a negative control with a cell viability of 100% Fig. 132 shows the percent viability of LN-229, HNGC2 and HepG2 cell lines after exposed to different concentration of astaxanthin for 24 h, 48h and 72 h respectively. In the case of LN-229 cells, at the end of the 24h, 48h, 72h the cytotoxicity was 85%, 78% and 55% was observed at 15 µmol/ml, whereas in 30 µmol/ml incubation it was 75%, 65% and 38%, at the end of the 72 h, the maximum activity was found to be 38% at 30 µmol/ml. The cell viability was decreased in all the tested concentration, whereas in HNGC2 cells it was 82%, 75%, 65% and 68%, 35% and 21%, at the end of the 72 h, the maximum cytotoxicity wsa found to be 21% at 30 µmol/ml. Similarly in HepG2 cells, after incubation of monoesters of astaxanthin at 24 h, 48 h and 72 h the cytotoxicity was found to be 75%, 64%, 58% and 55%, 48% and 28% at 15 and 30 µmol/ml. The highest activity was 28% observed at 30 µmol/ml after 72h incubation. At the end of the 24 h incubation, the 50% inhibition was found to be 23.86 µmol/ml. At the highest concentration 30 µmol/ml, the cell viability was decreased in all the cells.

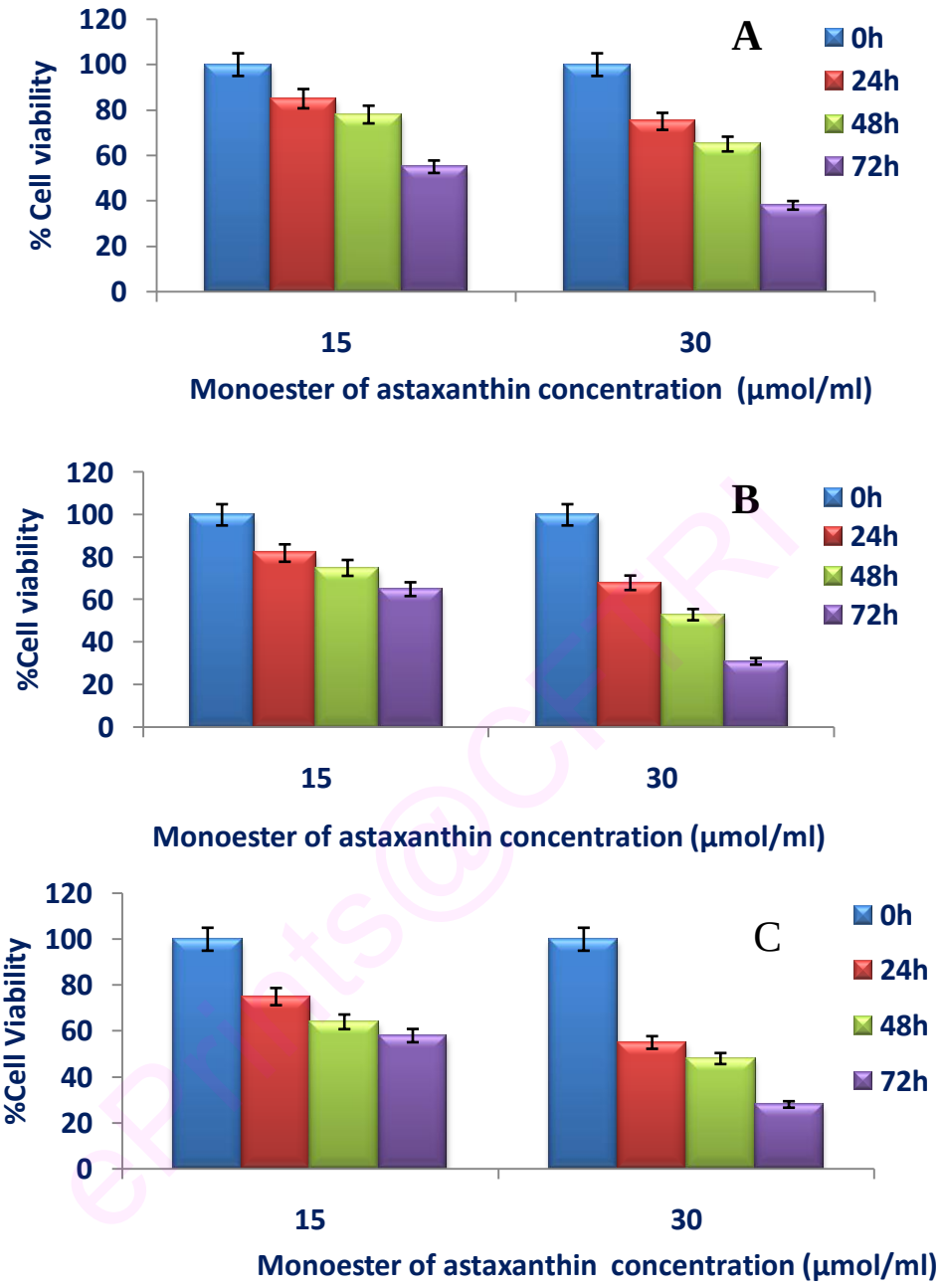


Fig. 131. Viability of LN-229 (A), HNGC2 (B), HepG2 (C) cell lines after 24 h, 48 h and 72 h exposure to different monoester of astaxanthin concentrations

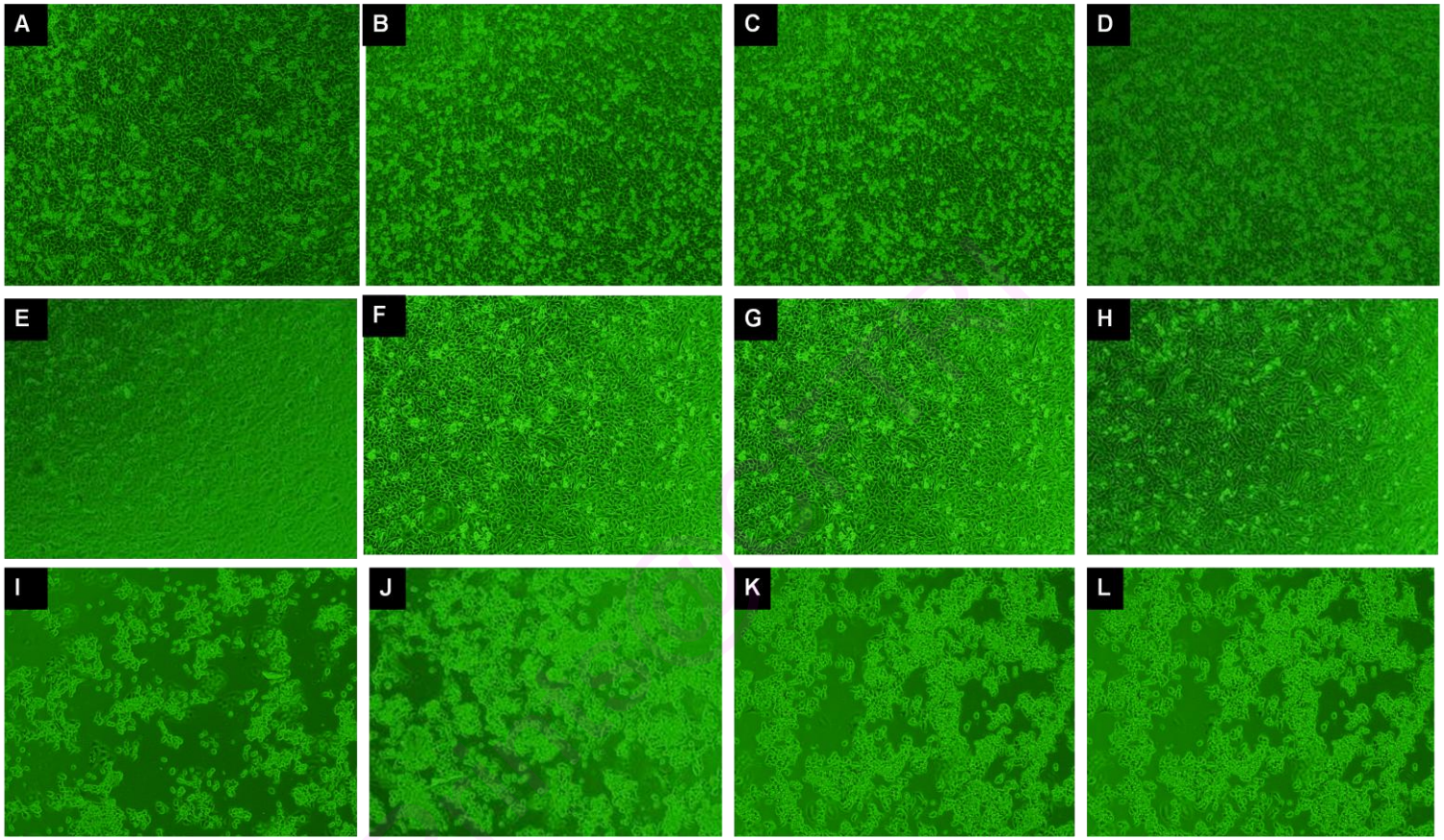


Fig. 132. Morphology of LN-229, HNGC2 humanglioma cells and $HepG_2$ liver hepatic cells treated with mono ester of astaxanthin and visualized under inverted microscope. A. Control LN-229 cells, (B-D) incubation of mono ester of astaxanthin in LN-229 cells at 24, 48 and 72 h, (E). Control HNGC2 cells, (F-H) Incubation of mono ester of astaxanthin in HNGC2 cells at 24, 48 and 72 h, (I) Control $HepG_2$ cells, (J-L). Incubation of monoester of astaxanthin in $HepG_2$ cells at 24, 48 and 72 h.

5.9.4. Antiproliferative properties of diester of astaxanthin from *H. pluvialis* on human glioma cell lines (LN-229, HNGC2) and liver hepatocellular carcinoma cell line (HepG2).

The antiproliferative properties of diesters of astaxanthin on human glioma cell lines (LN-229, HNGC2) and liver hepatocellular carcinoma cell line (HepG2) were studied. The *in vitro* cytotoxicity of diester of astaxanthin and only culture media for negative control, was investigated in human glioma cell lines (HNGC2, LN-229) and HepG2 liver cell line by using an MTT assay. The results after incubation of diester of astaxanthin at 24, 48, and 72 h were shown in Fig. 133-135. In the case of LN-229 cells, at the end of the 24h, 48h, 72h the cytotoxicity was 82%, 78 % and 60% was observed at 15 µmol/ml, whereas in 30 µmol/ml incubation it was 74%, 62 %and 34%, At the end of the 72 h, the maximum activity was found to be 34% at 30 µmol/ml. The cell viability decreased in all the tested concentration. In HNGC2 cells it was 75%, 68%, 65% at 15 µmol/m, whereas in 30 µmol/ml treated culture was 65%, 32% and 21%. At the end of the 72 h, the maximum cytotoxicity was found to be 21% at 30 µmol/ml. Similarly in HepG2 cells, after incubation of monoesters of astaxanthin at 24 h, 48 h and 72 h the cytotoxicity was found to be 70%, 68%, 45% and 59%, 51% and 25% at 15 and 30 µmol/ml. The highest activity of 58% was observed at 30 µmol/ml after 72h incubation.

5.9.5. Astaxanthin and its esters induced apoptosis on human glioma cell lines (LN-229, HNGC2) and liver hepatocellular carcinoma cell line (HepG2).

Analyzed the apoptosis cells, LN-229, HNGC2 and HepG2 induced to astaxanthin & its esters using confocal microscope techniques. For comparison, the morphology of the untreated cells was initially observed by phase contrast microscope. After incubation of both cell lines with astaxanthin, monoester of astaxanthin, blank DMSO, PBS buffer (concentration same as MTT cytotoxicity assay) for 24h, 48h, 72h, the morphology of both cell lines changed significantly as compared to the untreated cells (Fig. 136-138). The cells shrunk to a spherical shape and most of them were detached from the cover slip. The cytoplasm of the cell was distributed badly and formed the apoptotic bodies, and some of the cells were budding. Because of apoptosis most of the cells were detached from the cover slips and washing further removed the apoptotic cells, but the main

apoptosis features like cell shrinkage, chromatin condensation, and nuclei fragmentation were clearly observed with the help of confocal microscopy. CLSM images clearly demonstrated the apoptosis-induced cell death by astaxanthin on human glioma cell lines (HNGC2 and LN-229) and liver hepatocellular carcinoma cell line (HepG2).

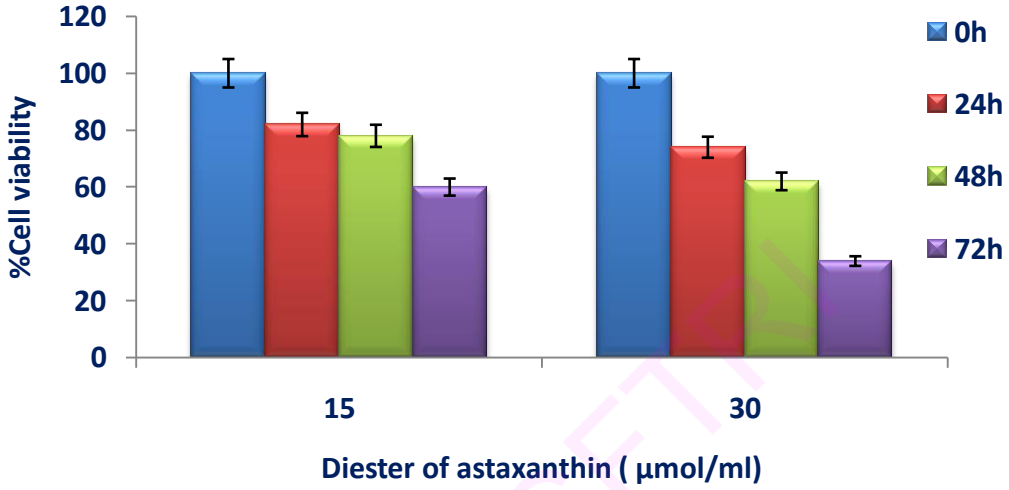


Fig. 133. Viability of LN-229 cell lines after 24 h, 48 h and 72 h exposure to diester of astaxanthin.

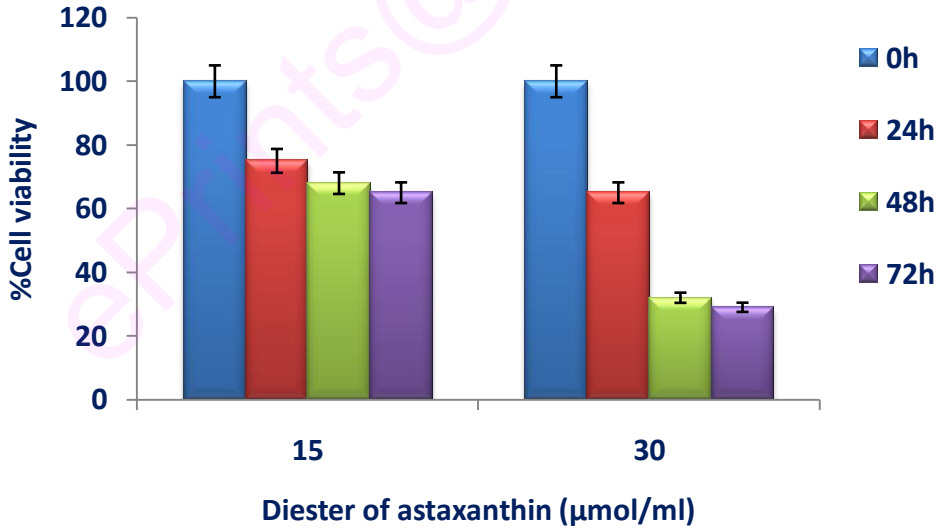


Fig. 134. Viability of HNGC2 cell lines after 24 h, 48 h and 72 h exposure to diester of astaxanthin.

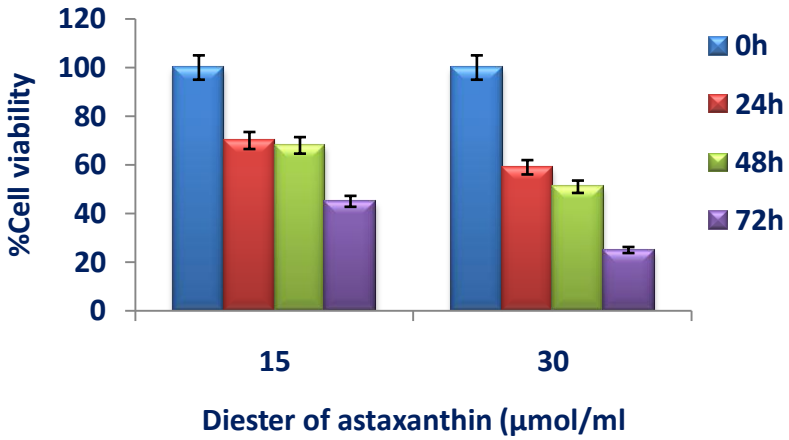


Fig. 135. Viability of HepG2 cell lines after 24 h, 48 h and 72 h exposure to diester of astaxanthin.

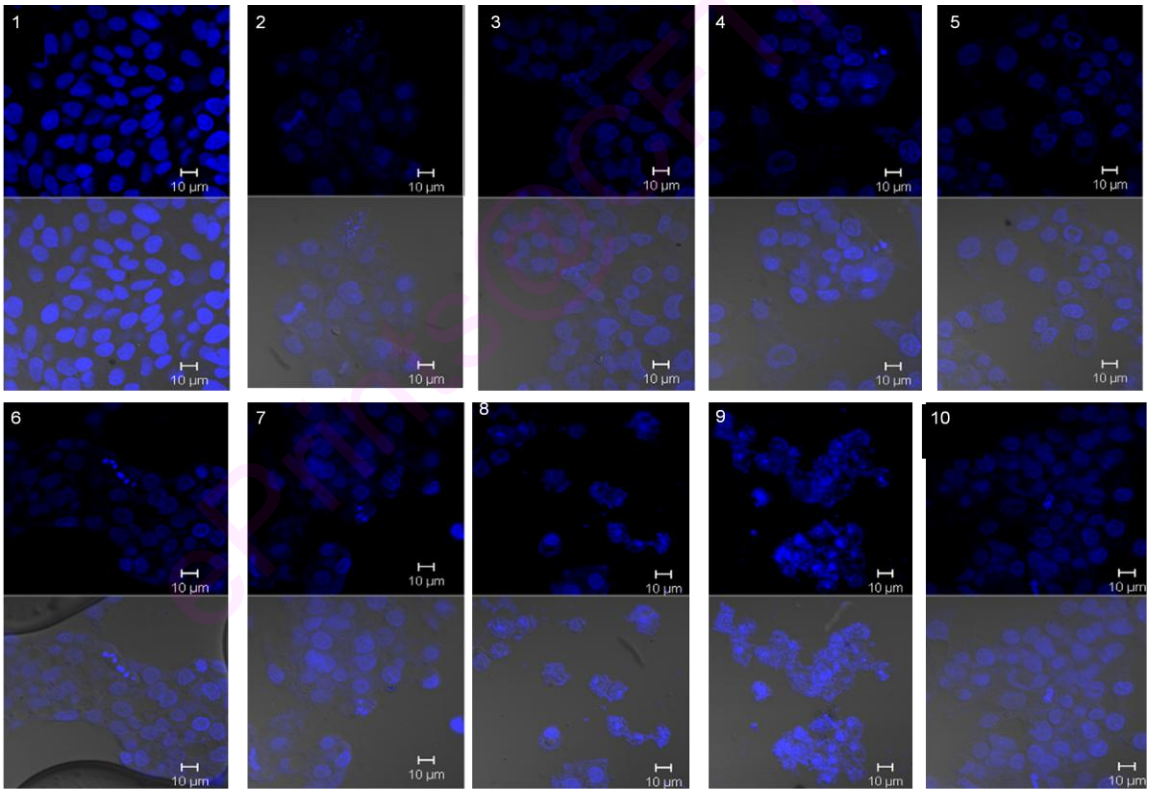


Fig. 136. Confocal microscopy observations of HepG2 cells apoptosis induced by astaxanthin. (1). HepG2 cells control, (2-10). Different concentration of astaxanthin.

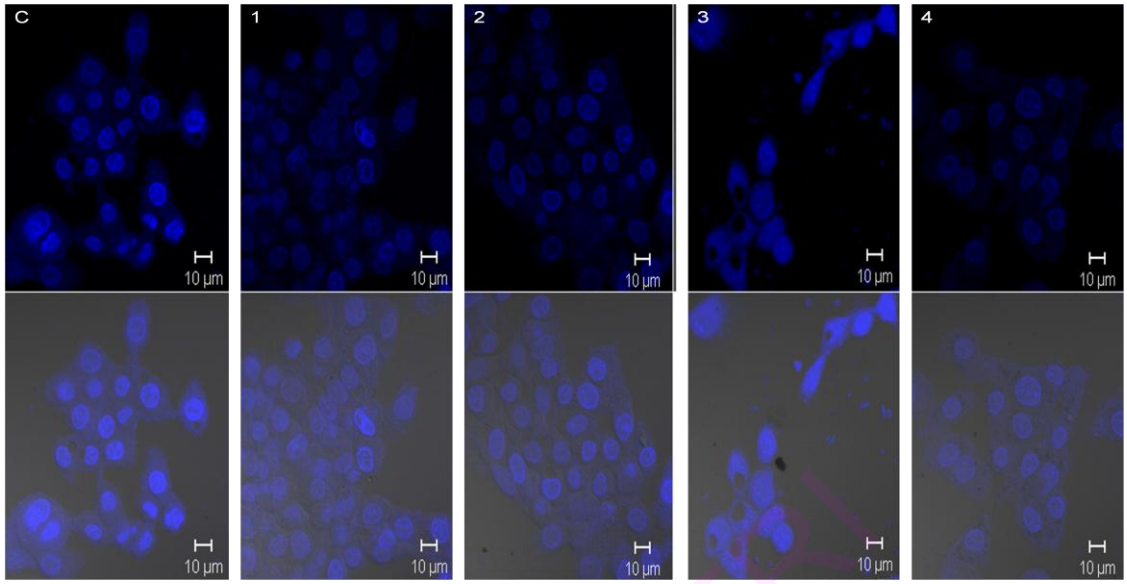


Fig. 137. Confocal microscopy images to demonstrate the apoptosis induced by monoester of astaxanthin. (C). Control $HepG_2$ cells (1-4). Treated with different concentration of astaxanthin monoester.

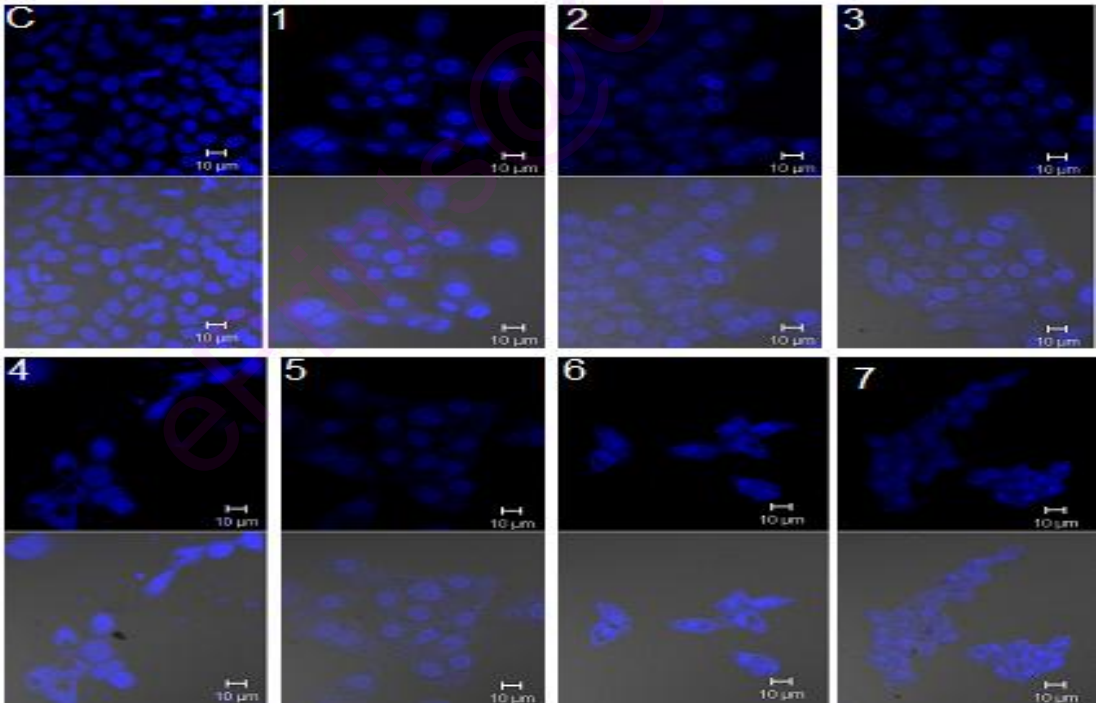


Fig. 138. Confocal microscopy images to demonstrate the apoptosis induced by diester of astaxanthin and synthetic astaxanthin. (C). $HepG_2$ cells control, (1-4). Treated with different concentration of astaxanthin diester, (5-7). Treated with different concentration of synthetic astaxanthin.

5.9.6. Inhibition of cell cycle progression and apoptosis in HNGC2 cells

The antiproliferative effect observed in astaxanthin esters treated cell lines was further verified by flow cytometric analysis of DNA content. Loss of DNA is a typical feature of apoptotic cells. Exploration of the intrinsic mechanism of the inhibitory effect on cell growth and cell cycle arrest was performed with 30 μmol/ml of the astaxanthin esters or without by FACS analysis after 24, 48 and 72 h of culture. The DNA content histograms obtained after staining permeabilized cells. The FACS analysis of control cells showed prominent G1, followed by S and G2/M phases. In the treated group, a typical subdiploid peak was observed after 72 h of culture, which implied the presence of cells with fragmented DNA. It was necessary to distinguish the source of cell death as either nectotic or apoptotic cells. The astxanthin esters induced the apoptosis of HNGC2 cells. From the results of flow cytometric analysis (**Fig. 139**). It was observed that there was a significantly increased percentage at the G2/M phase, along with a dramatically decreased cell population of the G1 Phase. The value of G2/M phase increased upto 85% in treated cells after 72 h of culture, whereas the value of G2/M phase in control cells was around 20%. These results suggested that the esters could inhibit cell proliferation by inducing the G2/M arrest in HNGC2 through regulating the G2 Check point.

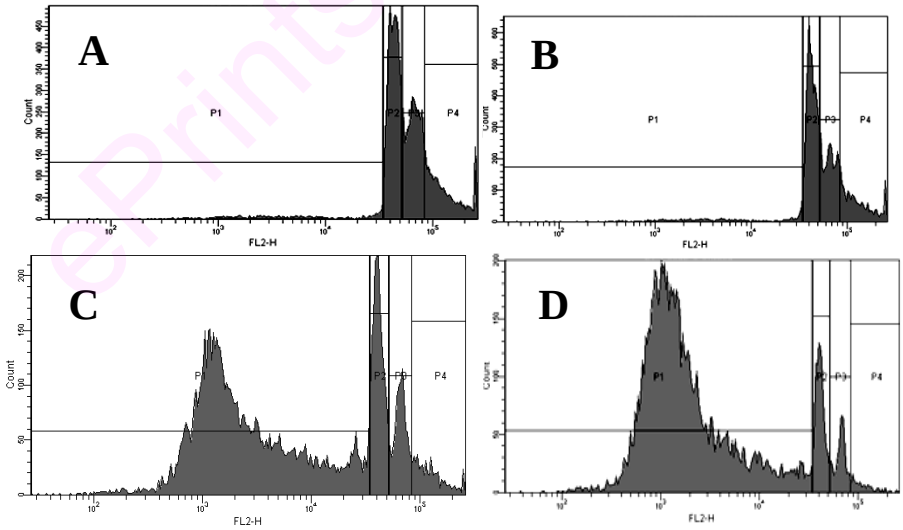


Fig. 139. Inhibition of cell cycle progression and apoptosis in HNGC2 cells by astaxanthin & its esters. Cell cycle progression (Phases of G0/G1, S and G2/M) and apoptosis (SubG1 phase) was measured by flow cytometry. This distribution of cells in different phases of the cell cycle. (A) control, (B) DMSO, (C and D) astaxanthin and its esters.

5.10. Discussion

The present investigation was on prevention of UV-DMBA induced skin carcinogen`s in rat model by astaxanthin & its esters isolated from cultured green algae *H. pluvialis*. The free radical scavenging and antioxidant activities play an important role in prevention of free radical–related diseases, including aging and ulcers (Packer, 1995). The current study addressed the identification of a potent antioxidant of astaxanthin and its esters from *H. pluvialis* and determined its antioxidant properties and inhibition of tyrosinase activity in *in vivo* rat models (Fig. 119 & 120). Skin is the largest organ in the human body and is exposed to a wide variety of environmental risk factors. It is well known that the skin function and appearance is affected by the nutritional status. Dietary supplementation in the animals with vitamins, minerals, essential fatty acids result in improved skin conditions. In this respect, the importance of the dietary source for photo protection has gained a great deal of interest. Many reports linked the beneficial properties of astaxanthin to their antioxidant activities. There are no reports on the anti-ageing efficacies of the dietary astaxanthin & its esters, UV-damaged skin cancer. In this present study we address the efficacy of astaxanthin & its esters in comparison with total carotenoid extract and standard astaxanthin from *H. pluvialis* at doses of 100 and 200 µg/kg b.w. against DMBA-UV induced skin carcinogenesis. It is well known that astaxanthin is highly lipophilic compound, therefore the function of astaxanthin as a free radical scavenger and antioxidants likely assisted by the ease with which it crosses morpho-physiological barriers. Tso and Lam (1996) had demonstrated that astaxanthin can cross blood retinal barrier in mammals and can extend its antioxidant benefits beyond that barrier. In addition to the fact that astaxanthin can readily enter into sub cellular compartments, where free radicals may be generated, it has no known toxic effects (Guerin et al., 2003).

H. pluvialis, in the presence of astaxanthin esters has an added advantage that, generally carotenoids, although potential antioxidants, they may lack such properties in *in vivo*, because of pro-oxidant effect. Esterified astaxanthin shows comparatively better stability than free astaxanthin, and hence it may exhibit more health beneficial effects than free astaxanthin. *H. pluvialis* may be a potential natural source for the isolation of

esterified astaxanthin and to deploy them for health beneficial effects against several disorders. Further, carotenoid esterification does not pose impediment for bioavailability in humans (Bowen et al., 2002).

The astaxanthin mono-di esters were showed potent inhibitory activity when compared to total carotenoid and standard astaxanthin. The process of oxidation of low-density lipoprotein is mediated by 15-lipoxygenase, and is believed to play a key role in mediating inflammatory reactions in ulcerous condition (Steinberg, 1999; Gundersen et al., 2003). Results may imply their beneficial role in the potential management of skin cancer. The present data on *in vivo* anticancer properties of total carotenoid and astaxanthin esters, thus suggest that astaxanthin esters may be a major anticancer properties. Ever since the role of carotenoids is known in the literature for their strong antioxidant and health beneficial properties against oxidative stress induced chronic diseases, several fruits and vegetable sources were explored. It is an attempt to use natural and synthetic compounds to intervene in the early stages of cancer, before invasive disease begins. Chemopreventive agents can act in two ways, they can prevent or stop the genetic mutations that lead to cancer, or they can prevent or stop the processes that promote proliferation (Chan et al., 2003). Various natural carotenoids have been shown to have chemopreventive activity, and some of them have been demonstrated to be more potent than β-carotene. The carotenoids including α-carotene, lutein, zeaxanthin, lycopene, β-cryptoxanthin, fucoxanthin, astaxanthin, capsanthin, crocetin and phytoene, as well as β-carotene, may be useful for cancer prevention (Nishino et al., 2002).

Carotenoids have also been shown to inhibit the proliferation of human breast cancer MCF-7 cell line *in vitro* (Li et al., 2002) and oral administration of astaxanthin has been shown to reduce the incidence of pre-neoplastic lesions and neoplasm in mice given with a bladder carcinogen. The carotenoids significantly reduced the incidence of bladder cancer. Astaxanthin is a possible chemopreventive agent for bladder carcinogenesis and that the effect may be partly be due to suppression of cell proliferation (Tanaka et al., 1994). Astaxanthin has also been shown to reduce the occurrence and development, of chemically induced oral cancer in rats (Tanaka et al., 1995a) and inhibit the growth of mammary tumors in mice (Chew et al., 1999). Mammary tumor growth

inhibition by astaxanthin was dose-dependent and was higher than that of canthaxanthin and β-carotene. Lipid peroxidation activity in tumors was lower in mice with fed 0.4% astaxanthin, but not in those fed with β-carotene and canthaxanthin. One of the mechanisms by which astaxanthin exerts antitumor activity may be via enhancement of immune responses. A study in mice found that astaxanthin suppressed transplanted fibro sarcoma tumor cell growth and stimulated immunity against tumor antigen. The astaxanthin-fed mice had significantly lower tumor size and weight than controls when supplementation was started one and three weeks before tumor inoculation (Jyonouchi et al., 2000).

Astaxanthin also improves antitumor immune responses by inhibiting lipid peroxidation induced by stress. Mice subjected to restraint stress had reduced total number of spleen cells and reduced NK cell activity. The stress also caused a significant increase in the lipid peroxidation of liver tissue. Astaxanthin improved the immunological dysfunction induced by restraint stress. The restraint stress also promoted hepatic tumors induced by inoculation. Daily oral administration of astaxanthin markedly attenuated the promotion of hepatic metastasis. These results suggested that astaxanthin improves antitumor immune responses by inhibiting of lipid peroxidation induced by stress (Kurihara et al., 2002). A recent review suggests that the antineoplastic properties appear tightly correlated to their ability to induce the gap junctional protein connexin-43 (Cx43). Up regulation of connexin-43 leads to decreased proliferation and decreased indices of neoplasia in animal and human cells (Hix et al., 2004). The present data on *in vivo* anticancer properties of SASX, ASX, TC, MASX and DASX, thus suggest that astaxanthin esters may be a major anticancer components present in the *H. pluvialis* extract. Further evaluation of biochemical changes like catalase, superoxide dismutase, glutathione peroxidase in control, UV-DMBA and treated animal groups revealed that the anti-cancer potency may be due to its showed *in vitro* and *in vivo* antioxidant, anti ulcer properties which would eliminate the oxidative stress condition during stress condition

Astaxanthin structure is very close to lutein and zeaxanthin and its protection from UV-light (Connor and Brien, 1998). However, an animal study Tso and Lam, (1996) demonstrated that astaxanthin can deposit in the retina of mammals, thus

protecting from light-induced damage and improving visual performances. The benefits of lutein and zeaxanthin carotenoids in human do not stop with eye health. Recent studies suggest that they may help to maintain heart health by reducing the risk of cardiovascular disease, and protect skin from UV induced damage (Mares-Perlman et al., (2002); Chen et al., (2002). Astaxanthin is believed to protect the skin and eggs of salmon against UV-light photo-oxidation (Meyers 1993). Astaxanthin supplementation helped in protecting the retinal photoreceptors in the eyes of rats exposed to acute UV-light injury (Tso and Lam, 1996) and the *in vitro* protective effect of astaxanthin against UV-induced photo oxidation (Connor and Brien, 1998) was stronger when compared with β-carotene and lutein. These findings suggest that astaxanthin has an excellent potential as an oral sun-protectant. More studies are needed to better understand the possible interactions between various antioxidants and their potential prooxidative role, to determine under which conditions supplementation with carotenoids such as astaxanthin can help reduce skin carcinogenesis.

H. pluvialis, being a microalga, there are multi-advantages, like its utilization as nutraceutical or food ingredient and it has been approved by the United State of Food and Drug Administration (USFDA) as a dietary ingredient. Presence of higher levels of astaxanthin esters in *Haematococcus* may also reduce the requirement in quantity, so that, an added advantage of supply to larger population can be anticipated. In conclusion, this is first evidence that isolated molecules such as SASX, FASX, TC, MASX and DASX from *Haematococcus* are having a potent anticancer properties, inhibition of tyrosinase enzyme in DMBA-UV induced rat skin models upon oral administration.

We have evaluated the inhibitory effects of astaxanthin and its esters on the proliferation of human glioma cell lines (HNGC2 and LN-229) and liver hepatocellular carcinoma cell line (HepG2). Astaxanthin and its esters inhibited the growth of cell lines and more potentially inhibited the growth of HNGC2 and LN-229 cell than the hepatocellular carcinoma HepG2 cell line. These findings suggest that astaxanthin esters has a higher potential for cancer prevention. The inhibition depended on the concentration of mono, diester of astaxanthin used and the duration of the treatment. No significant growth inhibitory or stimulating effects were found in cells treated with

various concentrations of astaxanthin esters in the first 24 h. At the end of the 72 h, 50% inhibition was observed at 35.71 µmol/ml, 20 µmol/ml and 25.86 µmol/ml of astaxanthin, whereas in the case of monoester it was 24.19 µmol/ml, 18.98 µmol/ml and 20.93 µmol/ml. Similarly in diester group the 50% inhibition was seen at 22.72 µmol/ml, 18 µmol/ml and 20 µmol/ml. The more inhibitory effect on HNGC2 cell lines found in this study is also consistent with the epidemiologic and clinical observations that astaxanthin and its esters. Antioxidant compounds can decrease mutagenesis, and thus carcinogenesis, both by decreasing oxidative damage to DNA and by decreasing oxidant-stimulated cell division (Ames, 1993). Effect of astaxanthin on improvement of the proliferative capacity as well as the osteogenic and adipogenic differentiation potential in neural stem cells (NSCs) was evaluated by Jeong et al., (2010). Joseph et al., 2010 suggested that astaxanthin administration may be beneficial in treatment of dogs for osteosarcoma. The effect of astaxanthin on cell proliferation, cell cycle progression and apoptosis was examined in the HepG2 human liver cancer cell line, it is significantly inhibited the proliferation of liver cancer cells in a dose-dependent manner and flow cytometric analysis demonstrated that astaxanthin restrained the cell cycle progression at G_1, and induced apoptosis. Astaxanthin has an affirmative and beneficial effect against chemically induced colonic pre-neoplastic progression in rats induced by DMH (Prabhu et al., 2009). Pre neoplasms and neoplasms induced by OH-BBN, and the antiproliferative potential, was greater for astaxanthin and canthaxanthin (Tanaka et al., 1995b). Methylcholanthrene-induced (Meth-A) mouse tumor cells grown in an astaxanthin supplemented medium had reduced cell numbers and lower DNA synthesis rates 1-2 days post incubation than control cultures (Sun et al., 1998). Similarly, astaxanthin inhibited murine mammary tumor cell proliferation by up to 40%, in a dose-dependent fashion, when included in the culture medium (Kim et al., 2001). In addition, of eight carotenoids tested, astaxanthin was the most effective at inhibiting the invasion of rat hepatoma cells in culture (Kozuki, et al., 2000). The growth of human cancer cell lines has also been inhibited by astaxanthin *in vitro*. Two human colon cancer cell lines were significantly less viable than control cultures after a four-day incubation with astaxanthin, although a stronger effect was seen from α-carotene, β-carotene or

canthaxanthin (Onogi, et al., 1998). Also, a weak effect of astaxanthin on human prostate cancer cell viability has been noted, but in this case neoxanthin and fucoxanthin appeared to be much more effective (Kotake-Nara et al., 2001). On the other hand, significant inhibition of androgen-induced proliferation of human prostate cancer cells was recently demonstrated in the presence of astaxanthin (Levy et al., 2002). Exposure to UVA radiation is believed to be the primary causative agent in skin tumor pathogenesis, both synthetic astaxanthin, human skin fibroblasts, melanocytes and intestinal CaCo-2 cells in culture (Lyons and Brien, 2002). The neuroprotective effect of astaxanthin is suggested to be dependent upon its antioxidant potential and mitochondria protection. It is suggested that treatment with astaxanthin may be effective for oxidative stress-associated neuro degeneration (Liu and Osawa, 2009, Ikeda et al., 2008). Growth-inhibitory effects by *H. pluvialis* were also observed in HT-29, LS-174, SW-480 cells (Palozza et al., 2009).

Many molecular targets, involved mainly in carcinogen metabolism, hormonal regulation, the cell cycle, apoptosis, DNA repair, cell signaling, and differentiation were reported to be independently associated with human prostatic carcinogenesis (Giovannucci et al., 1995 and Clinton, 1998). Although the mechanism by which astaxanthin inhibits the growth of human cancer cell lines are not well understood, protection against oxidative damage, induction of cellular processes controlling cell growth are considered to be possible mechanisms of astaxanthin action. The suppression of the proliferation of human glioma cell lines found in this study may be due in part to the direct effects of astaxanthin on cellular processes controlling cell growth and the induction of apoptosis. Astaxanthin and its esters induced growth arrest in the HNGC2 and LN-229 cells by increasing the accumulation of cells in the G0/G1 phase. Apoptosis creates as demonstrated by an increase in the sub G1 phase and decreasing DNA content in the G2/M phase, was affected by astaxanthin treatment in a dose dependent manner. This suggests that astaxanthin and its esters like many other chemopreventive agents follows a similar mechanism in inducing cell growth arrest and apoptosis. Molecular targets of astaxanthin in cell cycle check points and pathways of apoptosis in different stages of glioma and liver cancer cells will be investigated. In summary, we found a potent inhibitory effect of astaxanthin and its esters on the growth of human glioma cell

lines (HNGC2 and LN-229) and liver hepatocellular carcinoma cell lines. Results of this study also support epidemiologic findings that astaxanthin and its esters are effective chemo preventive agent for human glioma, liver cancer and may contribute to the reduced glioma, liver cancer risk observed in individuals who consume large amounts of nutraceutical based astaxanthin rich *H. pluvialis*

5.11. Salient features

The effect of the isolated astaxanthin & its esters on ultraviolet (UV) & 7, 12-dimethylbenz (a) anthracene (DMBA) induced skin carcinogenesis in i*n vitro* and *in vivo* rat models were evaluated. Astaxanthin & its esters influenced protection of Red Blood Cells by 70%, human buccals cells by 80% and DNA damage by 70% against the oxidant induced treatments. Total carotenoid (TC), free astaxanthin (FASX), monoester (MASX), diester of astaxanthin (DASX) and synthetic astaxanthin (SASX) were orally administered to rats at 100 and 200 µg carotenoid /kg b.w prior to UV-DMBA induced skin carcinogenesis. At 200 µg carotenoid /kg b.w dosage astaxanthin esters afforded significant protection on the UV-DMBA induced skin cancer. The significant results were obtained in 200 µg carotenoid /kg b.w treated group with effective decrease in the tumor incidence in comparison to the control group. Pretreatment with carotenoids containing astaxanthin esters significant increase in antioxidant enzyme levels-catalase (2.5 fold), superoxide dismutase (3 fold), glutathione (1.6 fold decrease) in serum whereas in liver the catalase (2.2 fold increased), superoxide dismutase (2.7 fold increased), glutathione (1.4 fold decreased) as in a dose dependent manner. Tyrosinase activity was inhibited nearly 75% in serum, liver and skin homogenates treated with astaxanthin esters at 200 µg/kg b.w. TBARS levels were recovered up to 60-65% upon treatment with astaxanthin esters at 200 µg/kg in plasma, liver and skin homogenates. This experiment shows protection offered by astaxanthin & its esters for decrease in tumor incidence in carotenoid treated animals. Further studies are needed to understand the mechanism & efficacy.

Inhibitory effect of astaxanthin & its esters on human glioma cell lines (LN-229, HNGC2) and liver hepatocellular carcinoma cell line (HepG2) was evident upon treatment for 24 h, 48 h and 72 h in (3-(4, 5-dimethyathiazol-2-yl)-2, 5-diphenyl

tetrazolium bromide) assay. The apoptosis of cells on LN-229, HNGC2 and HepG2 cells induced by astaxanthin and its esters was monitored using phase-contrast microscopic observations and confocal microscope techniques. These *in vivo* studies also corroborate the antiproliferative activity of cancer cell lines. Astaxanthin and retinol levels were quantified in serum and liver after administration of carotenoids to rats. The maximum astaxanthin (366 ng/ml) and retinol (72 ng/ml) were found in serum of DASX treated group, when compared to healthy control group. In the liver, the astaxanthin and retinol content were found to be 22.5 ng/g and 440 ng/g respectively.

SUMMARY AND CONCULSION

6.0. Summary and conclusion

There is a considerable interest, demand and search worldwide for novel and sustainable source of carotenoids in general and astaxanthin in particular. This is mainly due to its functional attributes viz., precursor for vitamin A, antioxidant potentials and also as natural color. With current consumer awareness regarding the benefits of naturally derived carotenoids over synthetic ones, extensive research is going on worldwide in utilizing of biotechnology as a tool for such compounds. Microalgae producing various pigments, have received the attention of food technologist. The green microalga *Haematococcus* produces astaxanthin and its esters. Among total carotenoids produced in this alga, major ones are astaxanthin and its esters, accumulating up to 80% of the carotenoids, with mono-ester constituting 70%, di-ester, 15-20% and free astaxanthin 4-5% under different cultural conditions. The aim of present investigation was to study the factors responsible for the culture of *Haematococcus* to support growth as well as astaxanthin production; investigations on the factors which maximize astaxanthin production with respect to various stress conditions, characterization of carotenoids produced in *Haematococcus,* stability of astaxanthin and its esters, antioxidant and anticancer properties of astaxanthin and its esters.

Objectives of the thesis are as follows:

- To study expression of astaxanthin production and enhancing the yield from cultured green alga *Haematococcus pluvialis.*
- To study the effect of expressed astaxanthin from *Haematococcus pluvialis* on biological activities, using *in vitro* and *in vivo* models.
- Evaluation of safety of *Haematococcus pluvialis* biomass in experimental animals.

The growth of *H. pluvialis* and total carotenoid production, with special focus on astaxanthin was evaluated under various culture regimes viz., nitrogen sources, carbon sources and salt types. After 21 days of culture with various nitrogen sources, its effects on algal biomass yield, total carotenoid, astaxanthin contents were monitored. The maximum biomass yield of 3.3 g/L, total carotenoid 2.9% and total astaxanthin with esters content of 2.5% were observed in case of sodium nitrate (10mM) treated culture; whereas in potassium nitrate treatment at 10 mM concentration the biomass yield was 2.7 g/L, total carotenoid 1.8% and total

astaxanthin 1.6%. Similarly, in the case of media with ammonium nitrate (10 mM) the biomass yield was 1.5 g/L, total carotenoid 1.7% and total astaxanthin 1.4% . At same level of calcium nitrate, the biomass yield 1.6 g/L, total carotenoid content 1.3% and total astaxanthin content 1.2%.. Another experiment was conducted to enhance the carotenoid content in *H. pluvialis* by studying the influence of different salt sources on growth of alga and carotenoid yields. After 21 days of culture with potassium chloride at 16 mM levels the maximum biomass yield of 2.5 g/L, total carotenoid 2.3% and total astaxanthin levels 1.95% was obtained which was an improvement in terms of overall productivity. The effect of different carbon sources on the biomass yield, total carotenoid and total astaxanthin content in *H. pluvialis* was studied. Of the carbon sources, ammonium carbonate(3 mM) treated culture resulted in maximum biomass yield 2.9 g/L, total carotenoid 2.6% w/w and total astaxanthin content 2.2% .

Algal biomass contained three different classes of pigments such as xanthophylls, chlorophylls and carotenoids. Carotenoids were quantified in *Haematococcus* biomass using HPLC method. *H. pluvialis* biomass contained astaxanthin esters AE, 77.58% as major, followed by neoxanthin 0.9%, violaxanthin 0.3%, astaxanthin 3.8%, lutein 1.4%, zeaxanthin 4.2%, β-cryptoxanthin 5.3%, β-carotene 1.7% and unidentified peaks 2.8% as confirmed by their retention times and the absorption spectra of the respective reference standards. LC-MS (APCI) was used for determination of molecular mass of each peak. Based on the mass fragmentation interpretation, violaxanthin, neoxanthin, astaxanthin, lutein, zeaxanthin, α-carotene, β-carotene, echinenone, 7, 8, 7', 8'-tetradehydroastaxanthin, and antheraxanthin were identified in *H. pluvialis* extract. Astaxanthin, mono-diesters were identified by liquid chromatography mass spectrum using APCI mode. Mass spectrum obtained from an astaxanthin mono-di esters such as ME $C_{16:0}$, ME $C_{17:2}$, ME $C_{17:1}$, ME $C_{17:0}$, ME $C_{18:4}$, ME $C_{18:3}$, ME $C_{18:2}$, ME $C_{18:1,}$ DE $C_{16:0}/C_{16:0}$, DE $C_{16:0}/C_{18:2}$, DE $C_{18:1}/C_{18:3}$, DE $C_{18:1}/C_{18:2}$ and DE $C_{18:1}/C_{18:1}$ were identified in *H. pluvialis*.

Further, the astaxanthin & its esters were confirmed by ^{13}C NMR, ^{1}H NMR, HSQC 2D, NOESY 2D and COSY 2D NMR. Based on the COSY and NOSEY spectrum confirmed the existenance of both *cis* and *trans* forms of astaxanthin & its esters. The NOESY spectra inturn provided through space interaction of hydrogens of the methyl group 19, 20 and also with protons of the adjacent carbon atoms in the chain. Based on the protons the compound-I was confirmed as astaxanthin. The long range spatial interaction of proton of the primary carbon with the hydrogen's at the

ring position is observed. The COSY and NOSEY spectrum confirms the existenance of both *cis* and *trans* forms of the centro symmetric astaxanthin. The NOESY spectra in turn provided space interaction of the hydrogen of the methyl group 19, 20 and 29 with neighboring protons and also the proton of the adjacent carbon atoms in the chain. These protons are there and confirmed the compound-II as centro symmetric monoester of astaxanthin. COSY and NOESY spectra show the existence of both the *cis/ trans* forms of astaxanthin monoester. The NOESY spectra in turn provide through space interaction of the hydrogen of the methyl group 19, 20, 29, and 29`with protons and also with the protons the adjacent carbon atoms in the chain. Based on the protons the compound-III was confirmed as centro symmetric diester of astaxanthin. COSY and NOSEY spectra confirmed the occurrence of both *cis*/*trans* forms of astaxanthin diester.

Astaxanthin was extracted from *Haematococcus* biomass using various edible oils and solvents. The maximum extractablility of carotenoids were found in palm oil with 51.29 µg carotenoid/g of oil and olive oil 47.26 µg carotenoid/g of oil. Among the solvents, ethylacetate, isopropyl alcohol:hexane followed by acetone could extract more carotenoids from *Haematococcus* biomass. Astaxanthin in its ester form was found to be fairly stable in all edible oils such as rice bran, mustard, groundnut, gingelly, coconut and palm oil at room temperature with variation in terms of its loss in content and colour during the 4 months period. Rice bran, gingelly and palm oil retained 84 - 90% of astaxanthin when heated at 70 ^{o}C for 8 h while palm oil was effective in retaining 90% of astaxanthin at 90 ^{o}C for 8 h without any change in its ester form in comparison to 90% carotenoid loss in aqueous form. At 120 and 150 ^{o}C, carotenoid loss was significant (60-90%) without change in the fatty acid profile of the edible oils. Antioxidant activity was found to be comparable with standard Butylated hydroxy anisole which is at nearly 95% in the carotenoid samples tested.

Haematococcus cyst cell extract was evaluated for antioxidant activity and anti lipid peroxidation in *in vitro* model systems using β-carotene linoleate model system (β-CLAMS), 1, 1-diphenyl-2-picryl hydroxyl (DPPH) method and hydroxyl radical scavenging model systems. The extract exhibited 89, 86 and 78% antioxidant activity in β-CLAMS, DPPH method and hydroxyl radical scavenging model systems. The extract showed 80- 85% antioxidant activity against lipid peroxidation in rat liver, brain, kidney tissues at 9 ppm levels. The extract also exhibited 87% inhibition of lipid

peroxidation in egg lecithin at 9 ppm levels. The algal extract also protected LDL from oxidation and exhibited 82% and 84% protection at 9 ppm levels of carotenoids.

The antibacterial properties of different solvent extracts of *H. pluvialis* were evaluated. The maximum phenolic contents 131 µg/mg was recorded in chloroform extracts of *H. pluvialis*. Hexane, chloroform, ethyl acetate, acetone and methanol extracts of *H. pluvialis* were tested against important bacterial isolates such as *Bacillus subtilus, Bacillus cereus, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus luteus, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, Shigella dysenteriae, Staphylococcus aureus, Streptococcus fecalis* and *Yersinia enterocolitica.* The antibacterial activity was determined by agar-well diffusion assay and minimum inhibitory concentration. Among the different solvent extracts of *H. pluvialis,* chloroform extract showed highest antibacterial effects followed by ethyl acetate, hexane, acetone and methanol. against the bacteria tested. Highest zone of inhibition was observed in chloroform extract against *B. subtilus* 17 mm followed by *E. coli* 14 mm. Acetone, ethyl acetate and methanol extracts showed highest zone of growth inhibition against *L. monocytogenes* 13 mm, *P. mirabilis* 13 mm and *S. aureus* 13 mm respectively. Chloroform extract of *H. pluvialis* recorded highest inhibition against *B. subtilus* MIC at 150 ppm and also inhibited the growth of *M. luteus* and *E. coli* with 250 ppm. All the solvent extracts exhibited varied degree of antibacterial activity. The chloroform extracts of *H. pluvialis* showed higher activity when compared with other extracts.

Antioxidant properties of astaxanthin & its esters were evaluated in *in vivo* model. The antioxidant enzymes such as catalase, peroxidase, superoxide dismutase and anti lipid peroxidation were studied. Among the groups, the astaxanthin diester at 250 µg/kg showed maximum antioxidant activity. Treatments of rats with toxin(CCl_4) at 2.0 g/kg body weight significantly reduced the levels of catalase, peroxidase and SOD by 31.65, 35.17 and 51.70%, whereas prefeeding of rats with 250 µg astaxanthin /kg b.w of carotenoids maintained the catalase, peroxidase and SOD activities at the levels comparable with control. Restoration of catalase in astaxanthin esters(250 µg/kg b.w) fed group was 42.44%, 49.40% and 58.06% higher when compared to toxin treated groups, whereas in rats treated with 100 µg/kg b.w synthetic astaxanthin catalase was higher by 44.32%. The similar observations were seen in the case of peroxidase and SOD enzymes. This shows the protection provided by feeding of

astaxanthin & its esters to the rats by restoration of the levels of these enzymes subsequent to treatment of toxin.

The hepatoprotective activity of astaxanthin & its esters from *H. pluvialis* was compared with that of synthetic astaxanthin. The CCl_4 treated group showed higher activity of serum glutamate pyruvate transaminase with a value of 153.39 units/ml and serum glutamate oxaloacetae transaminase at 186.35 units/ml. Whereas, astaxanthin monoester treated group showed lower serum glutamate pyruvate transaminase (GPT) activity of 98.45 units/mL and astaxanthin treated group 112.23 units GPT/ml. For synthetic astaxanthin, serum alkaline phosphatase activity was further less with 68.10 units/ml compared with 94.87 units/ml for the CCl_4 treated group, whereas on treatment with astaxanthin, astaxanthin ester activity was found to be 63.21 and 50.38 units/m1. Antioxidant properties of astaxanthin & its esters were evaluated in *in vivo* models. Among all the groups, the one which was treated with diester of astaxanthin at concentration of 250 µg/kg showed maximum antioxidant activity i.e protection when compared to control and synthetic astaxanthin treated group. Treatment of rats with toxin at 2.0 g/kg body weight significantly reduced the levels of catalase, peroxidase and SOD by 31.65, 35.17 and 51.70%. On the other hand, lipid peroxidation increased by 1.7 folds due to the CCl_4 treatment as compared to normal diet fed rats. However, pretreatment of rats with 250 µg/kg b.w of astaxanthin esters preserved catalase, peroxidase and SOD activities, which are comparable with control values of the enzyme. Restoration of catalase was 42.44%. 49.40% and 58.06% higher when compared to toxin treated groups respectively at 250 µg astaxanthin or esters /kg bw.

Bioavailability of astaxanthin from *Haematococcus pluvialis* was studied using single dose and repeated in rat model. Time course response to feeding biomass at various astaxanthin levels and the activity of antioxidant enzymes viz catalase, superoxide dismutase, peroxidase in plasma and liver of rats were studied. After gavage of *H. pluvialis* biomass, experimental rats did not show any variation in body weight when compared to control group. No clinical signs of any toxicity, mortality were noticed during the experimental period. Expectedly astaxanthin was not detected in plasma, liver and eye at 0 h group. However in the animals fed with the biomass , the astaxanthin levels reached maximum in plasma (128.49 ± 2.58 pmol/ml), liver (131.23 ± 4.58 pmol/g) and eye (281.45 ± 5.12 pmol/g) at respectively as measured at 2 h, 4 h and 6 h. Antioxidant enzymes catalase, SOD, peroxidase activity of 57%, 37%

and 51% compared with the control diet fed rats were found in plasma at 2 h; whereas in liver, at 4 h it was 34%, 39% and 54%. In repeated dose studies of feeding of *H. pluvialis* biomass for 15 days, the astaxanthin level was maximum in plasma (485.81 ± 34.58 nmol/ml) followed by liver (896.51 ± 101.76 nmol/g) and eye (508.41 ± 71.32 nmol/g)

Haemtococcus pluvialis cells were further evaluated for nutritional quality using albino rats. Treatment of *Haematococcus pluvialis* biomass at 2.5 g/kg and 5 g/kg b.w did not shown any death and toxic symptoms. *Haematococcus* treated group was very active similar to that of normal diet fed group. Body weight was measured in all the groups and the treated group gained higher body weight, when compared control groups. Gain in body weight was maximum upto 20% higher in 2.5 g/kg b.w. biomass treated groups, whereas in 5 g/kg b.w. *Haematococcus* biomass treated group showed 14% higher when compared to control group (normal diet). Treatment of *Haematococcus* biomass has not shown any adverse effect on the hematological profile of the experimental animals.

The inhibitory effect of astaxanthin & its esters on UV-DMBA induced skin carcinogenesis *in vitro* using cancer cell lines and *in vivo* rat models were evaluated. Astaxanthin & its esters offered protection against oxidation of DNA, Red blood cells and buccal cells in *in vivo* experiments. The oral administration of astaxanthin and its esters did not cause any apparent changes in clinical signs such as survivability, or any gross visible changes attributable to toxicity in the organs weight of rat. There was 73%, 74% and 78% inhibition of tyrosinase activity, respectively , in serum, liver and skin homogenates treated with astaxanthin esters group at 200 µg/kg b.w. The antioxidant enzymes were measured in UV-DMBA induced skin cancer and control groups. SOD and catalase levels increased in serum and GSH levels decreased during DMBA-UV induced carcinogenic conditions and reached normal levels upon treatment with astaxanthin & its esters in a dose dependent manner. Approximately 4 fold increase in TBARS levels was observed which depicts lipid peroxidation in DMBA-UV treated animals and which recovered up to 65% upon treatment with esters at 200 µg astaxanthin esters /kg b.w. Similarly, in the case of liver homogenates, SOD levels (2.7 fold increase), CAT (2.2 fold increase) and GSH (1.6 fold decrease) varied in DMBA-UV treated rats and were found to attain normal levels upon treatment with astaxanthin & its esters. Approximately 3.2 fold increase in TBARS levels depicts lipid peroxidation of DMBA-UV treated animals and was recovered up to 70% upon

treatment with esters at 200 µg/kg b.w. Similarly, in the case of skin homogenate, SOD levels increased in serum (2.7 fold), CAT (2.3 fold) and GSH decreased (2 fold) during DMBA-UV induced treated animals and were normalized upon treatment with astaxanthin and its esters. A 2.6 fold increase in TBARS levels depicts lipid peroxidation in DMBA-UV treated animals and which recovered up to 60% upon treatment with esters at 200 µg/kg b.w.

These studies show that astaxanthin and esters are degraded in the system by photochemical or oxidative reactions and the metabolites or oxidized products formed could be excreted. Further it is not clear, how the astaxanthin and its esters are transported. Degradation of astaxanthin in the body indicates that astaxanthin is involved in various reactions and also as an antioxidant in various oxidative reactions in tissues. Based on the metabolites formed tentative metabolic pathways for conversion of astaxanthin to its oxidation products was envisaged. This study emphasizes the essentiality of maintaining astaxanthin status especially in plasma and liver to function as an antioxidant since it is easily degraded into oxidation products. Metabolites of astaxanthin are highly effective in the human metabolism to exhibit protection against diseases. The aim of this investigation was to characterize the oxidation products of astaxanthin transformed biologically in plasma and liver of rats.

In vivo studies involved feeding of rats with astaxanthin from *Haemtococcus* cells for the period of five weeks. Rats were divided into six groups and received astaxanthin & its esters by gavages of supplementation with olive oil for five weeks. Astaxanthin metabolites isolated from serum and liver from *in vivo* studies were characterized by HPLC and LC-MS (APCI) techniques to elucidate their structure. Epoxy carotenoids were tentatively identified in liver and plasma. This study emphasizes the essentiality of astaxanthin to maintain its status in the metabolism.

In addition, the cytotoxicity of the astaxanthin & its esters was evaluated by using two different well-established glioma cell lines LN-229, HNGC2 and liver hepatocellular carcinoma cell line HepG2 by *in vitro* MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assays. Gliomas are the most common primary brain tumors in adults. They are highly invasive and are resistant to most conventional therapies including chemotherapy and radiation therapy. Astaxanthin treatments at the end of the 72 h the cytotoxicity was found to be 42%, 22%, 31% in LN-229, HNGC2, HepG2 cells at 30 µmol/ml concentration. Monoester of astaxanthin after exposed to LN-229, HNGC2, HepG2 cells for 72 h, the cytotoxicity was found to be 38%, 21%

and 28% at 30 µmol/ml. Diester of astaxanthin incubated to LN-229, HNGC2, HepG2 cells for 72 h, the cytotoxicity was found to be 41%, 21% and 28% at 30 µmol/ml. Confocal laser scanning microscopy images clearly demonstrated the apoptosis-induced cell death by astaxanthin & its esters on human glioma cell lines. These results indicated that astaxanthin & its esters showed better cytotoxic capacity in cancer cell lines.

The present investigation *Haematococcus* was cultivated using suitable media. The alga produced upto 2.25% carotenoid which comprises of up to 77% astaxanthin & its esters. Astaxanthin and its esters were identified and characterized using HPLC, LC-MS, and NMR techniques. Stability of astaxanthin was studied in various edible oils. Antioxidant and hepatoprotecitve activity against CCl_4 challenged animals reveals that both *Haematococcus* biomass as well as astaxanthin & its esters are significant protectants of vital organs like liver compared to the synthetic astaxanthin against oxidative damage. Bioavailability of astaxanthin was evaluated using single and repeated dose studied in animal models. Inhibitory effect of astaxanthin & its esters on UV-DMBA induced skin carcinogenesis in rats was found. Similarly anti proliferative effects of human glioma and liver hepatic cell lines were noticed in astaxanthin & its esters treatments. The biological properties of astaxanthin & its esters such as antioxidant, hepatoprotective activity, bioavailability and anticancer properties were evaluated which has a bearing in expanding the utility of astaxanthin and its esters rich biomass of *Haematococcus* for health food purposes.

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PUBLICATIONS AND PRESENTATIONS

8.0. Publications and presentations

Publications

- **Ranga Rao, A.,** Baskaran, V., Sarada, R., Ravishankar, G.A. 2010. Characterization of micro algal carotenoids by mass spectrometry and their bioavailability and antioxidant properties in rat model. Journal of Agricultural and Food Chemistry. 58: 8553-8559.
- **Ranga Rao, A.,** Sarada, R., Baskaran, V., Ravishankar, G.A. 2009. Identification of carotenoids from green alga *Haematococcus pluvialis* by HPLC and LC-MS (APCI) and their antioxidant properties. Journal of Microbiology and Biotechnology. 19(11): 1333–1341.
- **Ranga Rao, A.,** Sarada, R., Ravishankar, G.A. 2007. Stabilization of astaxanthin in edible oils and its use as an antioxidant. Journal of the Science Food and Agriculture. 87(9): 957-965.

Papers under preparation

- **Ranga Rao, A.,** Sarada, R., Ravishankar, G. A. Hepatoprotective activity of astaxanthin and its esters from green alga *Haematococcus pluvialis*.
- **Ranga Rao, A.,** Sarada, R., Ravishankar, G. A. *In vivo* antioxidant properties of astaxanthin and its esters from green *Haematococcus pluvialis*.
- **Ranga Rao, A.,** Sindhuja, H.N., Darmesh, S.M., Sarada, R., Ravishankar, G.A. Inhibitory effects of astaxanthin and its esters from *Haematococcus pluvialis* on UV-DMBA induced skin carcinogenesis in *in vitro* and *in vivo* rat model.
- **Ranga Rao, A.,** Sarada, R., Baskaran, V., Ravishankar, G.A. Ravishankar Bioavailability of carotenoids of microalgal biomass elucidated in rats- A repeated dose study.
- **Ranga Rao, A.,** Maheswara Reddy, E., Anjali Shiras, Darmesh, S.M., Sarada, R., Ravishanakar, G.A. Bioactive compounds from microalgal culture and Its effects on apoptosis, cytotoxicity of human glioma cell lines and liver hepatocellular carcinoma cell lines.

Presentations in symposium/conferences

Posters

- **Ranga Rao, A.,** Raghunath Reddy, R.L., Baskaran, V., Sarada, R., Ravishankar, G.A., Comparative bioavailability and antioxidant property of carotenoids of microalgae *Spirulina platensis*, *Haematococcus pluvialis* and

Botryococcus braunii biomass elucidated in experimental rats in 7th Gordon Research Conference on Carotenoids. Ventura Beach Marriott, Ventura, California, USA. January 2010.

- **Ranga Rao, A.,** Sandesh Kamath, B., Sarada, R. Stability of astaxanthin at different temperature in *Haematococcus* cells and edible oils in 16th Indian Convention of Food Scientists and Technologists (ICFOST-2004), Central Food Technological Research Institute, Mysore, India. December 2004.
- Sandesh Kamath, B., Sarada, R., Jagannath Rao, Vidhyavathi, R., **Ranga Rao, A.** Enhancement of egg yolk colour in layer chicken feed with microalga *Haematococcus pluvialis* in 16th Indian Convention of Food Scientists and Technologists (ICFOST-2004), Central Food Technological Research Institute, Mysore, India. December 2004.
- Sandesh Kamath, B., Brinda, B.R., Ravikumar, M.S., **Ranga Rao, A.,** Sarada R., Ravishankar, G.A. Scale up studies of green algae-*Haematococcus pluvialis* in National Symposium on Micro Algal Biotechnology, Bharathidasan University, Tiruchirapalli. March-2004.

Oral presentations

- **Ranga Rao, A.,** Sarada, R., Baskaran, V., Ravishankar, G.A. Bioavailability of astaxanthin and its esters from cultured green alga- *Haematococcus pluvialis* elucidated in experimental rats in 41st National Conference on Nutrition Society of India (NSI), National Institute of Nutrition, Hyderabad, Andhrapradesh, India. November-2009.
- **Ranga Rao, A.,** Sarada, R., Dayananda, C., Vidhyavathi, R., Ravishankar, G.A. 2005. Antioxidant activity of *H. pluvialis* extracts in *in vitro* models in National Seminar on Nutritional Status and Prospects, School of Biotechnology, Dr. G.R.D. College of Science, Coimbatore, Tamilnadu, India. September-2005.