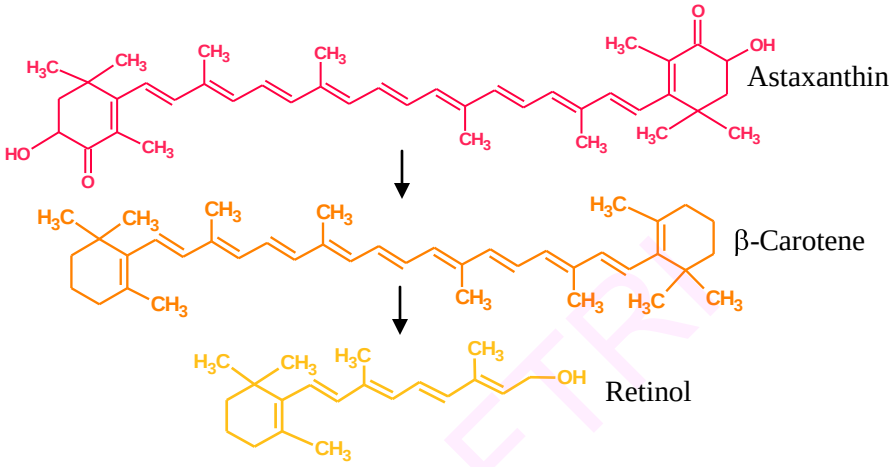
INFLUENCE OF SELECTED DIETARY CAROTENOIDS ON RETINOL DEFICIENCY INDUCED BIOCHEMICAL CHANGES IN TISSUE MEMBRANES OF RATS



Thesis submitted to the

UNIVERSITY OF MYSORE

For the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

By

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MARCH – 2011

Dedicated to,

My dearest family, for making me what I am.

My Guide, for recognizing and utilizing my capabilities.

My sweet friends, for making me smile, always.

ACKNOWLEDGEMENTS

I take this opportunity to thank Dr. V. Baskaran, my mentor and guide, for being my pillar of support and guiding beacon of light throughout my PhD programme. I am immensely grateful to him for his invaluable guidance, support, interest, sincere concern and faith in me.

I also thank Padmashree Dr. V. Prakash, Director, CFTRI, for granting me permission to pursue my Ph.D. at CFTRI, Mysore and for his many words of encouragement.

My gratitude is due to Dr. PV Salimath, Head, Department of Biochemistry and Nutrition, for his encouragement and support.

I am indebted to Dr. N. Bhaskar, Scientist, Department of Meat, Fish and Poultry Technology, for his many kindnesses, generosity, keen interest and best wishes for my progress and research work.

I also thank Dr. MC Varadaraj, Head, Human Resources Department and other staff members of the department for their help.

I express my gratitude towards Dr. TP Krishnakantha, Dr. S Divakar, Dr. GA Ravishankar, Dr. BR Lokesh, Dr. G Muralikrishna, Dr. NM Sachindra, Mrs. Revathi Baskaran, Mr. S Vishwanath, Mr. K Rathina Raj and Mr. BV Vijaya Kumar for their help and useful suggestions on various aspects pertaining to my research. I also thank all the staff members of the Department of Biochemistry and Nutrition for their help during my research programme.

I extend my thanks to the staff of Animal house for providing me facilities and aid for carrying out the experiments on animals, especially to Mrs. Rita Dass for her help. I also thank the staff of Central Instruments Facility and Services Department for their help in sample analysis and data collection. My thanks are also due to the administrative staff and staff members of FOSTIS (library) for their cooperation and help during my stay at CFTRI.

I thank the University Grants Commission, Government of India, New Delhi, for the award of Junior and Senior Research Fellowship from 2006-2011.

My special thanks to Prof UV Mani and Dr. (Mrs.) Mani of the Maharaja Sayajirao University of Baroda, Vadodara, who are a continuous source of encouragement and support, and Dr. R. Kannan, IAS and Mrs Seetha Kannan who have impacted my life in many ways and for their encouragement and belief in me.

Friends are my family away from home and I take this opportunity to thank my very special friends at CFTRI, Ms. Asha M, Mr. Amit, Mrs. Mamatha, Mrs. Aruna, Ms. Nidhi, Mr. Arunkumar, Mrs. Jamuna, Ms. Revathi, Ms. Shweatha, Ms. Shubhra, Mrs. Meesha, Mr. Vasu, Ms. Kavitha, Ms. Sinduja, Mr. Girish TK, Mr. Girish, Ms. Rajni, Ms. Ananya and Mr.

Debi Prasad. I also thank Mr. RLR Reddy, Mrs. Smita, Mrs. Fatima, Dr. Lakshminarayana, Dr. Raju, Dr. Ramprasad, Ms. Kanchan, Dr. Veerbala, Mr. Divakar, Mr. Ravikumar, Mr. Sathish, Dr. Rajini, Mrs. Bhumika, Mr. Ganesan and Mr. General whom I previously knew at CFTRI and who have moved onto greater avenues in life. I thank all my other friends at the Department of Biochemistry and Nutrition and other departments of CFTRI for lending a helping hand when I needed it.

Many friends have entered my life; some have become an integral part of it while the kindness of the others has left a lifelong impact. I profusely thank Prabhu, Shrikalaa, Ramandeep, Sukanya, Namrata, Maj. Neetu, Neetika, Sheetal, Subin, Neetu V., Rahana, Dheeraj, Poonam, Khevna, Ajay, Sweety, Amrita and Tathagat, for being the very special people that they are.

No words of thanks are enough for my lovely family, who have stood by me and believed in me, were understanding and have given me unconditional love and support in every endeavor. I thank my mom, Mrs. Geetha, dad, Lt.Col A Ravikumar (Retd.), beloved sister Ms. Kavitha, uncle, Mr. A Ramesh Kumar and aunt Mrs. Usha Rameshkumar for making my world beautiful.

The power of the cosmos controls us, makes us realize how insignificant we are and yet how significant an impact our actions can have. I thank God almighty for bestowing me with confidence and capability that have led to my achievements, a wonderful family, innumerable well wishers and fantastic friends.

Sangeetha Ravi Kumar

CERTIFICATE

I **Sangeetha Ravi Kumar**, certify that this thesis is the result of research work done by me under the supervision of **Dr. V. Baskaran** at **Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore**. I am submitting this thesis for possible award of Doctor of Philosophy (Ph.D.) degree in **Biochemistry** of the University of Mysore.

I further certify that this thesis has 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 1st March, 2011

Signature of Guide *Date:*

Date: *Counter signed by*

.....

Signature of Chairperson/Head of Department
Institution with name and official seal

Department of Biochemistry and Nutrition
Central Food Technological Research Institute

Date: 1st March, 2011

Dr. V. Baskaran, M.Sc., M.Ed., M.Phil., Ph.D.
Scientist
Department of Biochemistry and Nutrition.

CERTIFICATE

This is to certify that the thesis entitled "INFLUENCE OF SELECTED DIETARY CAROTENOIDS ON RETINOL DEFICIENCY INDUCED BIOCHEMICAL CHANGES IN TISSUE MEMBRANES OF RATS" submitted by Ms. SANGEETHA RAVI KUMAR, is the result of research work carried out by her in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore under my guidance during the period of 2006-2011.

V. BASKARAN
Guide

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DECLARATION

I hereby declare that the thesis entitled **"INFLUENCE OF SELECTED DIETARY CAROTENOIDS ON RETINOL DEFICIENCY INDUCED BIOCHEMICAL CHANGES IN TISSUE MEMBRANES OF RATS"** submitted to the University of Mysore for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOCHEMISTRY** is the result of research work carried out by me under the guidance of **Dr. V. Baskaran,** Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore during the period 2006-2011.

I further declare that these results have not been submitted for any other degree or fellowship.

Place: Mysore
Date: 1st March, 2011

SANGEETHA RAVI KUMAR

CONTENTS

LIST OF TABLES

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

LIST OF FIGURES

CHAPTER 1 **Page No.**

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
β	Beta
^{0}C	Degree Celsius
AAx	Amarouciaxanthin
AUC	Area under the curve
CAT	Catalase
CRALBP	Cellular retinal binding protein
CRBP	Cellular retinol binding protein
dl	Decilitre
ELISA	Enzyme linked immunosorbent assay
FUCO	Fucoxanthin
FUOH	Fucoxanthinol
g	Gram
GC	Gas chromatography
GSH	Glutathione, reduced
GST	Glutathione-S-transferase
HDL	High density lipoprotein
HPLC	High Performance Liquid Chromatography
h	Hour(s)
IRBP	Interphotoreceptor retinoid-binding protein
IU	International unit
kg	Kilogram
LC-MS	Liquid chromatograph-mass spectrometry
LDL	Low density lipoprotein
Lpx	Lipid peroxidation
l	Litre
ND	Not detected
µg	Micro gram
µl	Micro litre
µM	Micromolar
µmol	Micromol
MDA	Malondialdehyde

min	Minutes
ml	Milliliter
mg	Milligram
mM	Millimolar
MUFA	Monounsaturated fatty acids
ng	Nanogram
nm	Nanometer
nmol	Nanmomol
OD	Optical density
pmol	Picomol
PUFA	Polyunsaturated fatty acids
RAR	Retinoic acid receptors
RBP	Retinol binding protein
RD	Retinol deficient
RE	Retinol equivalent
RXR	Retinoid "X" receptors
SD	Standard deviation
SFA	Saturated fatty acids
SOD	Superoxide dismutase
TG	Triglyceride
U	Unit
UNICEF	The United Nation’s Children’s Fund
VAD	Vitamin A deficiency
VLDL	Very low density lipoprotein
v/v	Volume by volume
WHO	World Health Organization

SYNOPSIS

Chapter 1: General Introduction

Vitamin A deficiency (VAD) is a major nutritional problem in India as well as almost 60 other countries (WHO, 2009). There are ~127 million and 4.4 million preschool children with VAD (serum retinol < 0.70 μmol/L) and xerophthalmia (dryness of the conjunctiva and cornea of the eye), respectively. WHO (2005) has reported that >6 million women develop night blindness during pregnancy annually. Approximately 45% of VAD and xerophthalmic children and pregnant women with low-to-deficient vitamin A status live in South and South-East Asia. Prevalence of VAD in India among preschool children is estimated to be 30.8%, while that of xerophthalmia is 1.6%. Pregnant women (1.6%) in India are deficient in vitamin A while 12.1% suffer from night blindness (West et al, 2002). VAD and its progress involve a variety of physiological changes. Many functions of vitamin A such as vision, growth, reproduction, skin health, immunity and erythropoiesis are affected in VAD. A general alteration in the physical characteristics of cell membranes may be induced by VAD. Retinol deficiency has prooxidative effect and increases the oxidative stress in cell membranes of rats (Kaul and Krishnakantha 1997). The Governments of affected countries are trying to exterminate VAD by various means such as administration of high but safe doses of synthetic vitamin A. Although VAD prophylaxis programmes are in place in most of the affected countries, greater effort is needed to assess and prevent VAD and its disorders (WHO, 2007). It is now widely accepted that alternate dietary sources of nutrients are the sustainable answers to eradicate VAD and the associated disorders.

Vitamin A or retinol is available either in its preformed form (animal sources) or as provitamin A carotenoids (plant sources) Provitamin A carotenoids include β, α- and γ-carotenes and are cleaved to retinol by the carotenoid cleavage enzyme. Carotenoids with polar groups such as hydroxyl, epoxide and keto groups are classified as xanthophylls and usually do not possess provitamin A activity (except β-cryptoxanthin) but are beneficial to health due to their antioxidant properties (Krinsky, 1993). Even though plant foods containing carotenoids have been used as dietary sources of vitamin A, its deficiency continues to be one of the major nutritional problems in developing countries. This is mainly due to inadequate intake, improper processing techniques, poor intestinal absorption and bioavailability. In addition, epidemiological studies have established an inverse relationship between consumption of fruits and vegetables rich in carotenoids and chronic degenerative diseases such as cancer, cardiovascular diseases, neurodegenerative disorders, diabetes and age related macular degeneration (Williams et al.,

2002; Bone et al., 2003). However, although the beneficial effects of the carotenoids are well known, their absorption, metabolism and mechanism of action are poorly understood. To understand the health benefit of carotenoids against VAD, it is important to have a better insight into the bioavailability and metabolism of carotenoids under condition like VAD.

Carotenoid bioavailability is poor and only 10-20% of the carotenoids in a meal are absorbed into circulation. Carotenoid bioavailability is affected by a variety of dietary and non-dietary factors, such as the matrix in which it is incorporated, the processing methods involved in the preparation of the food, the nutrient status of the individual, amount and origin of dietary fat, etc. (Erdman et al, 1993). In view of their multiple health benefits, research by many is focused on determining factors that can improve carotenoid bioavailability (Deming et al., 2000; van het Hoff et al., 2000). It is also important to determine the mechanism of action of different carotenoids and to compare their effectiveness in modulating the changes caused by nutrient deficiency.

β-Carotene is cleaved by the intestinal β-carotene monooxygenase either centrally or excentrically to yield retinol. Besides retinol, apocarotenols are also reported to be formed from β-carotene (Barua and Olson, 2000). In lower vertebrates such as fish, xanthophylls such as lutein, zeaxanthin, canthaxanthin, astaxanthin have exhibited provitamin A activity with the formation of 3'-dehydroretinol via reductive pathways (Moren et al., 2003; Matsuno, 1991; Goswami and Barua, 1986). β-Carotene is the most widely studied carotenoid by virtue of its provitamin A and antioxidant properties. However, other carotenoids have also been of interest in recent times and understanding their metabolism is of importance as well. Lutein for instance, plays an important role in the prevention and management of age related macular degeneration (Krinsky and Johnson, 2005). However, the mechanism of antioxidant action of lutein and its metabolites is still largely unknown. Since VAD results in increased oxidative stress (Anzulovich et al., 2000) in the cells, and carotenoids are potent antioxidants, it would be of interest to study their effect on various biochemical parameters at the cell membrane and tissue level. In view of this background, it was proposed to investigate the possible provitamin A activity and antioxidant property of xanthophyll carotenoids such as astaxanthin, lutein and fucoxanthin in comparison with β-carotene in retinol deficient (RD) rats in terms of formation of retinol and/or its related compounds, and various biochemical parameters at the membrane and tissue level. The objectives of the work carried out are as follows:

1. Isolation and purification of carotenoids (β-carotene, lutein, astaxanthin and fucoxanthin) from selected leafy greens and algae.

2. Investigating the effect of vitamin A deficiency on bio-accessibility, bioavailability and bio-efficacy of dietary carotenoids (β-carotene, lutein, astaxanthin and fucoxanthin).
3. Determining the influence of dietary carotenoids on vitamin A deficiency induced biochemical changes on the kinetics of carotenoid metabolizing enzyme and membrane bound enzymes in intestine and liver of rats.
4. Evaluating the influence of dietary carotenoids on vitamin A deficiency induced changes in lipoproteins and retinol binding proteins in plasma and tissue.
5. Effect of dietary carotenoids on vitamin A deficiency induced changes in lipid profile, lipid peroxidation and enzymes involved in antioxidant defense mechanisms in tissue membrane.
6. Effect of dietary carotenoids on vitamin A deficiency induced changes in tissue membrane of rats with reference to structure and function.

The results of these studies constitute the subject matter for the thesis and are organized into six chapters.

Chapter 2: Materials and Methods

This section provides the list of all the materials and chemicals used for the laboratory experiments and animal studies as well as the name of the suppliers of the chemicals. A description of each method employed is presented. Conditions maintained for the rats used in the study are also described. Methods for extraction of carotenoids from plant and feed sources and their analysis by HPLC and LCMS, purification of carotenoids by open column chromatography (OCC) and confirmation by TLC, method standardized for the extraction of fucoxanthin from seaweeds and its purification by OCC and HPLC, chemical preparation of fucoxanthinol and amarouciaxanthin from fucoxanthin and their analysis by HPLC, calculation of vitamin A activity of food sources, procedure for the preparation of vitamin A free casein and diet, preparation of control and experimental diets including retinol-free and retinol-supplemented diets for animal feeding studies and dispersion of carotenoids for gavage studies are provided. The experimental design for the single and repeated dose and dietary studies are described in detail. Biochemical analysis of blood and tissue samples obtained at the end of the animal experiments such as preparation of plasma, serum, liver homogenates and liver and brain microsomes, protein analysis, extraction of carotenoids from biological samples, their HPLC and LCMS analysis are outlined. Assay for the β-carotene cleavage enzyme, intestinal ß-carotene, 15-15'-monooxygenase, lipid peroxidation assay, assays for activities of antioxidant molecules such as

superoxide dismutase, catalase, glutathione-S-tansferase and glutathione levels are described. Assays for membrane bound enzymes in liver and brain microsomes such as $Na^{+}K^{+}$-ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase and acetylcholine esterase are explained. Methodology for biochemical parameters such as extraction of lipid from biological samples, analysis of fatty acids by GC, estimation of triglycerides, phospholipids, cholesterol in various tissues, HDL- and LDL+VLDL-cholesterol in serum and qualitative separation of retinol binding protein (RBP) by gel electrophoresis and quantitation of RBP by ELISA are provided. At the end of the chapter, the statistical analyses employed for the data obtained from the various experiments is discussed.

Chapter 3: Isolation and purification of carotenoids (β-carotene, astaxanthin, lutein and fucoxanthin) from leafy greens, medicinal plants, vegetables and algae.

This chapter begins with a brief introduction on the beneficial effects of greens, vegetables and fruits with regard to carotenoid composition and their functional benefits towards treatment for VAD. The literature survey also attempts to cover the available methods for the extraction, purification and HPLC analysis of carotenoids from plant sources. This chapter describes the extent of purification achieved for different carotenoids using different solvent systems on silica packed OCC. For example, the purity of β-carotene, lutein+zeaxanthin obtained from *P. sowa* was 91 ± 3% and 94 ± 2% respectively, while it was 93 ± 2% and 90 ± 4% for neoxanthin and violaxanthin, respectively. The purity of the fucoxanthin obtained from the brown seaweed *P. tetrastromatica* was 85 ± 3%. The characteristic spectra of the carotenoids extracted from leafy greens (n=23), medicinal plants (n=33), vegetables (n=25) and algae (n=18) were monitored for confirmation of their identity. The absorption maxima (λ_{max}) of the carotenoids eluted were 426, 454, 480 (ß-carotene), 421, 448, 475 (α-carotene), 421, 447, 475 (lutein), 415, 440, 468 (neoxanthin), 425, 449, 476 (violaxanthin), 432, 454, 480 (zeaxanthin) and 447, 470 (fucoxanthin).

Concentration (mg/100g dry weight) of ß-carotene and lutein in green leafy vegetables ranged between 1.5 to 120.2 and 11.7 to 185.2 respectively. α-Carotene (mg/100g dry weight) was detected in 8 leafy greens (0.3-35.6) and was highest in jio (*C. benghalensis*, 35.6). Lamb's quarters (*C. album*), prickly amaranth (*A. spinosus*), jio, Indian dill leaf (*P. sowa*), colocasia leaves (*C. anti-quorum*) and amaranth leaves (*Amaranth sp.*) were the better sources of β-carotene. The highest levels of lutein content were found in lamb's quarters, jio, chilli leaves (*C. annuum*), dill leaves, *I. pestigridis*, prickly amaranth and colocasia leaves. Lamb's quarters, jio, prickly amaranth and colocaia leaves were thus rich sources of both lutein and ß-carotene. RE values (in mg) were highest for Lamb's quarters (20) followed by jio (19), prickly amaranth (16),

amaranth (11), Indian dill leaf (10), colocasia leaves (10) and lowest for green cabbage (0.01) and red cabbage (0.03).

Concentration (mg/100g dry weight) of ß-carotene and lutein in medicinal plants was ranged between 0.4 to 34.7 and 11.8 to 679 respectively. α-Carotene (mg/100g dry weight) was detected in 25 medicinal plants (0.1-15.7) and was highest in Indian borage (*C.aromaticus,* 15.73). The highest levels of ß-carotene (mg/100 g dry weight) were for butterfly pea (*C. ternatea*), thyme leaved gratiola (*B. monnieri*) and holy basil (*O. sanctum*). The highest levels of lutein content were found in butterfly pea, conch grass (*C. dactylon*) and hoary basil (*O. canum*). Thus, butterfly pea was the richest source of both lutein and ß-carotene among the 33 medicinal plants analyzed. RE values (in mg) were highest for thyme leaved gratiola (5.7) followed by butterfly pea (5.3) and holy basil (5.3).

Amongst the 25 vegetables analyzed, kenaf (*H. cannabius*), red/green lettuce (*L. sativa*), carrot (*D. carota*) and yellow zuchchini (*C. pepo*) were found to be better sources of β-carotene (> 1 mg/100g dry weight). Carrot, yellow zuchchini, tomato (*L. esculentum*), and kenaf contain appreciable amounts of α-carotene (> 1 mg/100 g dry weight). Yellow zuchchini, kenaf and carrot contain higher levels of lutein (>10 mg/100 g dry weight). β-cryptoxanthin was detected only in bitter orange while lycopene was detected only in tomato. RE values (in mg) were highest for carrot (26.8) followed by kenaf (8.6), yellow zucchini (2.0) and tomato (1.5).

Concentration (mg/100g dry weight) of ß-carotene and lutein in the seaweeds was ranged between 0 to 18.9 and 0 to 2.9 respectively while the level of fucoxanthin ranged from 0 to 9.2. α-Carotene (mg/100g dry weight) was detected in 6 algae (0.04 to 7.1) and was highest in *C.sertularciocles* (7.07). Amongst the 18 algae analyzed for their carotenoid composition, the highest levels of ß-carotene (mg/100 g dry weight) were found in *Enteromorpha* (18.9), *C. racemosa* (16.9), *T. connoides* (15.5) and Palmaria (15.3). The highest levels of lutein (mg/100 g dry weight) were found in *S. cristaefolium* (2.9), *H. stipulacia* (1.42) and *Enteromorpha* (1.33). *D. dichromata, S. tenerrium, S. cristaefolim* and *P. tetrastromatica* are found to be good sources of fucoxanthin (3.6-9.2 mg/100g dry weight). *Enteromorpha* was thus a good source of lutein, zeaxanthin and ß-carotene among the algae analyzed. RE values (in mg) were highest for *Enteromorpha* (3.2) followed by *C. racemosa* (2.9), *T. connoides* (2.6) and *Palmaria* (2.6).

Interestingly, from the results it was seen that some of the lesser known and consumed leafy greens such as *C. album, A. spinosus, C. benghalensis, P. sowa, C. anti-quorum,* and *Amaranth sp.* (keerai) showed highest vitamin A activity. RE values of medicinal plants were lower than leafy greens. However, the total carotenoid content of these plants were significantly higher (1.5 fold) than the other plants screened. This could be one of the reasons for the medicinal

properties of these plants and their use in Ayurvedic medicine. Moreover, although the RE values of vegetables, in general, was lower than the leafy greens, consumption of vegetables may still be recommended as β-carotene source, as it is speculated that carotenoids from vegetables and fruits are more bioavailable as compared to leafy greens. Algae like *Enteromorpha, C. racemosa, T. connoides, Palmaria, S. tenerrium,* and *S. cristaefolium* showed good vitamin A activity (RE >1 mg) and thus their consumption can be recommended to obtain retinol.

Chapter 4: Effect of vitamin A deficiency on bioavailability of dietary carotenoids (β-carotene, astaxanthin, lutein and fucoxanthin).

This chapter begins with an introduction and a brief review of literature on the bioavailability and bioefficacy of carotenoids *in vitro* and *in vivo.* Retinol deficiency was induced in rats, which resulted in reduced food intake and consequently stunted growth (weight gain) as compared to control group (received diet with sufficient retinol). Gavage study with a single dose of β-carotene, astaxanthin, lutein and fucoxanthin was carried out while repeated gavage study (for 7 and 15 days) was carried out for β-carotene, astaxanthin and lutein using retinol deficient (RD) rats. Dietary study was carried using carrot and dill leaf powder as a source of β-carotene and lutein, and purified astaxanthin, for 20 days. Fucoxanthin was not included for the repeated dose and dietary studies because it failed to yield retinol in the single dose study. In addition, there is close similarity between the structures of astaxanthin, lutein and β-carotene, unlike fucoxanthin.

On gavage to RD rats with a single dose of β-carotene, retinol levels increased in the plasma (0.6-0.96 μmol/l), liver (6.5-20.5 nmol/g) and intestine (12.7-5.2 nmol/g) accompanied by enhanced intestinal monooxygenase (55-69%) activity over 8h as compared to RD (0h, baseline) group (0.38 μmol/l, 3.1 nmol/g, 2.9 nmol/g). No intact β-carotene was detected in plasma, but it was detected in liver after 8h (10.7 pmol/g). Whereas, gavages of astaxanthin, lutein and fucoxanthin were not associated with increased retinol levels and monooxygenase activity indicating that they did not undergo bioconversion to retinol. On comparison of AUC values of carotenoids in plasma (pmol/ml/8h) and liver (nmol/g/8h), it was found that astaxanthin (356.6, 197.1) was more bioavailable as compared to lutein (140.8, 203.1), while the lutein level was more than fucoxanthin (2.9, 31.1). Gavages of carotenoids resulted in elevated plasma triglycerides level (155.6-205.1 mg/dl) as compared to RD group (120.8 mg/dl) which supports the reason for the higher carotenoid levels than control, as triglycerides are a carrier for carotenoids.

Repeated gavages with β-carotene and astaxanthin for 7 and 15 days resulted in elevated retinol levels in the plasma which was supported by enhanced intestinal monooxygenase activity (69-72%, 68-70%) when compared to RD (baseline) group. The rise in retinol level was higher for β-carotene than astaxanthin group and corresponded with lower levels of intact β-carotene as compared to astaxanthin. Results show that the postprandial plasma and liver levels of astaxanthin were lower than lutein which may be due to a part of astaxanthin being converted to β-carotene and thereafter to retinol. The elevated retinol levels in plasma and liver of the astaxanthin group supports the above results. Interestingly, along with increased retinol levels, β-carotene was also detected in the plasma and liver of astaxanthin group after 7 and 15 days of repeated gavages with astaxanthin. Whereas, no retinol was detected in the lutein group as compared to RD group (baseline). The intact lutein levels were significantly higher than β-carotene and astaxanthin. The influx of the carotenoids and resultant rise in retinol levels in the β-carotene and astaxanthin groups was accompanied by higher triglycerides level indicating that the carotenoids were bioavailable.

Dietary feeding of carrot powder (β-carotene source), purified astaxanthin and dill leaf powder (lutein source) for 20 days resulted in elevated retinol levels in plasma and intestinal monooxygenase activity as compared to RD group, and was in the order of β-carotene > lutein > astaxanthin. Carotenoids of interest detected in plasma, liver and intestine of carrot powder fed group included β-, α-carotene and lutein, while astaxanthin fed group showed the presence of astaxanthin and β-carotene . The dill leaf powder fed group showed lutein and β-carotene in plasma, liver and intestine. A corresponding increase was observed in plasma triglycerides, indicating the absorption of carotenoids into the biological system. Results indicate that, the repeated dose and dietary feeding of astaxanthin to RD rats resulted in increased retinol levels along with detectable levels of β-carotene, unlike the single dose. Further, on repeated feeding of astaxanthin to RD rats, it was found that astaxanthin may be converted to β-carotene which in turn cleaved to retinol. Single/repeated dose and dietary feeding of β-carotene or carrot powder resulted in elevated retinol levels in plasma and liver. Lutein and fucoxanthin gavages were not associated with change in the retinol levels, indicating that they do not yield retinol even under retinol deficiency.

Chapter 5: Elucidation and characterization of metabolites of β-carotene, astaxanthin, lutein and fucoxanthin in vitamin A deficient rats

This chapter begins with an introduction that briefly describes the reported metabolites of β-carotene, astaxanthin, lutein and fucoxanthin. Plasma and liver extracts of the RD rats gavaged with a single dose of β-carotene were analyzed by HPLC and LCMS to characterize their metabolites under RD condition. The major metabolite of β-carotene (m/z 537 $(M+H)^+$) detected in plasma and liver was retinol (m/z 287 $(M+H)^+$). Few other minor peaks were not considered as their magnitude was too low for confirmation. No apocarotenals were detected as metabolites of β-carotene in this study. Either the β-carotene or apocarotenals were rapidly degraded to retinol or else the centric cleavage of β-carotene may have been predominant resulting primarily in retinol. On feeding β-carotene to RD rats, there was a rise in plasma and liver retinol levels which corresponded with a lower level of β-carotene indicating its conversion to retinol.

Plasma and liver extracts of rats after repeated gavage with astaxanthin showed the presence of its native form (m/z 597 $(M+H)^+$) along with β-carotene (m/z 537 $(M+H)^+$) and retinol (m/z 287 $(M+H)^+$). In addition, an unknown compound identified in liver was characterized as echinenone (m/z 551 $(M+H)^+$), the reduced metabolite of astaxanthin/canthaxanthin, usually noticed in algae and lower vertebrates that feed on algae. RD rats fed on astaxanthin showed an increase in plasma and liver retinol levels along with detectable level of β-carotene supporting the speculation that the bioconversion of astaxanthin to β-carotene via echinenone, resulted in retinol. Astaxanthin and its hydroxyl derivatives may undergo reduction reactions leading to dehydroxylation with the removal of two hydroxyl groups and one keto group to form echinenone. It is probable that the intermediate compounds of these reductive reactions are unstable and short-lived and therefore were not detected. Echinenone in turn may have been reduced to β-carotene and further cleaved to retinol by the action of monooxygenase. This study strongly suggests that under clinical RD condition in rats, in contrast to previous reports, astaxanthin may have been reduced to echinenone and β-carotene for the rapid formation of retinol. Based on the results metabolic pathway of astaxanthin is proposed.

Lutein gavages to RD rats did not result in change in the plasma and liver retinol levels, while lutein (m/z 551 $(M+H-H_2O)^+$), zeaxanthin (m/z 569 $(M+H)^+$) and other metabolites designated as A (m/z 552 $(M+H)^+$), anhydrolutein, B (m/z 601.8 $(M+H)^+$), lutein diepoxide, C (m/z 382.5 $(M+H)^+$), D (m/z 201.3 $(M+H)^+$) and E (121.2 $(M+H)^+$) were detected in the plasma and liver. Lutein oxidation in liver may have resulted in the formation of the metabolites designated as B (lutein diepoxide) and C. Fragmentation of A (anhydrolutein) may have resulted

in the formation of the metabolites designated as D and E. Based on the results, the metabolic pathway for lutein is proposed.

Fucoxanthin intubation to RD rats showed the presence of major metabolites fucoxanthinol (m/z 617 $(M+H)^+$) and amarouciaxanthin (m/z 615 $(M+H)^+$). Unidentified metabolites are designated as A (m/z 603 $(M+H)^+$), B (m/z 579 $(M+H)^+$), C (m/z 551 $(M+H)^+$), D (m/z 523 $(M+H)^+$), E (m/z 369 $(M+H)^+$), F (m/z 641 $(M+H)^+$), G (m/z 629 $(M+H)^+$), H (m/z 623 $(M+H)^+$), I (m/z 596 $(M+H)^+$), J (m/z 574 $(M+H)^+$), K (m/z 517 $(M+H)^+$), L (m/z 429 $(M+H)^+$), M (m/z 339 $(M+H)^+$) and N (m/z 311 $(M+H)^+$) in plasma and liver. Amarouciaxanthin was a major metabolite of fucoxanthin in liver whereas, it was fucoxanthinol in plasma, demonstrating that liver enzymes may play a role in hydrolyzing the fucoxanthinol to amarouciaxanthin. The conversion of fucoxanthin to fucoxanthinol and/or amarouciaxanthin was higher in liver, which may be due to dehydrogenation and/or isomerization of 5,6-epoxy-3-hydroxy-2,4-dihydro-β end group of fucoxanthinol to 6-hydroxy-3-oxo-ε end group of amarouciaxanthin by liver dehydorgenase. Metabolites found in plasma (A & B) and liver (A, F, H, I & K) could have resulted from either fucoxanthinol or amarouciaxanthin by removal of water molecules due to enzymatic reactions involving oxygenases. Metabolites (E, L, M & N) could partly be due to enzymatic retroaldol cleavage. Enzymatic demethylation of fucoxanthinol or amarouciaxanthin could be the reason for the metabolites C, D, G, J, K, L, M & N. The possible metabolic pathway of fucoxanthin in RD rats is proposed.

Chapter 6: Influence of dietary carotenoids on vitamin A deficiency induced biochemical changes

This chapter begins with an introduction detailing the effect of carotenoids on various biochemical parameters. There were three experiments, viz., single dose study (gavage of β-carotene, astaxanthin, lutein and fucoxanthin) repeated dose study (gavages of β-carotene, astaxanthin and lutein for 7 and 15 days) and dietary study (dietary feeding of carrot powder, dill leaf powder (sources of β-carotene and lutein) and purified astaxanthin) to RD rats. These studies mainly dealt with biochemical parameters like 15-15', β-carotene monooxygenase, lipid peroxidation, activities of antioxidant molecules such as superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione (GSH) levels, lipid profile parameters such as fatty acids, total cholesterol, phospholipids, triglycerides, HDL-cholesterol and LDL+VLDL-cholesterol in blood and liver tissue. Separate baseline (RD) and control groups were maintained for each experiment.

Results showed that retinol deficiency resulted in altered biochemical parameters when compared to control in all experiments. There was a significant increase in lipid peroxidation which ranged from 72-89% (plasma) and 76-88% (liver) and a decrease in the activities of SOD (66-77%, 51-77%), CAT (55-89%, 63-77%), GSH levels (55-67%, 64-78%) in plasma and liver and liver GST (54-60%). The fatty acid profile revealed an increase (34-50%) in saturated fatty acids (SFA) and decreased mono-unsaturated (MUFA, 40-52%) and poly-unsaturated (PUFA, 44-55%) fatty acids in the plasma as a result of retinol deficiency. The cholesterol, phospholipid and triglyceride levels in the plasma and liver was decreased when compared to control group.

Results of the single dose study revealed that gavage of β-carotene to RD rats resulted in enhanced monooxygenase activity over 8h (56-70%) which corresponded with increased plasma retinol levels (Chapter 2) when compared to RD group, unlike astaxanthin, lutein and fucoxanthin, whose monooxygenase activity and retinol levels were not significantly different from RD group (44.4 pmol retinal/h/mg protein, 0.38 μmol/l). In contrast, repeated gavages of carotenoids for 7 and 15 days resulted in enhanced monooxygenase activity in β-carotene (70, 73%) and astaxanthin (68, 70%) groups as compared to RD group. No such alterations were found in lutein and fucoxanthin groups. Dietary feeding study also demonstrated that the activity of monooxygenase was elevated on feeding diet supplemented with carrot powder (73%) as β-carotene source, pure astaxanthin (69%) and dill leaf powder (73%) as lutein source, as compared to RD group and corresponded with the elevated retinol levels in respective groups (Chapter 2).

Single and repeated gavages with carotenoids to RD rats resulted in significantly lowered lipid peroxidation in plasma (>40%) and liver (>25%) and increased activities of SOD (>20%, >22%), CAT (>29%, >29%), GSH (>11%, >13%) in plasma and liver and activity of GST (>25%) in liver. The results show that the level of intact carotenoids was in the order of lutein > astaxanthin > β-carotene. Effect of carotenoids in reducing oxidative stress by lowering lipid peroxidation and increasing activity of antioxidant molecules was in the order of astaxanthin $\simeq$ fucoxanthin > lutein > β-carotene. The reason for the differential effects exhibited by the carotenoids may be due to their chemical structures, which vary in terms of polarity and functional groups. Plasma and liver lipid peroxidation was lower in groups fed diet supplemented with carrot powder (50, 38%), astaxanthin (20, 60%) and dill leaf powder (43, 44%) as compared to RD group.

Gavage with carotenoids to RD rats also exhibited an ameliorative effect on the plasma fatty acid profile with decrease (20-47%) in the SFA and increase in the MUFA (23-48%) and PUFA (31-57%) levels. A decrease in the SFA was in the range 47-48% on feeding diet supplemented with carrot powder, astaxanthin and dill leaf powder and simultaneously, an

increase in MUFA by 41-42% and PUFA by 51-52% was recorded. The order of the ameliorative effect of the carotenoids on fatty acid profile was: β-carotene > astaxanthin > lutein. On feeding carotenoids, serum and liver cholesterol and phospholipids were increased when compared to RD group. The triglycerides showed a significant increase in plasma by 1.4 to 2.1 fold and in liver by 1.1 to 1.8 fold indicating the influx of carotenoids. Based on the biochemical parameters studied, the protective effect of the carotenoids was in the order of astaxanthin $\underset{\sim}{}$ fucoxanthin > lutein > β-carotene.

Chapter 7: Effect of dietary carotenoids on vitamin A deficiency induced changes in biochemical constituents in tissue membrane of rats with respect to structure and function

This chapter commences with an introduction that succinctly describes the retinol deficiency mediated oxidative stress on the tissue membrane as well as membrane related biochemical parameters. Effect of retinol deficiency and subsequent carotenoid ingestion on the retinol levels, lipid peroxide levels, activity of Na^+K^+-ATPase, Ca^{2+}-ATPase, Mg^{2+}-ATPase, superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione levels (GSH), fatty acid profile, cholesterol, phospholipids and triglycerides levels in liver microsomes and acetylcholine esterase (AChE) activity in brain microsomes was studied.

Retinol deficiency resulted in depleted retinol levels (0.8-2 nmol/ml) in the liver microsomes as compared to control (66.5-874 nmol/ml). A single dose of respective carotenoids resulted in increased retinol levels in the liver microsomes of β-carotene group over 8h (3.5-20.3 nmol/ml) as compared to RD group (1.6 nmol/ml) while no such increase was observed in the astaxanthin, lutein and fucoxanthin groups. Whereas, a rise in retinol levels was observed in the liver microsomes on repeated gavages (7 and 15 days) with β-carotene (31, 93.4 nmol/ml) and astaxanthin (5.5, 15.4 nmol/ml) as compared to RD group (0.8 nmol/ml). In contrast, no significant change was observed in the retinol levels of liver microsomes of lutein group (1, 2.1 nmol/ml). The presence of β-carotene in the liver microsomes of astaxanthin group (15 days) indicated its formation from astaxanthin. In addition to β-carotene, intact astaxanthin was also detected in the liver microsomes of astaxanthin group, whereas, lutein and zeaxanthin were detected in the liver microsomes of rats gavaged with lutein. Detectable levels of β-, α-carotene and lutein were detected in the liver microsomes of rats fed diet supplemented with carrot powder, while astaxanthin and β-carotene were detected in groups fed on diet with astaxanthin and β-carotene.

Retinol deficiency resulted in significantly elevated activities of membrane bound Na^+K^+-ATPase (69-76%), Ca^{2+}-ATPase (69-75%) and Mg^{2+}-ATPase (51-67%) than the control group. On the other hand, AChE activity in brain microsomes was significantly lowered (73-84%) as compared to the control group. Retinol deficiency also resulted in elevated lipid peroxide levels (84-95%) and decreased activities of SOD (61-70%), CAT (70-73%), GST (48-55%) and GSH levels (51-63%) as compared to control. Fatty acid profile of RD rats revealed elevated saturated fatty acids (SFA) that ranged between 20 to 33% and lowered mono- and poly-unsaturated fatty acids (MUFA and PUFA) by 43-60% and 60-70% respectively in comparison with control group. Estimation of the cholesterol: phospholipid ratio revealed increased ratio values for RD group (0.91-0.92) as compared to control group (0.69-0.7), indicating altered fluidity and thus integrity of the microsomal membranes in retinol deficiency.

Single/repeated gavages and dietary feeding of carotenoids resulted in amelioration of the alterations caused by retinol deficiency. The elevated Na^+K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase activities in RD group were lowered by 17-73%, 21-73% and 17-63% respectively. Whereas, the lowered AChE activity caused by retinol deficiency was elevated on feeding carotenoids by 20-83%. In the single dose study, the order of the effectiveness of the carotenoids with respect to the membrane bound enzymes was β-carotene $\simeq$ fucoxanthin > astaxanthin $\simeq$ lutein. Whereas, in the repeated dose study, the effect was in the order of astaxanthin > β-carotene > lutein. Efficacy of dietary feeding of carotenoid sources on the membrane bound enzymes was as follows: carrot powder $\simeq$ dill leaf powder > astaxanthin. A significant reversal in the activity of Na^+K^+-ATPase in astaxanthin and β-carotene groups was probably due to the rise in retinol levels.

Administration of carotenoids as single/repeated gavages and dietary feeding to RD rats significantly ameliorated the changes caused by retinol deficiency with suppression of lipid peroxidation (22-89%) and increased SOD (10-68%), CAT (11-74%), GST (9-57%) activities and GSH (16-66%) levels. The order of suppression of lipid peroxidation by carotenoids after a single dose was astaxanthin $\simeq$ fucoxanthin > lutein > β-carotene while their effect on the antioxidant molecules was in the order of fucoxanthin > β-carotene $\simeq$ lutein > astaxanthin. The protection to membrane by repeated dose of carotenoids was in the order of astaxanthin > lutein > β-carotene. Protective effect of feeding diet supplemented with carotenoid sources was fond to be alike. Results show that lipid peroxidation was suppressed in carotenoid fed groups, especially in liver microsomes of the astaxanthin group. The possible reason for this could be due to the dual presence of intact astaxanthin and newly formed retinol. The reason for differences in the potencies of carotenoids used in this study may lie in their localization in the membrane.

Otherwise, retinol formed from β-carotene or astaxanthin may associate with cell membrane and provide protection.

Gavage studies as well as dietary feeding of carotenoid sources to RD rats ameliorated the changes occurred in the fatty acid profile by retinol deficiency. The SFA were decreased by 5 to 32%, while MUFA and PUFA were elevated by 13-60% and 40–69%, respectively. The ameliorative effect in the case of a single dose of astaxanthin and lutein was greater than the β-carotene and fucoxanthin. The effect of repeated gavages of carotenoids on the fatty acids was in the order of astaxanthin > β-carotene $\underset{\sim}{}$ lutein. Effect of feeding carrot powder, astaxanthin and dill leaf powder supplemented diets was similar to each other. Further, gavage of astaxanthin and lutein resulted in lowered cholesterol: phospholipid (C: P) ratio (0.77, 0.79) in liver microsomes as compared to RD (0.92) group and the effect of β-carotene was greater than astaxanthin and lutein. Whereas, repeated gavages for 7 and 15 days with β-carotene and astaxanthin was found to decrease their ratio further. Feeding carrot powder, astaxanthin and dill leaf powder supplemented diet to RD rats resulted in similar decrease of the cholesterol: phospholipid ratio and the values were 0.7, 0.74 and 0.72 respectively. Hence, β-carotene was more effective in lowering the cholesterol: phospholipid ratio after a single dose as compared to astaxanthin and lutein while both β-carotene and astaxanthin were more effective than lutein when the dose was administered repeatedly.

In conclusion, many of the leafy greens, vegetables and medicinal plants analyzed in this study were rich in lutein, β-carotene, zeaxanthin, neoxanthin, violaxanthin and α-carotene. Fucoxanthin was detected in many of the marine seaweeds analyzed. More than 50% of the plants screened are unexplored and have been studied for the first time. The results could be useful to health workers and persons practicing Ayurveda and naturopathy in the selection of plants for their antioxidative properties for alleviation of diseases. Results of the studies with rats show increased lipid peroxidation and decreased activity of antioxidant molecules, altered liver microsomal ATPases and brain microsomal acetylcholine esterase activities as a result of retinol deficiency. Astaxanthin, β-carotene and lutein resulted in amelioration of these biochemical changes. Interestingly, β-carotene detected in the plasma of astaxanthin group along with elevated retinol levels indicates its conversion to retinol via β-carotene. Increased activity of intestinal β-carotene monooxygenase further supports the above results. Although the level of astaxanthin was lower than lutein, its efficacy in suppressing lipid peroxidation in plasma and liver microsomes was higher. On the basis of the protection afforded to the membranes with respect to

lipid peroxidation, antioxidant and membrane bound enzymes, the order of the effect was astaxanthin > lutein > β-carotene.

Results further suggest that rats metabolize nonprovitamin A carotenoid, astaxanthin to retinol in retinol deficiency, maybe to meet the retinol requirement for maintenance of structure-function of membranes. Retinol deficiency resulted in depleted retinol levels in liver microsomes and induced alteration in the activities of membrane bound and antioxidant enzymes, lipid peroxide levels and lipid parameters indicating altered structure and function of the membranes. β-Carotene, astaxanthin, lutein and fucoxanthin were found to ameliorate retinol deficiency induced alterations in the rats. When administered a single dose, β-carotene was the most effective, while on repeated gavages, β-carotene and astaxanthin were found to have greater effect. Dietary feeding of carotenoid sources was also found to ameliorate the alterations caused by retinol deficiency. Retinol deficiency in rats resulted in depleted retinol levels and activity of antioxidant enzymes, while, Na^+K^+-ATPase activity and lipid peroxides level in plasma, liver and microsomes was elevated. Carotenoid gavage ameliorated these effects.

Chapter 8: General discussion and summary

In this chapter, the findings of the present investigation are discussed. Results of investigations of similar nature reported in literature are also discussed, highlighting the important findings of the present investigation. The major findings of the investigation are briefly summarized. At the end of the thesis, a collective list of references, which forms the basis for interpretation of the data obtained in comparison with earlier and contemporary published results, has been given.

CHAPTER 1: General Introduction

India is being heralded as the future leader of the world and is rapidly progressing in various fields like medicine, computers and software, nuclear technology and so on. However, to be on par with the developed nations, the growth and development of the country has to be holistic and in all fields. The most important resource of a nation is its people. And, maximum input is possible only by people who enjoy good health. Hence, the health and nutritional status of every individual in the country is an integral part of progress.

Eradication and cure of communicable diseases, nutritional deficiency conditions, and chronic disorders along with improving the standard of living is the primary goal of medical and health research. The essential micronutrients include the vitamins A, B, C, D, E and K and minerals. Vitamin A has long been recognized as vital for well being and has been associated with vision. It is now well established that vitamin A not only plays an essential role in vision health, but in a number of other physiological functions as well. The chemistry, dietary sources, functional properties, metabolism and deficiency related health problems of vitamin A (retinol) and various strategies to prevent its deficiency are outlined below.

Retinol (Vitamin A)

Retinol is a fat-soluble vitamin. It is the free-alcohol form of vitamin A and can be reversibly converted by enzymatic activity to the visually active aldehyde form of vitamin A (retinal), in eyes. Except in retina, retinal concentrations are normally very low in other tissues. This is due to enzyme kinetics that favor either its re-conversion to retinol, or transformation to retinoic acid (Palace et al., 1999). The term vitamin A now includes all β-ionone derivatives (other than carotenoids) that have the biological activity of all-trans retinol (Mc Larsen and Frigg, 2001). Chemical formulae and structures of retinol, retinal and retinoic acid are given in Table 1.1. Excellent dietary sources of preformed retinol are given in Table 1.2.

Functions of Vitamin A

Vitamin A performs multitude functions and plays an important role in maintaining the homeostasis of the body. Some of the major functions along with the mechanism of action of vitamin A are given in Table 1.3. The prime function of vitamin A is regulation of vision cycle (Rando, 1994).

Table 1.1. Chemical structure and formulae of retinol, retinal and retinoic acid

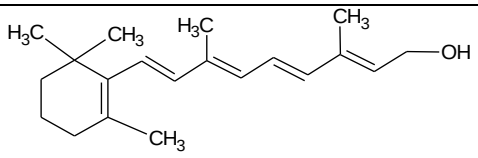
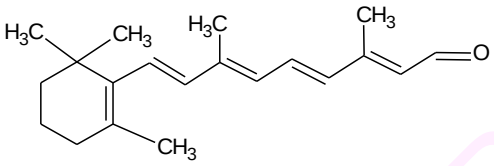
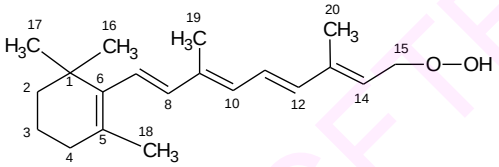
Molecule	Chemical Structure	Chemical Formula
Retinol		$C_{20}H_{30}O$
Retinal		$C_{20}H_{29}O$
Retinoic Acid		$C_{20}H_{30}O_2$

Table 1.2. Dietary sources of preformed retinol

Dairy Products	Organ Meats
▪ Milk	▪ Liver
▪ Cheese	▪ Kidney
▪ Butter	▪ Red meat
▪ Margarine	

(Source: Palace et al., 1999)

Major functions performed by vitamin A are its role in vision or visual phototransduction. Phototransduction is a process by which light is converted into electrical signals in the rod, cone and photosensitive ganglion cells of the retina of the eye. The visual cycle is the biological conversion of a photon into an electrical signal in the eye. The mechanism involved is described in the visual cycle (Figure 1.1). All-trans retinol reaches the retinal pigment epithelium via the blood stream, where it is esterified and stored, or isomerized to 11-cis retinol, which is further oxidized to 11-cis retinal. This is transported to the rod (in low illumination) and cone (colour and

Table 1.3. Functions and mechanisms of actions of vitamin A.

Function	Mechanism
Normal vision	Scotopic and colour vision via Wald's Visual Cycle.
Cell growth	Regulation of fibroblasts, collagen synthesis and inhibition of collagen-degrading matrix metallo-proteinases.
Cell differentiation	Signaling molecule that causes progenitor cells to take the first step towards differentiating into specific organ cells.
Cell mediated defence (immune system)	Boost production of antibodies and white blood cells (macrophages and T-cells), regulate T-lymphocytes and thymocytes, increase natural killer cell numbers and activity and regulate growth and activity of B-cells.
Non specific defence (epithelium)	Lining of skin, respiratory, urico-genital and gastrointestinal tract.
Gene expression and transcription	Regulation via retinoic acid nuclear receptors: retinoic acid receptors (RAR) or the retinoid "X" receptors (RXR).
Glycoprotein synthesis	Regulation of lipid linked oligosaccharides.
Haemopoiesis	Iron metabolism.
Fertility	Male (spermatogenesis and testes health), Female (reproductive organs' health and embryogenesis).
Embryogenesis	Development of heart, embryonic circulatory and central nervous systems.
Bone/skeletal development	Activities of epiphyse**a**l-c**a**rtil**a**ge cells, required for time sequence of bone growth, m**a**tur**a**tion, **a**nd degener**a**tion.
Teeth metabolism	Formation of enamel.
Gap junctional communication	Increase gap junctional communication to reduce uncontrolled cell growth.
Energy balance	Thyroid metabolism and oxygen consumption.
Antioxidant	Lipophillic chain breaking antioxidant by combining with peroxyl radical.

(Source: Mc Larsen and Frigg, 2001)

bright illumination) cells where it combines with opsin to form light sensitive rhodopsin or ionopsin respectively. On light exposure, 11-cis retinal is isomerized back to all-trans retinal that is reduced to all-trans retinol and transported back and recycled. These complex biochemical reactions in the retina result in the generation of a nerve pulse. The inter-photoreceptor retinoid-binding protein (IRBP) lies in the layer between the epithelium and photoreceptor cells and is responsible for the transport of the isomers. The cellular retinal-binding protein (CRALBP) binds closely to 11-cis retinal and 11-cis retinol and protects the former from photoisomerization.

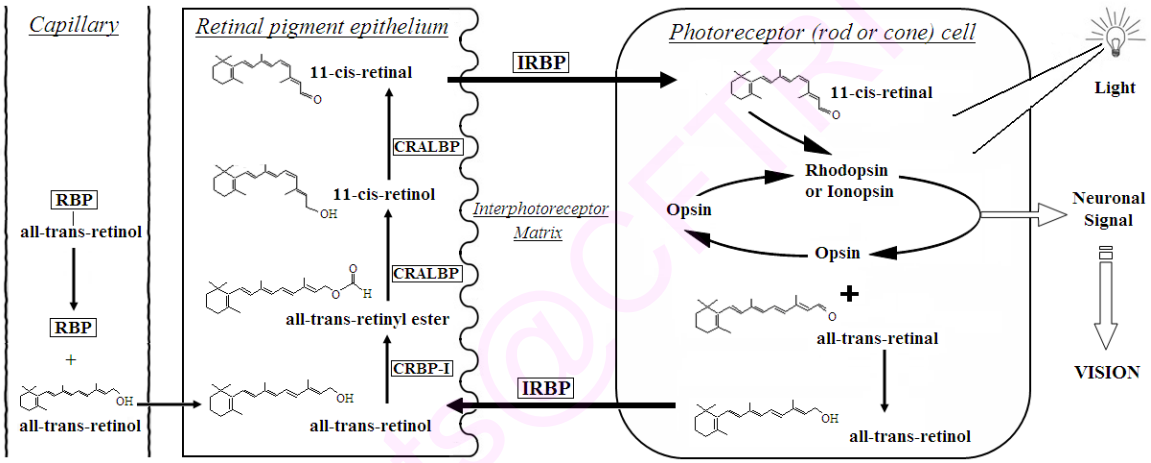


Figure 1.1: Role of vitamin-A in the visual cycle in eyes. CRALBP = Cellular retinal-binding protein, CRBP = Cellular retinol-binding protein, IRBP = Interphotoreceptor retinoid-binding protein, RBP = Retinol binding protein.

Antioxidant function

Retinol also acts as an antioxidant in the body in different ways. It can act as a chain breaking antioxidant by combining with peroxyl radicals, before these radicals can propagate peroxidation in the lipid phase of the cell and generate hydroperoxides. Retinol is superior in scavenging peroxyl radicals than tocopherol when the initiating radical species are present within the lipid bi-layer and do not originate from the aqueous environment. This property of retinol may have been due to its short polyene chain, allowing it to have a higher mobility and a better opportunity to interact with peroxyl radicals in the membrane phase (D'Aquino et al., 1989). Using pulse radiolysis techniques, it has been reported that retinol can scavenge the potentially

damaging glutathione radical (D'Aquino et al., 1989). Antioxidant activities of retinoids have been ranked as retinol > retinal > retinyl palmitate > retinoic acid (Das, 1989).

Other Functions of Vitamin A

Retinoic acid via the retinoic acid receptor influences the process of cell differentiation, growth and development of embryos. Retinoic acid is also an influential factor used in differentiation of stem cells to more committed fates, echoing retinoic acid's importance in natural embryonic development pathways. Vitamin A is essential for the correct functioning of epithelial cells. Glycoprotein synthesis requires an adequate vitamin A status. In severe vitamin A deficiency (VAD), lack of glycoprotein synthesis may lead to corneal ulcers or liquefaction. In VAD, mucus-secreting cells are replaced by keratin producing cells, leading to xerosis. Vitamin A is essential for maintenance of the epithelial tissue as a physical barrier to infection and is also involved in maintaining a number of immune cell types including lymphocytes and melanoytes.

Metabolism of Vitamin A

The pathways involved in the metabolism of retinol in blood and liver are summarized in Figure 1.2. Retinol and retinyl esters released from food are hydrolyzed to retinol in the intestinal lumen and enter the blood circulation after re-esterification to retinyl esters, which bind to chylomicrons and reach the liver. Retinol is transported in the plasma bound to retinol binding protein (RBP), which is further complexed with transthyretin, the protein that transports the thyroid hormone, triiodothyronine (Green et al., 1993). Retinol in other tissues, including the heart, is obtained from the plasma transport complex. Retinol and retinyl esters bound to RBP and chylomicrons are taken up by the hepatocytes bound to cellular retinol binding protein (CRBP). CRBP is responsible for creating a thermo-chemically favorable gradient for retinol uptake by the cell when its binding sites are unoccupied by the ligand (Noy and Blaner, 1991). In addition to binding to CRBP intracellularly, vitamin A can be found embedded in the lipid bilayer of cellular and sub cellular membranes (Ciaccio et al., 1993). Retinol appears to orient its cyclic carbon ring near the polar-group region of the lipid bilayer with its polyene chain extended into the medial non-polar region of the bilayer (De and Zidovetzki, 1988). The retinol in the hepatocytes may be stored as retinyl esters in the stellate cells and alternately, the retinol isomerizes to retinal, giving rise to retinoic acid and its metabolites. The kinetic behavior of retinol metabolism in the blood appears to be similar for humans and rats (Burri and Clifford, 2004).

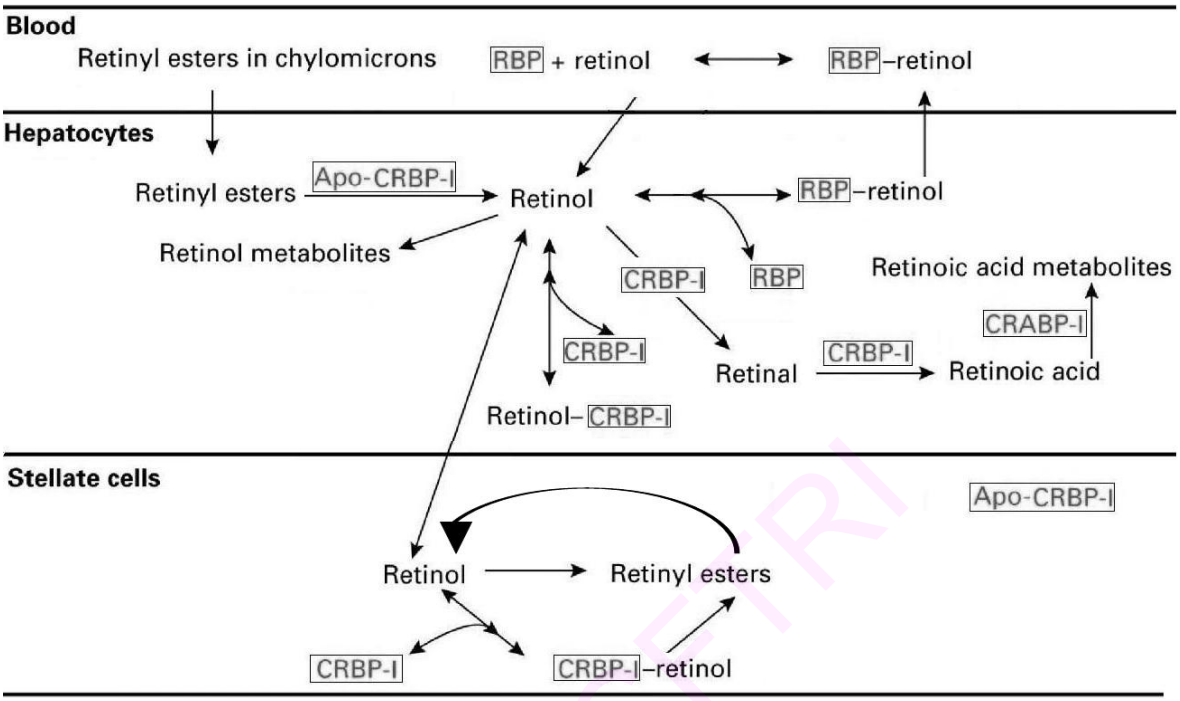


Figure 1.2: Metabolic pathway of retinol in blood, hepatocytes and stellate cells in liver. (Source: Noy, 2000)

Assessment of Vitamin A status

Since serum retinol concentration is tightly regulated and 90% of body stores are in tissues such as liver and kidney, it is difficult to assess retinol status using non-isotopic methods (Clagett-Dame and DeLuca, 2002). Changes in the dietary intake results in only small alteration in the serum retinol concentrations. Therefore, except in severe retinol deficiency, serum retinol concentration provides uncertain estimates of retinol status. Previously, serum retinol level lower than 0.35 µmol/l was considered "deficient" and <0.7 µmol/l as "low". However, WHO (1996) has recommended <0.7 µmol/l as sub-clinical state of retinol deficiency.

The nutritional status is assessed using various tests and these are effective depending upon the stage of retinol deficiency. Physical symptoms such as ocular signs (Bitot's spot, conjunctival and corneal xerosis, corneal scar, etc.), physiological tests (dark adaptation for night blindness), sub-clinical structural change (conjunctival impression cytology), serum retinol levels, enzyme activity such as erythrocyte transketolase, estimation of serum retinol-binding protein

(RBP) and indirect methods such as dietary intake have been utilized to assess the vitamin A status.

Vitamin A deficiency

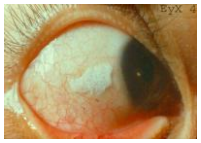
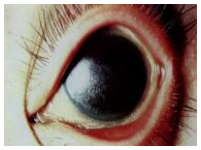
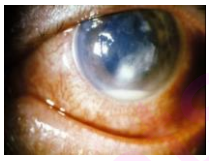
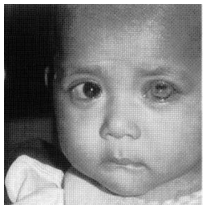
VAD and its progress involve a variety of physiological changes. Many functions controlled by vitamin A such as vision, growth, reproduction, skin health, immunity and erythropoiesis (due to impaired mobilization of iron from stores to bone marrow) are affected in VAD. Since rhodopsin in rod cells are more sensitive to VAD than iodopsin in cone cells, rod function is affected early and reflected in impaired night vision, leading to night blindness. All the clinical signs and symptoms that affect the eye in VAD are classified under Xerophthalmia and are listed in Table 1.4.

Prevalence of vitamin A deficiency

The World Health Organization in 1995 (West et al., 2005) had estimated that 254 million children have VAD and 2.8 million are afflicted by xerophthalmia. Subsequently, estimates were changed to 75-140 million and 3.3 million, respectively. The present analysis indicates there are ~127 million and 4.4 million preschool children with VAD (serum retinol < 0.70 µmol/L or displaying abnormal impression cytology) and xerophthalmia, respectively. More than 7.2 million pregnant women in the world are vitamin A-deficient (serum or breast-milk vitamin A concentrations < 0.70 µmol/L), and another 13.6 million have low vitamin A status (0.70-1.05 µmol/L). Further, WHO has reported that >6 million women develop night blindness during pregnancy annually. Roughly, 45% of VAD and xerophthalmic children and pregnant women with low-to-deficient vitamin A status live in South and South-East Asia. These regions harbor > 60% of all cases of maternal night blindness, three fourths of whom seem to live in India. Prevalence of VAD in India among preschool children is estimated to be 30.8%, while that of xerophthalmia is 1.6%. Pregnant women (1.6%) in India are deficient in vitamin A while 12.1% suffer from night blindness (West et al., 2002).

Reports from different parts of India also show the high prevalence of VAD. Laxmaiah et al. (2002) reported 51.7 and 21.5% prevalence of VAD in infants/pre-schoolers and school-going children, respectively, in the state of Punjab, while Feldon et al. (2005) reported 3.7% incidence of VAD (corneal xerosis, night blindness and bitot's spots) in Delhi. Rao et al. (2005) have estimated that 1.6% of school going children of the Gond tribal population in Jabalpur and 23% of slum children in Bhopal, Madhya Pradesh are affected by VAD (Dwivedi et al., 1992). Studies

Table 1.4: Xerophthalmia classification by ocular signs and WHO classification.

Classification	Pictorial representation	Description
Night Blindness (XN)		Rod cells-more sensitive to VAD than cone cells. Onset of night blindness (Xerophthalmia).
Conjunctival Xerosis (X1A)		Xerotic change in conjunctiva such as abnormal impression cytology, dryness of conjunctiva, keratinization and accumulation of material.
Bitot's Spot (X1B)		Final stage of xerosis, in exposed part of conjunctiva, heaped-up desquamated, keratinized epithelial cells forming a raised area.
Corneal Xerosis (X2)		Hazy appearance for 1-2 days, advancing into keratomalacia. Prompt treatment up to this stage can preserve sight without residual impairment.
Corneal ulceration or keratomalacia (X3A: < $^1/_3$ of corneal surface and X3B: > $^1/_3$ of corneal surface)	 	Softening of corneal substance (colliquative necrosis) and increased xerosis of epithelium, stroma becomes oedematous. Usually one ulcer per eye, typically in infero-nasal position, Collection of sterile pus in anterior chamber (hypopyon) and infection is common.
Corneal Scar (XS)		Visual impairment, damage confined to cornea may be overcome by surgery (not when internal structures are involved).
Xerophthalmic fundus (XF)		Rare condition, results from prolonged VAD, impairment of rod function succeeded by structural damage to retina.

(Source: Mc Larsen and Frigg, 2001)

by Choudhary et al. (2003) and Pathak et al. (2003) have estimated 13.7 and 15.9 % prevalence of VAD in the adolescent aged individuals in India. Pregnant women in Southern districts (2.9%) and 4.8 % in Delhi are reported to fall under the VAD category (Toteja et al., 2002). Vinutha et al. (2000) reported that nearly 30% of the 109 pregnant women examined exhibited VAD. Feldon et al. (2005) found a prevalence of 1.1, 1.9, 14.3, 11.4, 1.6, 2.8, 10.7 and 7.2% in Nanded (Maharashtra), Goalpara, Dhubri, Guwahati (Assam), Farrukabad (Uttar Pradesh), Badaun (Uttar Pradesh), Bareilly (Uttar Pradesh), Bellary, Bagalkot (Karnataka), Ranchi (Jharkhand), and Khagaria (Bihar) respectively and an overall prevalence of 3.7% in children. Although VAD prophylaxis programmes are in place in most of the affected countries, greater effort is needed to assess and prevent VAD and its disorders (WHO, 2009).

Effect of retinol deficiency

Retinol is found in all cells as an integral part of the complex membrane structure (Britton, 1995). Therefore, retinol deficiency may provoke a general alteration in the physical characteristics of cell membranes. Retinol deficiency has pro-oxidative effect and increases the oxidative stress in rats (Kaul and Krishnakantha 1997). Cellular and sub-cellular membranes are susceptible to lipid oxidation due to high concentration of polyunsaturated fatty acids and close proximity to oxygen, transition metals, and peroxidases. Increased lipid peroxidation (Lpx) due to retinol deficiency is reported to be indicative of damage to cell membrane structure-function, in turn contributing to pathological abnormalities in tissues as well (Anzulovich et al., 2000). Membrane bound ATPases and antioxidant molecules are intimately associated with the regulation of Na^+, K^+, Ca^{2+}, and Mg^{2+} ions across the membrane and defense activities (Selvendiran and Sakthisekaran, 2004). Retinol deficiency induced oxidative stress leads to an elevated activity of Na^+K^+-ATPase and alters membrane fluidity in rat liver and kidney (Kaul and Krishnakantha, 1997). Anzulovich et al. (2000) and Oliveros et al. (2000) have reported the change in liver histoarchitecture and heart tissue as well as elevated oxidative stress as a result of retinol deprivation for 3 months in rats.

Strategies to prevent and cure VAD and its related disorders

Strategies in practice to prevent and cure VAD include:

1. Nutrition programmes for prophylaxis and treatment of VAD and related symptoms, including food fortification.

2. Proper immunization against measles, which is strongly correlated with the occurrence of VAD in infants and children.
3. Improvement of nutrition and health awareness with respect to identification of VAD and its symptoms, administration of synthetic vitamin A and foods rich in vitamin A.

Proposed strategy to prevent VAD:

4. Increased incorporation of foods that naturally contain precursors of vitamin A such as β-carotene, α-carotene etc. (provitamin A carotenoids) and/or other carotenoids such as lutein, astaxanthin, neoxanthin, violaxanthin, lycopene, etc. (non-provitamin A xanthophylls) which can render protection against VAD and its associated symptoms and disorders.

Amongst the strategies, the first three have been undertaken by the Government of India and implemented by means of various programmes. The National Prophylaxis programme for prevention of infant and childhood blindness due to VAD by the Government of India involves administration of 100,000 IU retinol at 9 months of age (infants) and 200,000 IU every 6 months to children aged 1-3 years. An immediate dose of vitamin A is administered in case of severe malnutrition or if measles is present and more than one month has elapsed since the last dose. Retinol fortified edible oil is used to treat moderately advanced stages of VAD characterized by ocular signs such as xerophthalmia. Although, this programme benefits infants and children, it does not answer the cause for pregnant and lactating mothers, who are a vulnerable group as well. Other programmes that address the vitamin A status of an individual include the Integrated Child Development Services (ICDS) that provides supplementary nutrition with vitamin A, iron, folic acid and covers immunization as well. The Reproductive and Child Health (RCH) programme that deals with infants and women and provides vitamin A supplementation to children aged 6-36 months. The Kishore Shakti Yojana (KSY) deals with prophylactic measures against anaemia, goitre and VAD and immunization for adolescent girls. UNICEF (2007) has reported 50-79% implementation of two doses of preformed vitamin A to children in the Indian subcontinent (Figure 1.3). The first dose consists of administration of 100,000 IU at 9 months of age (with measles immunization) and the second dose is 200,000 IU at 15 months of age (with DPT booster vaccine). Although these programmes have been implemented with varying levels of success at the community level, the outcomes of the programmes have not been very encouraging. The reason probably lies in the inefficient compliance rate of the interventions. Therefore, the strategy that deals with high consumption of provitamin A rich foods as an alternative to vitamin A or

antioxidant carotenoids can be used as a VAD preventive tool. Thus, the results of this research work will ultimately help in understanding the importance of dietary carotenoids (provitamin A and nonprovitamin A carotenoids) in VAD and will also help in determining their relative superiority as a source of vitamin A and therefore aid in implementation of this strategy at the community level.

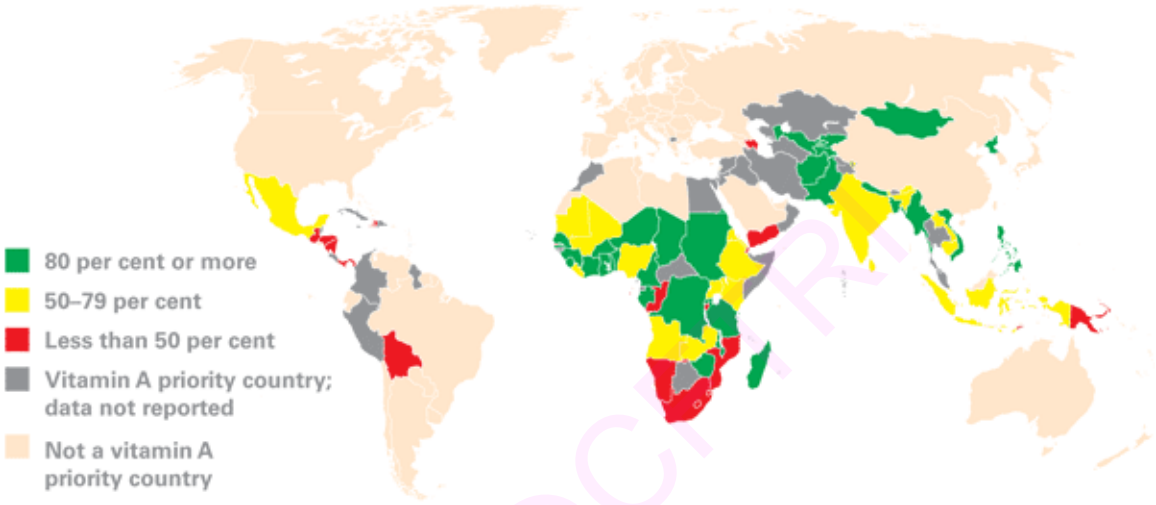


Figure 1.3. Country-wise coverage of two doses of supplemental vitamin A (Source: UNICEF, 2007).

Carotenoids

Carotenoids are isoprenoid compounds, biosynthesized by tail-to-tail linkage of two C_{20} geranylgeranyl diphosphate molecules. They are highly lipophilic and are sparingly soluble or insoluble in water. This produces the parent C_{40} carbon skeleton from which all the individual variations are derived by either cyclization of end groups or by changes in hydrogenation level or addition of oxygen-containing functional groups. Carotenoids that contain oxygen functional groups are called xanthophylls while the parent hydrocarbons are carotenes. Addition of polar groups alters the polarity of the carotenoids and affects their interaction with other molecules. The most characteristic feature of all carotenoids is the long chain of alternating double and single bonds forming the central polyene chain (Figure 1.4). The conjugated double bonds generally are 9 to 13 in number that gives the carotenoids distinctive molecular shape, chemical reactivity, and light-absorbing properties (Britton, 1995; Krinsky, 1992). In recent years,

carotenoids have created interest by virtue of their provitamin A activity and antioxidant property in animals.

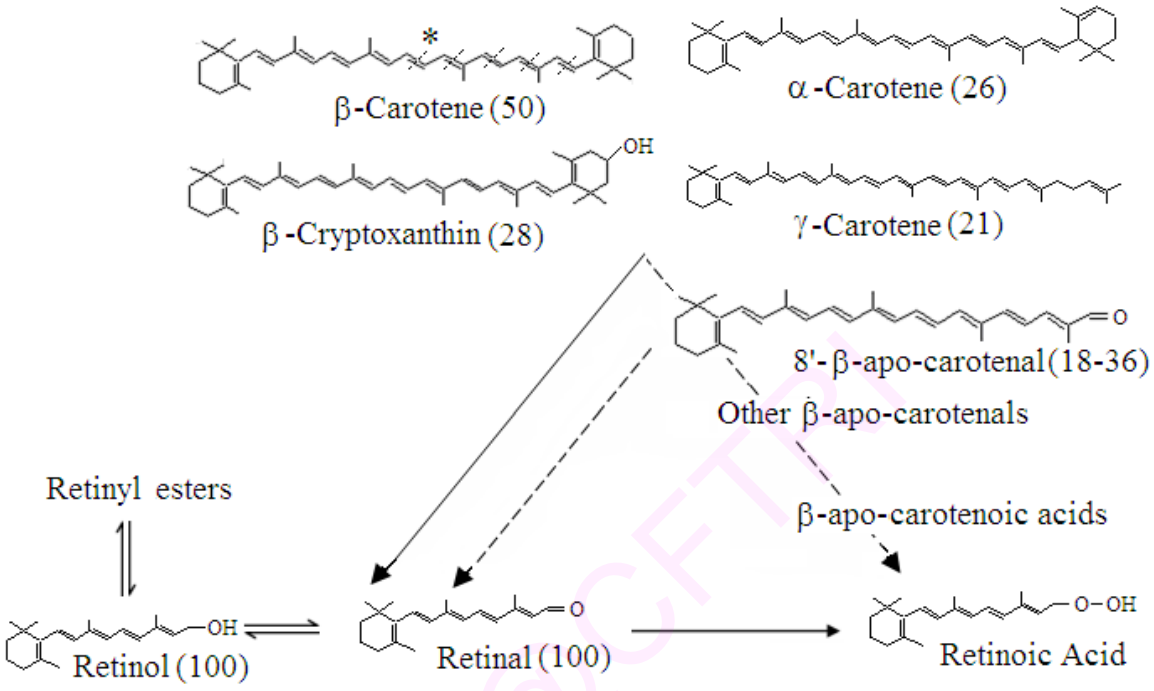


Figure 1.4: Formation of retinoids from carotenoids via the centric (solid lines) and eccentric (dashed lines) cleavage (Source: Stahl et al., 1994). Values in parenthesis indicate relative vitamin A activity of carotenoids (Combs, 1992).

* Centric cleavage of β-carotene that yields two molecules of retinol.

Provitamin A carotenoids

Of the 600 known carotenoids, nearly 50 are found to exhibit provitamin A activity. Provitamin A activity of a carotenoid is defined as the ability of the carotenoid to yield vitamin A (retinol or retinal) as a result of cleavage by the enzyme β-carotene-monooxygenase. Depending upon the structure of the carotenoid and the point of cleavage, one or two molecules of retinol may be formed. Amongst the provitamin A carotenoids, β-carotene, α-carotene, γ-carotene and β-cryptoxanthin are commonly present in fruits and vegetables. The structures of these carotenoids are shown in Figure 1.4. The common feature of all provitamin A carotenoids is the presence of at least one β-ionone ring attached to a polyene chain with alternating double bonds at one end of the structure, which is identical to the structure of retinol. β-Carotene when cleaved at the centre

yields two molecules of retinol (Figure 1.4). Unlike β-carotene, α-carotene, γ-carotene, β-cryptoxanthin etc. are identical in structure to retinol at only one end (β-ionone ring end) and therefore yield only one molecule of retinol when they undergo cleavage.

β-Carotene

β-Carotene (β-β-carotene) in addition to its pro-vitamin A activity exerts other properties such as antioxidant and immunological functions (Iannone et al., 1998). Of the carotenoids that can be metabolized into vitamin A, β-carotene has the highest provitamin A activity. Major sources of dietary β-carotene include green leafy vegetables (spinach, coriander, amaranth, lettuce, etc.), orange and yellow fruits (papaya, orange, mango, pineapple, etc.) and vegetables (pumpkin, carrot, capsicum, baby corn, etc.) (Raju et al., 2007; Krinsky and Johnson, 2005). Factors such as food vehicle, chopping, cooking, presence of dietary fat, protein-energy malnutrition, and β-carotene intake can influence the bioavailability of β-carotene from foods (van het Hof et al., 1998; Poor et al., 1993). The β-carotene in fruit, grains, and oils may be more effective as a source of vitamin A than that in dark-green leafy vegetables (Castenmiller et al., 1999; de Pee et al., 1998). The chemical structure of β-carotene and the central and eccentric points of cleavage are shown in Figure 1.5.

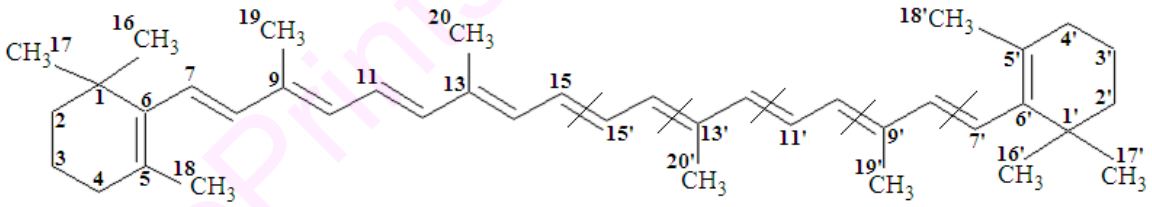


Figure 1.5: Chemical structure of β-carotene, showing central cleavage at 15-15’ bond to yield 2 molecules of retinol or eccentric cleavage at other points (13-14, 13’-14’, 11-12, 11’-12’, 9-10, 9’-10’, 7-8, 7’-8’) to yield 1 molecule of retinol.

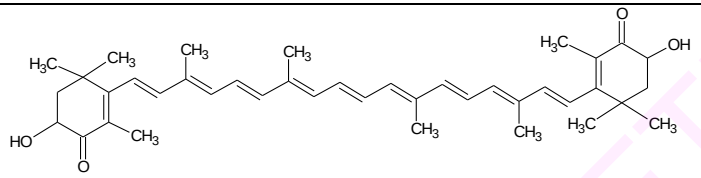
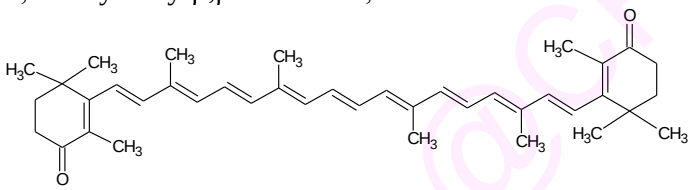
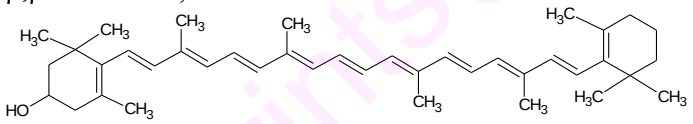
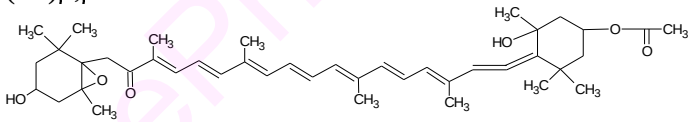
β-Carotene has a unique structure with two identical β-ionone rings at each end with a polyene chain with alternate double bonds between them (Figure 1.5) such that if folded or cut at the 15-15’ bond, the two parts can be superimposed onto each other perfectly. Hence, if there is a central cleavage at the 15-15’ position, it results in formation of 2 retinol molecules. On the other hand, cleavage at other positions (eccentric) on the polyene chain (13-14, 13’-14’, 11-12, 11’-12’,

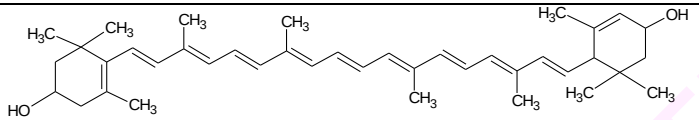
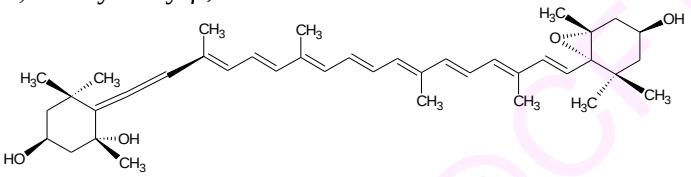
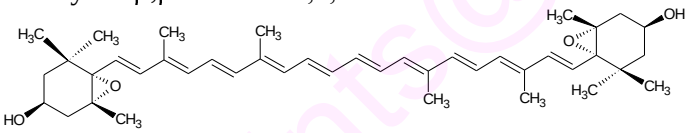
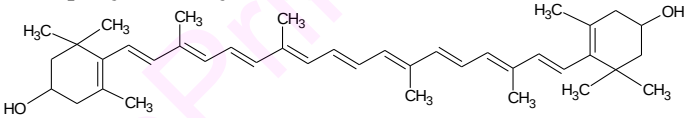
9-10, 9'-10', 7-8, 7'-8') results in only 1 molecule of retinol. The number of double bonds on the polyene chain and the functional groups present determine the antioxidant property of carotenoids (Britton, 1995). By virtue of 9 double bonds in its polyene chain, β-carotene exhibits significant antioxidant activity and is thought to protect against carcinogenesis by scavenging radicals involved in tumor formation (Ames, 1983). β-Carotene reacts chemically with peroxyl radicals to produce epoxides and apo-carotenal products and protects from oxidative damage (Iannone et al., 1998). Due to its highly non-polar and lipophilic nature, β-carotene associates well with the lipids in the cell membrane and thus prevents lipid peroxidation and provides protection at the membrane level by participating in reactions occurring in the lipid layer (McNulty et al., 2007). Additionally, β-carotene is critical to normal embryonic development, growth, and reproduction of birds and fish, irrespective of its ability to form vitamin A (Matsui et al., 2003; Koutsos et al., 2003).

Nonprovitamin A carotenoids

Carotenoids that do not possess provitamin A activity but are known to exert health benefits are the xanthophyll carotenoids. Xanthophylls are more polar than β-carotene due to the presence of oxygen containing functional groups such as hydroxyl, ketone and epoxides on the β-ionone rings or polyene chain. The chemical structures of common hydroxyl (lutein, zeaxanthin), ketone (astaxanthin, canthaxanthin) and epoxide (neoxanthin, violaxanthin, fucoxanthin) xanthophylls are given in Table 1.5. Xanthophylls provide protection against oxidative stress in various chronic and degenerative diseases such as cancer, cardiovascular diseases, neurodegenerative diseases, etc. by virtue of their strong antioxidant property. The potent antioxidant effects of xanthophylls arise from two factors, viz., polyene chain with 9 or more alternating double bonds and presence of polar groups. The lipophilic, yet polar nature of xanthophylls helps them to participate in reactions of both aqueous and lipid phases. Hence, xanthophylls are often more effective than the hydrocarbon carotenes as antioxidants (McNulty et al., 2007; Gruszecki and Strzalka, 2005). Due to the variation in the structure from carotenes, the localization of the xanthophylls in the cell membrane also varies. Xanthophylls are often arranged perpendicular or at an angle, spanning the length of the cell membrane (McNulty et al., 2007; Woodall et al., 1997), enabling the positioning of the polyene chain towards the lipophilic core and polar groups towards the periphery and thus affording protection at both levels. Xanthophyll carotenoids used in the present study are discussed below.

Table 1.5: Xanthophylls commonly found in the diet and the functional groups associated with their chemical structure.

Carotenoids	Chemical structure and systemic name	Functional groups	Chemical formula
Astaxanthin	 3,3'-dihydroxy-β,β-carotene-4,4'-dione	Hydroxyl (OH), Ketone (C=O)	$C_{40}H_{52}O_4$
Canthaxanthin	 β,β-carotene-4,4'-dione	Ketone (C=O)	$C_{40}H_{52}O_2$
β-Cryptoxanthin	 (3*R*)β,β-caroten-3-ol	Hydroxyl (OH)	$C_{40}H_{56}O$
Fucoxanthin	 (3*S*,5*R*,6*S*,3'*S*,5'*R*,6'*R*)-5,6-Epoxy-3'-ethanoyloxy-3,5'-dihydroxy-6',7'-didehydro-5,6,7,8,5',6'-hexahydro-β,β-caroten-8-one	Acetyl (CH3COO), Hydroxyl (OH), Ketone (C=O), Epoxode ()	$C_{42}H_{58}O_6$

Lutein	 3,3'-dihydroxy-β,ε-carotene	Hydroxyl (OH)	$C_{40}H_{56}O_2$
Neoxanthin	 3*S*,5*R*,6*S*,3'*S*,5'*R*,6'*R*)-5,6-Epoxy-6,7-didehydro-5,6,5',6'-tetrahydro-β,β-carotene-3,5,3'-triol	Hydroxyl (OH) Epoxode (C–O–C)	$C_{40}H_{56}O_4$
Violaxanthin	 5,8-Epoxy-5,8-dihydro-β,β-carotene	Hydroxyl (OH) Epoxode (C–O–C)	$C_{40}H_{56}O_4$
Zeaxanthin	 3,3'-dihydroxy-β,β-carotene	Hydroxyl (OH)	$C_{40}H_{56}O_2$

Astaxanthin

Astaxanthin is a symmetric ketocarotenoid (3,3'-dihydroxy-β, β-carotene-4,4'-dione) and naturally occurs in a wide variety of marine and aquatic organisms. The bright red to pink color of crustaceae (shrimp, krill) and salmonidae (salmon, rainbow trout) results from accumulation of astaxanthin or/and its esters. Because animals are not capable of synthesizing astaxanthin *de novo*, micro algae, *Haematococcus* sp. serve as their natural dietary source. Apart from coloration, astaxanthin is considered to be essential for growth processes and as a vitamin A precursor in fish (Moren et al., 2002; Matsuno, 1991).

Similar to other carotenoids, astaxanthin is known to be a potent antioxidant, approximately 10 times higher than β-carotene and 100 times greater than α-tocopherol (Shimidzu et al., 1996). Amongst other diverse biological functions of astaxanthin, an important role is its involvement in cancer prevention (Tanaka et al., 1994; Kotake-Nara et al., 2001) and free radical quencher (Miki et al., 1991). The effect of dietary astaxanthin on mammary cancer was evaluated and it was found to reduce growth of induced mammary tumors by 50% compared to β-carotene and canthaxanthin (Chew et al., 1999). Astaxanthin is reported to significantly influence immune function in several *in vitro* and *in vivo* assays. Astaxanthin enhances *in vitro* antibody production by mouse spleen cells and can also partially restore decreased humoral immune responses in old mice (Jyonouchi et al., 1994). The antioxidant properties of astaxanthin are believed to have a key role in several other properties such as protection against UV-light photo-oxidation, gastric inflammation, cancer, ulcer's Helicobacterpylori infection, aging and age-related diseases, or the promotion of the immune response, liver function and heart, eye, joint and prostate health (Guerin et al., 2003). In an animal model study, astaxanthin supplementation led to an increase in blood levels of HDL (Miki et al., 1998), the form of blood cholesterol inversely correlated with coronary heart disease. The presence of the hydroxyl- and keto- groups on each ionone ring gives astaxanthin the ability to esterify with fatty acids and renders higher anti-oxidant activity than other carotenoids (Guerin et al., 2003). The structure of astaxanthin is identical to β-carotene except for the presence of two keto groups at 4, 4' positions and two hydroxyl groups at 3, 3' positions (Table 1.5). These functional groups render it more polar than other carotenoids.

Lutein

Lutein (3,3'-dihydroxy-β,ε-carotene), found in fruits and vegetables, and zeaxanthin, are often referred to as macular pigments that are found in the macula of retina. Lutein

supplementation results in its selective accumulation in ocular tissues with increase in macular pigment concentration, and improvement in visual function under conditions like age related macular degeneration (Krinsky et al., 2003). Lutein displays biological activities that are evidenced by the inverse association between high intake of lutein and lower risk for age-related maculopathy. Lutein and zeaxanthin are thought to protect the eyes in two ways. The first hypothesis is that the macular pigments filter blue light that damages the photoreceptors and the retinal pigment epithelium. The second hypothesis is that these carotenoids act as antioxidants to limit the oxidative stress of the eye tissue that results from metabolism and light. Investigators from the Eye Disease Case-Control study (Eye Disease Case-Control Study Group, 1993) reported that patients with highest level of plasma lutein/zeaxanthin had decreased age related macular degeneration. Since humans cannot synthesize lutein and zeaxanthin, they must be ingested through the diet. Lutein is found copiously in fruits (corn, peaches, oranges, persimmons) and vegetables (spinach, kale, broccoli, peas, brussel sprouts, leafy greens, etc.). Egg yolk is also a source of lutein and zeaxanthin. Lutein and zeaxanthin from these food sources are highly bioavailable (Chung et al., 2004).

Lutein is similar in structure to α-carotene (Figure 1.4, Table 1.5), differing only by the presence of two hydroxyl groups at 3 and 3' positions. β- and α-Carotenes are identical except for the 'ε' bond in one of the β-ionone rings of α-carotene, unlike β-carotene that has 'β' bonds in both β-ionone rings. Zeaxanthin, on the other hand is similar to β-carotene in that respect. Both lutein and zeaxanthin have a polyene chain with 9 alternating double bonds and 2 hydroxyl groups at 3, 3' positions (Table 1.5). The presence of these polar groups renders them more polar than the hydrocarbon carotenes. Due to the difference in the structure from β-carotene, their localization in the cell membrane also varies (McNulty et al., 2007; Gruszecki and Strzalka, 2005). While β-carotene lies in the lipid rich core of the cell and participates in reactions of the lipid phase and thus protects the lipids, lutein and zeaxanthin span along the length of the cell membrane with the polyene chain close to the lipid core and the polar functional groups extending towards the periphery. This results in its participation in many reactions of both the aqueous and lipid phases and may be the reason for the potent antioxidant activity exhibited by these two carotenoids.

Fucoxanthin

Fucoxanthin (Acetic acid [(1S,3R)-3-hydroxy-4-[(3E,5E,7E,9E,11E,13E,15E)-18-[(1S,4S,6R)-4-hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]heptan-1-yl]-3,7,12,16-tetramethyl-

17-oxooctadeca-1,3,5,7,9,11,13,15-octaenylidene]-3,5,5-trimethylcyclohexyl] ester) is a major xanthophyll carotenoid found in brown algae (Haugan et al., 1992). Brown algae, such as hijiki (*Sargassum fusiforme*), kombu (*Laminaria japonica*), and wakame (*Undaria pinnatifida*), are staples in the diet of East Asians. In animal experiments and cell culture studies, the administration of brown algae powders or fucoxanthin extracts was reported to suppress carcinogenesis (Das, 2005; Funahashi et al., 2001; Kotake-Nara et al., 2001). Kotake-Nara et al. (2001) demonstrated that epoxy xanthophylls such as fucoxanthin and neoxanthin reduce the viability of prostate cancer cells by inducing apoptosis to a greater extent than other carotenoids. The anti-proliferative effect of fucoxanthin is stronger than β-carotene and lycopene. However, the mechanism by which fucoxanthin suppresses colon carcinogenesis is not fully understood (Hosakawa et al., 2004). Maeda et al. (2007) have reported lower weight gain and glucose levels in obese and diabetic mice on consumption of fucoxanthin.

Fucoxanthin has a unique structure including an unusual allenic bond and 5,6-monoepoxide in its molecule. It has 8 alternate double bonds on the polyene chain and several functional groups including two 'hydroxyl' groups on the β-ionone rings, an 'epoxide' group and 'acetyl' group on separate β-ionone rings and a 'keto' group in the polyene chain (Table 1.5). These functional groups render fucoxanthin more polar than carotenes and may be responsible for its reactivity and antioxidant properties, resulting in its biological effects. Fucoxanthin is reported to be metabolized to fucoxanthinol *in vivo* (Asai et al., 2004; Sugawara et al., 2002) and consequently to amarouciaxanthin A (Asai et al., 2004). Fucoxanthinol is similar to fucoxanthin in structure with only the 'acetyl' group of fucoxanthin being replaced by a 'hydroxyl' group in fucoxanthinol. Thus, most of the biological effects of fucoxanthin are expressed by the action of fucoxanthinol *in vivo*.

Absorption and metabolism of carotenoids

Carotenoids, being lipid-soluble, follow the same intestinal absorption process as dietary fat. Release from the food matrix and dissolution in the lipid phase are important initial steps in the absorption process. β-Carotene and other carotenoids are thought to diffuse from mixed micelles in the small intestine through the unstirred water layer into the enterocyte. The presence of fat is essential for carotenoid absorption as bile is secreted in response to it in the small intestine. Bile acids and salts in turn are essential for the formation of mixed micelles, which help in entrapping carotenoids and crossing the aqueous unstirred water layer. The type of dietary fat also affects the rate of absorption as reported by Raju and Baskaran (2009), Lakshminarayana et

al. (2009; 2007) and Raju et al. (2006) who found that olive oil (oleic acid) enhances bioavailability compared to sunflower oil (linoleic acid). Moreover, the chemical nature and structure of carotenoids results in varied localization in the mixed micelles. The highly non-polar and hydrophobic β-carotene lies in the core centre of the micelle while xanthophylls being more polar lie closer to the periphery. The localization of carotenoids in the mixed micelles may also affect the bioavailability of the carotenoids as it may affect the transfer of carotenoids, especially when multiple carotenoids are present and there is competitive absorption. It is believed that the uptake is by passive diffusion and carotenoids appear to compete with one another for absorption (Harrison and Hussain, 2001). Fatty acid esters of xanthophylls are cleaved in the lumen of the small intestine prior to uptake by the mucosa. Most of the provitamin A carotenoids are cleaved to retinol by β-carotene cleavage enzyme monooxygenase and reductases, either in the lumen or in the brush border cells of the intestine (Krinsky et al., 1993). The retinal formed is re-esterified in the brush border cells. Carotenoids and retinoids are taken up by the mucosa of the small intestine, packaged into triacylglycerol-rich chylomicrons and secreted into lymph (Parker, 1996). Chylomicrons are then partially degraded by the action of lipase in extra-hepatic tissues to yield chylomicron remnants. The esters are then taken up from the remnants by liver cells and cleaved to yield free vitamin A (Harrison et al., 1995). Free vitamin A can be re-esterified, in the endoplasmic reticulum, and transferred to stellate cells for storage or excreted from the cell. The absorption and metabolism of carotenoids is summarized in Figure 1.6. The metabolic pathways involved in retinol metabolism are shown in Figure 1.2.

Carotenoids in the human plasma are bound with lipoprotein (75% in LDL and 25% in HDL). Nonpolar carotenoids like α- and β-carotene are transported in LDL (60%) and HDL (25%) or VLDL (15%) whereas; polar carotenoids are transported evenly in HDL and VLDL (Parker, 1996). The carotenes, being lipophilic, are located in the core of lipoproteins, which may explain why they do not transfer between lipoproteins at an appreciable rate. The xanthophylls, being more polar, may be located on the surface of lipoproteins, and are likely to undergo more rapid surface transfer, resulting in the observed apparent equilibration between LDL and HDL. In terms of the total body pool of carotenoids, major organs of storage include the liver and adipose tissue. Some carotenoids are stored selectively in other tissues, lutein in macula and corpus luteum, lycopene in prostate (Ferreira et al., 2000), and β-carotene in bovine corpus luteum (Haliloglu et al., 2002).

Theoretically, β-carotene is metabolized (cleaved) to yield 2 retinal molecules. However, it provides less than 50% of the biological activity of vitamin A (Olson, 1989). In humans, the accepted conversion ratio for β-carotene to retinol is 6:1 (Wang, 1994). The absorption and

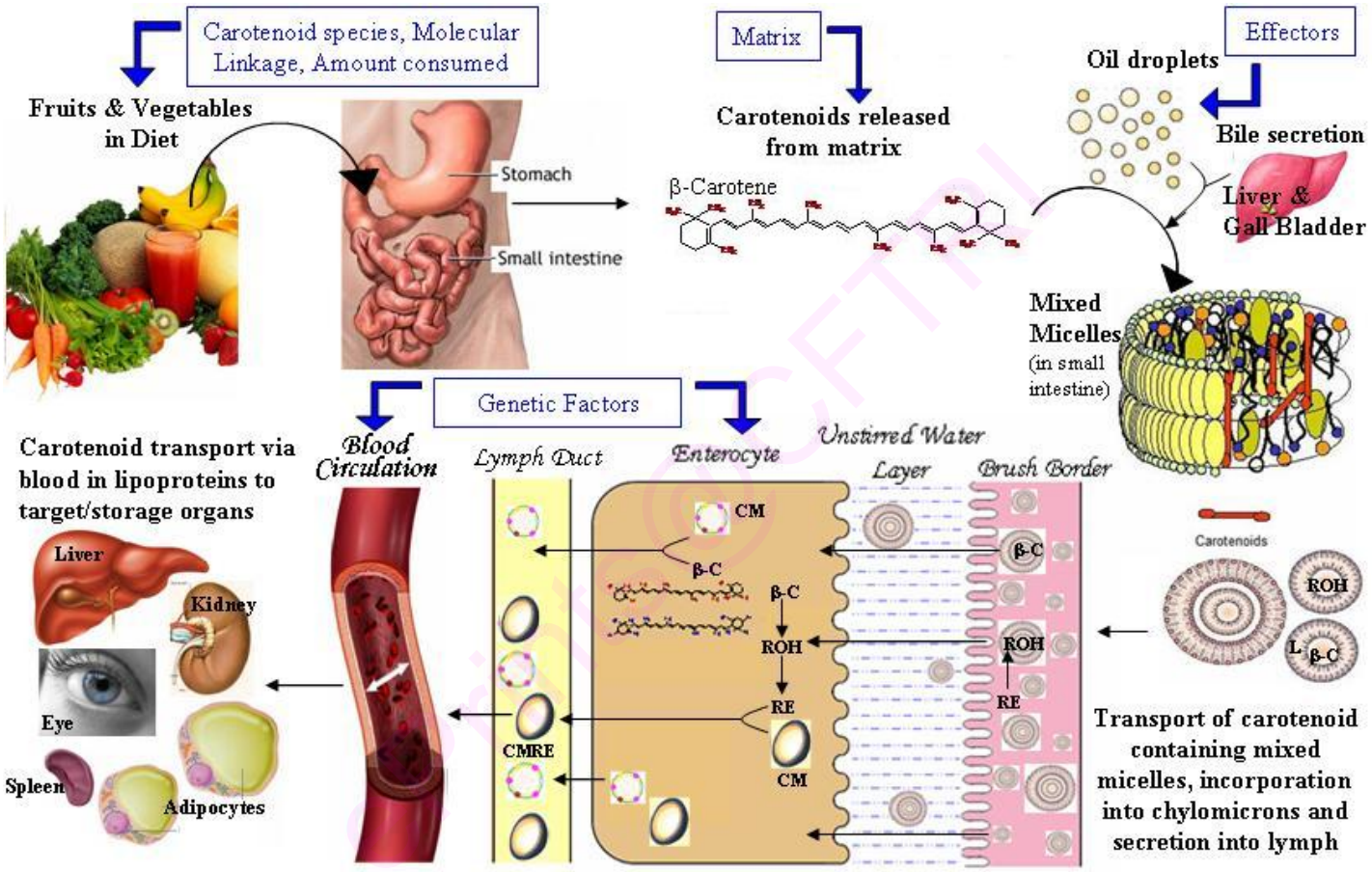


Figure 1.6: Absorption and metabolism of carotenoids *in vivo*. Blue boxes and arrows indicate some of the factors that affect carotenoid bioavailability, for example β-carotene. β-C, β-carotene; CM, chylomicron; CMRE, chylomicron containing retinyl esters; RE, retinyl ester; ROH, retinol.

bioavailability of β-carotene, and its conversion to vitamin A are varied in rodents (Tang et al., 2003; Furr and Clark, 1999). Reasons for the variability might include environmental factors, nature of food matrix, genetic factors, and/or pathologic conditions (van Het Hof et al., 2000).

The main excretion route of carotenoids is via feces (20–60%), while urinary excretion accounts for 10–30% (Lemke et al., 2003). There is very little information available about other routes of excretion. For example, retinoids are excreted in the breath, but the extent to which retinoids are excreted in saliva, sweat or tears is unknown even though both retinoids and retinoid transport proteins are present in tears (Wiggert and Chader, 1985). Much less is known about carotenoid absorption and metabolism compared to that of retinoids.

Factors affecting the absorption and bioavailability of carotenoids

In general, the bioavailability of carotenoids is very poor and only 10-20% of the total carotenoids in the food material are bioavailable. Intestinal absorption of carotenoids depends upon several dietary and non-dietary factors including level and origin of dietary fat, amount of carotenoids, digestibility of food, presence of antioxidants or dietary fibres and in the case of β-carotene, vitamin A status (Erdman et al., 1993). The various factors that can affect the bioavailability of carotenoids have been included into a mnemonic (SLAMENGHI- 'S'pecies, molecular 'L'inkage, 'A'mount of carotenoid consumed, 'M'atrix in which incorporated, 'E'ffectors of absorption and bioconversion, 'Nutrient status, 'G'enetic factors, 'H'ost related factors, Mathematical 'I'nteractions), coined by the Wageningen group, Netherlands (Mc Larsen and Frigg, 2001). Some of these factors have been shown in Figure 1.6.

Most carotenoids occur naturally in plants in the all-*trans* form and it is more easily absorbed than the 9-*cis* forms. The 9-*cis* forms of carotenoids are converted to all-*trans* form before entering the blood circulation. Carotenoids are often present in food (fruits, vegetables, seafood, etc) in the complex ester forms but the bioavailability of the carotenoid esters is inadequately studied. Nearly 10-20 % of carotenoids in a meal may be absorbed into the blood stream. However, the body has a threshold for absorption and after a certain level, no matter how high the content in the food, the absorption remains a plateau. The duration of supplementation or presence of mixed carotenoids in the diet may also affect the bioavailability. The effect of food matrix and processing on bioavailability of carotenoids is shown in Figure 1.7. Fat is essential for absorption as it triggers the bile secretion which is essential for micellization in the small intestine. A minimum of 5g fat in the daily diet is essential for adequate micelle formation. The type of dietary fat also plays an important role in bioavailability of carotenoids. Lakshminarayana

et al. (2009; 2007), Raju et al. (2006) and Baskaran et al. (2003) have found that β-carotene and lutein bioavailability is enhanced in the presence of oleic acid and linoleic acid respectively. Whereas, Nidhi and Baskaran (2010) studied the role of vegetable oils on lutein bioavailability. Adequate protein and zinc assist in maintaining vitamin A status (Russel, 2000). Presence of dietary fibre tends to reduce the bioavailability of lutein and β-carotene (Mamatha and Baskaran, 2010; Omaye et al., 1997). The presence of multiple types of carotenoids may lead to competitive absorption (Raju and Baskaran, 2009; Tyssandier et al., 2003). Alcohol ingestion interferes with bioconversion of β-carotene to retinol (Albanes et al., 1997). Absorption of carotenoids is also influenced by vitamin A status of the individual (Barua and Olson, 2000). If vitamin A status is low, bioconversion is likely to be higher. Host related factors like sex of the individual appears to influence the carotenoids bioavailability from the diet.

Health Benefits of Carotenoids

Carotenoids afford several health benefits. Provitamin A carotenoids are precursors of retinol (vitamin A) which is an essential micronutrient. Moreover, most of the carotenoids are potent antioxidants. With regard to possible mechanisms of action, a leading hypothesis is that carotenoids serve as singlet oxygen quenchers and as antioxidants in preventing free-radical damage to cellular components (Krinsky 1992). Carotenoids may also be important for enhancing immune responses, gap junction communication and carcinogen-metabolizing enzyme activity (Stahl et al., 1997; Wang, 1994). Besides cell-mediated and humoral immune responses, β-carotene has been shown to regulate nonspecific cellular host defense (Chew and Park, 2004). A greater effectiveness is reported at low oxygen tensions with respect to antioxidant effect of carotenoids (Burton and Ingold, 1984). Antioxidant activities other than scavenging singlet oxygen and peroxyl radicals have also been attributed to carotenoids. Dietary β-carotene could mediate an increase in the activities of the superoxide dismutase and catalase (Blakely et al., 1988). Studies have shown that lutein is able to scavenge sulfur radicals (Chopra et al., 1993) and β-carotene quenches glutathione (thiyl), sulfonyl and nitrogen dioxide radicals (Everett et al., 1996).

On the basis of intake or biomarkers of intake, carotenoids have been postulated to play a protective role in angina pectoris (Riemersma et al. 1991), cardiovascular disease (Gaziano et al., 1995) and cancer (van Poppel, 1996), particularly cancer of lung (Dartigues et al., 1990; Knekt et al., 1990) and stomach (Chen et al., 1992). It has been shown that when retinoids or carotenoids

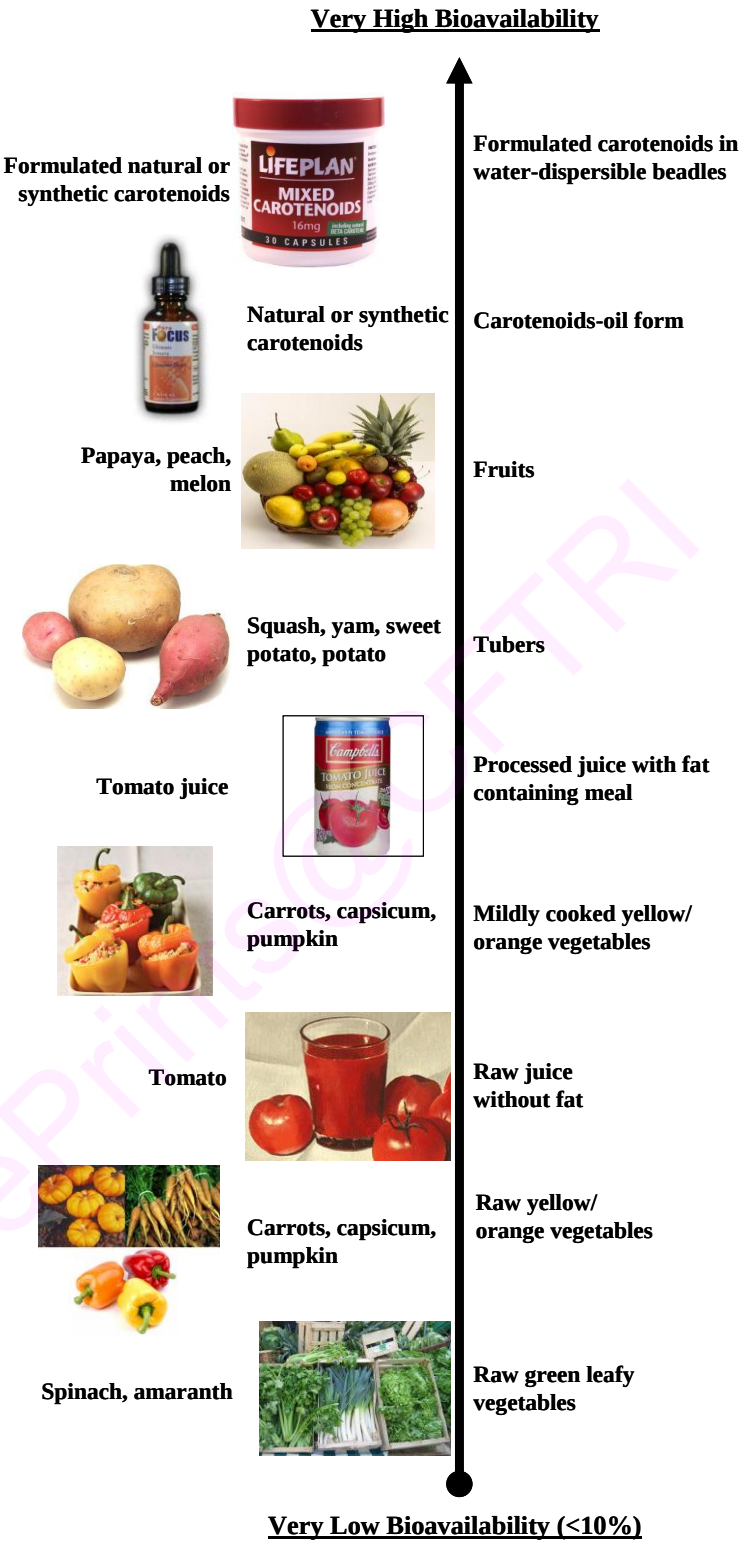


Figure 1.7: Effect of food matrix and processing on bioavailability of carotenoids (Source: Boileau et al., 1999).

are added after carcinogen exposure they are capable of suppressing the formation of transformed foci (Bertram et al., 1990). Because of their antioxidant property, carotenoids have been suggested to protect against coronary vascular disease. One contributor to the development of coronary vascular disease is the oxidation of low-density lipoproteins (LDL). The fact that LDL is a major transporter of β-carotene in the circulation and it has the capacity to trap peroxyl radicals and quench singlet oxygen lends support to this hypothesis (Clevidence and Bieri, 1993).

Carotenoids in cell membranes

Carotenoids are incorporated in the lipid bilayer of the cell membranes (Britton, 1995). Their orientation in the membrane depends upon their chemical structure (Figure 1.8). They are incorporated in the lipid bilayer in such a way that the chromophore is entirely embedded in the hydrophobic core of the membrane. Xanthophylls with polar groups (located at two opposite sides of the molecule), orient in the membrane in such a way that these groups remain anchored in opposite polar zones of the bilayer. This is due to the hydrogen bond formation with the hydrophilic groups of lipid molecules (Gruszecki and Strzalka, 2005). Thus, due to varied localization of the carotenoids in the membrane, their antioxidant property is varied. Asai et al. (1999) have reported reduced membrane phospholipid peroxidation in red blood cells and liver of mice that were fed with turmeric (contains curcumin, a compound very similar to carotenoids in structure) and capsicum (contains xanthophyll carotenoids). Cantrell et al. (2003) studied the ability of different carotenoids to quench singlet oxygen species in DPPC liposomes. Lancrajan et al. (2001) have studied the uptake of carotenoids into plasma, mitochondrial and cell membranes and reported that β-carotene is preferentially incorporated in mitochondrial membrane while lutein is taken up by the cell membrane. Differences in the orientation of the carotenoid can also have a substantial effect on the properties of the membrane. Thus, carotenoids such as zeaxanthin, which have two polar end groups and span the membrane, can act as a "rivet" in the membrane structure and increase its rigidity and mechanical strength. The presence of carotenoids will have effect on the thickness, strength, and fluidity of membrane (Subezynski et al., 1991). Kaul and Krishnakantha (1997, 1994) have studied the effect of retinol deficiency on lipid peroxidation, membrane structure and associated enzymes in liver and brain microsomes. Although, many reports are available on the carotenoids in membranes, studies on their effect at the membrane level in VAD are scarce. The above literature survey demonstrates that work related to the influence of dietary carotenoids on reverting VAD induced biochemical changes, possible

conversion of xanthophylls to retinol under condition and comparative antioxidant property with respect to down regulation of lipid peroxidation induced by retinol deficiency is lacking.

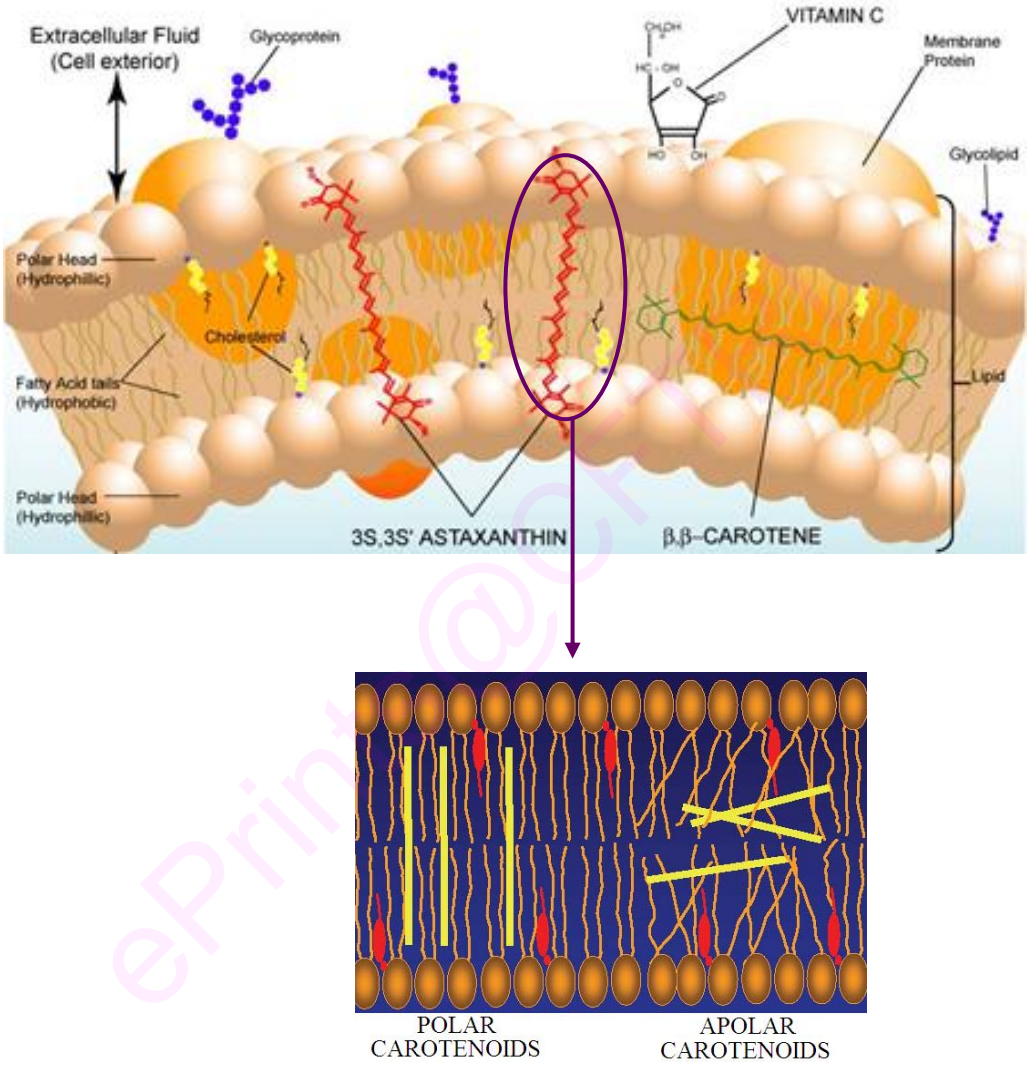


Figure 1.8. Localization of carotenoids in the cell membrane (Source: www.fujihealth science.com, www.cardaxpharma.com).

Hence, the aim of this work was to study the bioavailability of dietary carotenoids (provitamin-A and non-provitamin-A carotenoids) and their influence on modulating the retinol

deficiency induced biochemical changes in tissue membranes of rats. The objectives of the work carried out in this study are as follows:

1. Isolation and purification of carotenoids (β-carotene, lutein, astaxanthin) from selected leafy greens and algae.
2. Investigating the effect of vitamin A deficiency on bio-accessibility, bioavailability and bio-efficacy of dietary carotenoids (β-carotene, lutein, astaxanthin and fucoxanthin).
3. Determining the influence of dietary carotenoids on vitamin A deficiency induced biochemical changes and kinetics of carotenoid metabolizing enzyme and membrane bound enzymes in intestine and liver of rats.
4. Evaluating the influence of dietary carotenoids on vitamin A deficiency induced changes in lipoproteins and retinol binding proteins in plasma and tissue.
5. Effect of dietary carotenoids on vitamin A deficiency induced changes in lipid profile, lipid peroxidation and enzymes involved in antioxidant defense mechanisms in tissue membrane.
6. Studying association of vitamin A level in tissue membrane and the effect of dietary carotenoids on vitamin A deficiency induced changes in biochemical constituents in tissue membrane of rats with reference to structure and function.

Aim and scope of the study

Vitamin A or retinol is an essential micronutrient involved in various important physiological functions such as vision, reproduction, immune functions, growth and development. Since it cannot be synthesized in the body, it must be supplied in the diet either as preformed vitamin A (retinoids) from animal origin or as provitamin A carotenoids from fruits and vegetables. Provitamin A carotenoids, in particular, β-carotene rich fruits and vegetables are the major sources of vitamin A. Vitamin A deficiency (VAD) is a serious public health problem in most of the developing countries including India and more prevalent in infants, children, pregnant and lactating mothers. The main cause of the VAD is low or inadequate intake of vitamin A rich foods. Various prophylaxis programmes are being implemented in the countries with compromised vitamin A status, but have not been completely successful in eliminating the problem. Food based strategies using dietary sources of vitamin A rather than administration of synthetic vitamin A, are recommended, as they can be effective in the prevention and control of VAD. Green leafy vegetables, yellow vegetables and fruits are considered to be good sources of vitamin A due to their provitamin A content. However, studies have shown poor efficiency of conversion of dietary β-carotene to retinol due to various factors demonstrating lower bioavailability of β-carotene than synthetic β-carotene. Bioconversion of provitamin A carotenoids into retinol is inversely proportional to the vitamin A status. Besides vitamin A precursors, it has been reported that nonprovitamin-A carotenoids such as fucoxanthin, astaxanthin and lutein afford protection against the retinol deficiency induced changes in murine model. Bioavailability of the carotenoids is influenced by the food matrix in which they are present, the extent of their release and incorporation into mixed micelles in the intestinal lumen. With respect to provitamin A carotenoids, the term bioefficacy is defined as the product of the fraction of the ingested amount that is absorbed (bioavailability) and the fraction of that is converted to retinol (bioconversion). Thus, determination of provitamin A carotenoids in familiar and less familiar green leafy vegetables, plants used traditionally in folk medicine, vegetables and algae, their retinol equivalent and their possible role in ameliorating retinol deficiency induced changes in membrane are of importance, so that they can be exploited for prevention of VAD and its related disorders. Hence, the present study was undertaken to assess the carotenoid composition and vitamin A activity of common agri-horticultural produce such as leafy greens, vegetables, medicinal plants and algae (marine seaweeds). The β-carotene rich sources can be explored as dietary sources of vitamin A for prevention of VAD and to ensure vitamin A security.

The bioavailability of carotenoids is affected by their chemical structure as it influences their incorporation in the mixed micelles, their uptake in the intestine enterocytes and transportation by lipoproteins. Hence, this study aimed to determine the bioavailability and bioefficacy of dietary carotenoids (β-carotene, astaxanthin, lutein and fucoxanthin) in retinol deficiency. In addition, bioefficacy of carotenoids was determined by estimating the retinol levels in circulation and liver tissue. The carotenoid metabolizing enzyme, 15-15' β-carotene monooxygenase activity was also estimated for confirmation of cleavage of carotenoids to retinol. The results of the study would help to find possible provitamin A activity of xanthophylls in retinol deficiency. In addition, the difference in the bioavailability of the carotenoids in retinol deficiency would be ascertained.

It is speculated that metabolites of carotenoids may exhibit superior functional properties. However, it is evident from the literature that studies on possible provitamin A activity and metabolism of lutein, astaxanthin and fucoxanthin in VAD condition are lacking. Hence, studies were undertaken to elucidate and characterize the metabolites formed as a result of feeding β-carotene, astaxanthin, lutein and fucoxanthin to retinol deficient rats. It is also aimed to elucidate the structural aspects of the metabolites by LC-MS and possible metabolic pathways based on the metabolites.

Retinol is an essential micronutrient and is an integral part of the cell membrane. It is involved in maintaining the membrane structure and function. Its efficiency may affect the biochemical constituents of the tissues and membranes. Hence, the objective of the study was to determine the effect of retinol deficiency and subsequent administration of single/repeated doses and dietary feeding of carotenoids on biochemical parameters such membrane bound enzymes (Na^+ K^+ ATPase, Ca^{2+} ATPase, Mg^{2+} ATPase, acetylcholine esterase), antioxidant molecules (superoxide dismutase, catalase, glutathione-S-transferase and glutathione levels), fatty acid profile, lipid profile (HDL-cholesterol, LDL+VLDL-cholesterol, total cholesterol, phospholipids, triglycerides, cholesterol: phospholipid ratio) and retinol binding protein (RBP) levels. The result will help in understanding the efficiency of nonprovitamin A carotenoids in comparison with retinol and provitamin A carotenoids in ameliorating the alterations in biochemical constituents caused by retinol deficiency in rats.

In brief, from the proposed study the following aspects shall be achieved:

- Possible provitamin A activity of xanthophylls in VAD and thus their use as alternate source of vitamin A.

- Protective effect of the carotenoids on the biochemical constituents of the membrane and tissue in retinol deficiency and the comparison of the beneficial effect to determine the more effective carotenoid.
- Possible biodegradation of astaxanthin, lutein and fucoxanthin and characterization of their metabolites.

The results of present investigation will help to disseminate the research information to common people about the importance of carotenoids in agri/horticultural produce, and their protective effect against VAD, possible alternate sources of retinol, possible metabolism of astaxanthin, lutein and fucoxanthin *in vivo* and their health benefits with reference to retinol deficiency.

CHAPTER 2: Materials and Methods

Materials

Vitamin free casein, vitamins, minerals, cellulose, glucose, DL methionine, calcium phosphate, potassium citrate monohydrate, manganese carbonate, ferric citrate, sodium selenate, zinc carbonate, cupric carbonate, potassium iodate, potassium chromate, heparin, histidine, sucrose, thymine hydrochloride, riboflavin, pyridoxyl hydrochloride, nicotinic acid, D-calcium pantothenate, folic acid, D-biotin, cyanocobalamine, cholecalciferol and menadione were purchased from HiMedia Laboratories Pvt. Ltd. (India) were purchased from Hi-Media Laboratories Ltd. (Mumbai, India). Choline chloride was purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). Vitamin A palmitate dispersed in oil for animal diets was purchased from Roche (Switzerland). Cornstarch and peanut oil were obtained from the local super market. Freshly harvested green leafy vegetables, vegetables and medicinal plant materials were collected from local farms or vegetable markets or open fields (Mysore, Karnataka) and used immediately for extraction and analysis. Algae were collected at Mandapam, Tamil Nadu, off the eastern coast of India, dried and then used for analysis of carotenoids.

Chemicals

All-trans retinol (95%), retinal (98%), retinylpalmitate, DL-α-tocopherol, all-trans β-carotene (97%), α-carotene (95%), lutein (90%), astaxanthin (98%), DL-α-tocopherol, fatty acids standards (methyl esters), thiobarbituric acid (TBA), adenosine triphosphate (ATP), ouabain, 1,1,3,3-tetramethoxypropane (TMP), cytochrome-C, xanthine oxidase, boron trifluoride-methanol solution, BF_3), β-nicotinamide adenine monohydrate (NAD^+), sodium taurocholate, acetylthiocholine chloride, 5,5'-dithiobis (2-nitrobenzoic acid), porcine cholesterol esterase, dipalmitoyl phosphatidylcholine (DPPC), triolein, dithionitro benzoic acid (DTNB), bovine serum albumin (BSA) and tricine were purchased from Sigma-Aldrich (St. Louis, MO). Neoxanthin, violaxanthin and zeaxanthin were kindly donated by Dr. Akhiko Nagao (NFRI, Japan). Ethylene glycol tetra acetic acid (EGTA), glutathione reduced (GSH), 1-chloro-2, 4-dinitrobenzene (CDNB), trichloroacetic acid (TCA), xanthine, cholesterol, anhydrous sodium sulphate, ammonium acetate, potassium hydroxide, sodium hydroxide, magnesium chloride, anhydrous ferric chloride, ammonium molybdate, anhydrous monobasic sodium phosphate, anhydrous dipotassium hydrogen phosphate, anhydrous dibasic sodium phosphate, potassium

dihydrogen orthophosphate, potassium chloride, ethylene diamine tetra acetic acid (EDTA), ethylene diamine tetra acetic acid (disodium salt), dithiothreitrol (DTT), Tween 20, Tween 40, sodium carbonate, copper sulphate, alumina (60-120 mesh size), blue R-250, TEMED, ammonium persulphate, tris (hydroxymethyl) amino methane, tris-HCl, acrylamide, bisacrylamide, Folin Ciocalteu's reagent, acetyl acetone, N-[2-hydis-acrroxyethyl] piperazine-N'-ethanesulphonic acid (HEPES), glycine, ascorbic acid, glycerol, bromophenol blue, brilliant blue R and sodium chloride were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India).

Analytical reagent grade acetone, acetonitrile, dichloromethane, methanol, hexane, chloroform, ethyl acetate, di ethyl ether, N-butanol, isopropanol, benzene and HPLC grade acetonitrile, hexane, methanol, and dichloromethane were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Hydrogen peroxide solution (50%) glacial acetic acid, sulphuric acid and hydrochloric acid were procured from Merck (Mumbai, India) while pre-coated silica TLC plates were procured from Merck (Germany). Magnesium oxide, sodium dodecyl sulphate and formaldehyde (37-41%) were purchased from SD Fine-Chem Ltd. (Mumbai, India). Sodium potassium tartarate, ammonium thiocyanate, silica gel (60-120 mesh) and sodium metaperiodate were procured from RFCL (Rankem chemicals India Ltd.). Sephadex G-25 was obtained from Amersham Biosciences (Sweden). Retinol binding protein 4 (Mouse/Rat) EIA (ELISA) kit was procured from Alpco Diagnostics (USA). Whatman No. 1 filter paper was obtained from Whatman Ltd (England).

Animals

Animal experiments were conducted after due clearance from the Institutional Animal Ethics Committee (IAEC No. 126/08). Weanling male albino rats (OUTB-Wistar, IND-CFT (2c)) weighing 30 ± 2g were used throughout the study and they were housed in individual stainless steel cages in the institute animal house facility at room temperature (28 ± 2°C). A 12-h Light/Dark cycle was maintained and the rats had free access to food and water *ad libitum*. These animals were used for various experiments.

Diets

Diets for animal feeding experiment were prepared according to the guidelines of AIN-76 (AIN, 1977) while vitamin A deficient diet was prepared according to the modified AIN-76 guidelines (Smith, 1990). The composition of vitamin mixture and mineral mixture used in the

diet is given in Table 2.1 and 2.2 respectively. Vitamin A was not included in the preparation of vitamin mixture. Control diet had sufficient amount of vitamin A (400,000 IU) dispersed in the refined peanut oil prior to mixing. The vitamin and mineral mixtures were prepared separately by mixing all the ingredients using sucrose as base material and stored in airtight containers at 4°C. Diets were prepared as per the composition given in Table 2.3. Respective diets were prepared separately by mixing together all the ingredients and repeatedly passing through a sieve (25 pores per cm^2) to ensure proper mixing and homogeneity. The peanut oil used as a fat source was first checked for its vitamin A content by HPLC analysis. The prepared diets were stored in airtight containers at 4°C. Known quantity of the diet was provided to each animal daily and diet intake and gain in body weight was recorded on a daily basis.

Composition and preparation of control diet

Composition of the control diet is described in Table 2.3. The following ingredients were mixed for preparation of diets (g/kg): casein (200), DL-methionine (3), cellulose (50), corn starch (325), glucose (324), mineral mixture (35), vitamin mixture (10), choline chloride (2), ascorbic acid (1), refined peanut oil (50) containing retinyl palmitate as vitamin A (400,000 IU, AIN-76). Vitamin mixture and mineral mixture were the same as those described in Tables 2.1 and 2.2.

Composition and preparation of vitamin A deficient diet

Composition of the vitamin A deficient diet is given in Table 2.3. To ensure that the diet was devoid of vitamin A, vitamin-free casein was refluxed for 16 hours with 2.5 volumes (w/v) of 95% ethanol and 0.0025 volumes of concentrated HCl to remove traces of retinol (Sundaresan et al., 1967). Thereafter, the refluxed casein was thoroughly washed twice with 95% ethanol. Excess ethanol was drained and the casein was sun dried for complete removal of ethanol. The retinol level of casein was checked by HPLC analysis and then used for diet preparation. Utmost care with respect to cleanliness and hygiene was maintained to prevent contamination of the diet with retinol from other sources. The following ingredients (g/kg) were mixed thoroughly and passed through a sieve (25 pores per cm^2) several times to ensure homogeneity: vitamin-A free casein (200), DL-methionine (3), cellulose (50), corn starch (325), glucose (324), mineral mixture (35), vitamin A free vitamin mixture (10), choline chloride (2), ascorbic acid (1) and peanut oil (50). The diet was stored in airtight containers at 4°C.

Table 2.1 Composition of vitamin mixture used to prepare animal diets.

Vitamin	mg/100g	Vitamin	mg/100g
Thiamine hydrochloride	60	D-biotin	2
Riboflavin	60	Cyanocobalamine	0.5
Pyridoxine hydrochloride	70	Vitamin E (α-tocopherol acetate, 5000 IU)	335.5
Nicotinic acid	300	Cholecalciferol	0.25
D-calcium pantothenate	160	Menadione	5
Folic acid	20	Sucrose (fine powder, to make to 100 g)	

Source: AIN, 1976

Table 2.2 Composition of mineral mixture used to prepare animal diets.

Mineral	g/100g	Mineral	g/100g
Calcium phosphate	50	Ferric citrate	0.6
Sodium chloride	7.4	Sodium selenate	0.001
Potassium citrate monohydrate	22	Zinc carbonate	0.16
Potassium sulphate	5.2	Cupric carbonate (55% Cu)	0.03
Magnesium oxide	2.4	Potassium iodate	0.001
Manganese carbonate	0.35	Potassium chromate	0.0213
Sucrose (fine powder, to make to 100 g)			

Source: AIN, 1976

Table 2.3. Composition (g/kg) of the experimental (Retinol deficient) and control (Retinol sufficient) diets[a].

Ingredients	Retinol deficient diet	Retinol sufficient diet
Vitamin A free casein	200	200
DL-Methionine	03	03
Cellulose	50	50
Corn starch	325	325
Glucose	324	324
AIN-76 Vitamin mixture[b]	10	10
AIN-76 Mineral mixture[c]	35	35
Choline chloride	02	02
Ascorbic acid	01	01
Fat (peanut oil)	50	50
Retinyl Palmitate	00	400,000 IU

[a]AIN, 1976; Smith, 1990.

[b]Composition given in Table 2.1.

[c]Composition give in Table 2.2.

Dispersion of carotenoids in peanut oil for gavage studies

β-Carotene, astaxanthin, lutein and fucoxanthin were dispersed in refined peanut oil (1.6 μmol/ml oil for single dose, 0.2 mg/ml for repeated dose), vortexed and sonicated till they were homogenously dissolved in the oil. The volume of individual gavages (0.5 ml) contained the required amount of carotenoids (0.8 μmol for single dose, 0.1 mg for repeated dose).

Diet supplemented with β-carotene

For the dietary study, *Dacota carota* (carrot) was used as a source of β-carotene (42 mg/Kg diet). Fresh carrots were chopped fine, dried in a cross-flow hot air oven (Magnus, Mumbai, India) at 42 $\pm$ 2°C for 10 hours and powdered in a hammer mill (Cadmac, New Delhi, India). Carotenoid composition of the carrot powder was determined by HPLC according to the procedure described elsewhere. Based on the results obtained, the carrot powder was added to the diet and thus, the concentration of β-carotene per Kg of diet was 42 mg. The carrot powder was

mixed along with other ingredients (Table 2.4) to obtain homogenous distribution of the powder in the diet. The diet prepared was stored in airtight opaque containers at 4°C. Prior to feeding, the β-carotene content of the diet was checked by HPLC.

Table 2.4. Composition (g/kg) of the carotenoid supplemented diets.

Ingredients	Experimental diets		
	β-Carotene diet	Astaxanthin diet	Lutein diet
Vitamin A free casein	200	200	200
DL-Methionine	03	03	03
Cellulose	50	50	50
Corn starch	283	325	312.5
Glucose	282	324	311.5
AIN-76 Vitamin mixture[a]	10	10	10
AIN-76 Mineral mixture[b]	35	35	35
Choline chloride	02	02	02
Ascorbic acid	01	01	01
Fat (peanut oil)	50	50	50
Carrot powder[c]	84	-	-
Astaxanthin powder	-	0.042	-
Dill leaf powder[d]	-	-	25

[a]Composition given in Table 2.1.

[b]Composition given in Table 2.2.

[c]Carrot powder (84 g) provides β-carotene (42 mg), α-carotene (29 mg) and lutein (7.6 mg).

[d]Dill leaf powder (25 g) provides lutein (42 mg) and β-carotene (16.9 mg).

Diet supplemented with astaxanthin

For the dietary study, commercially available astaxanthin (42 mg/Kg diet) was mixed with other ingredients of vitamin A deficient diet (Table 2.4). Quantity of diet required for each day (100 g) was sealed under nitrogen in separate aluminum foil covers to protect from oxidation by light and air and stored in airtight opaque containers at 4°C. Each day, a fresh packet was

opened for feeding the animals. Prior to feeding, the astaxanthin content of the diet was checked by HPLC as per the method described elsewhere.

Diet supplemented with lutein

For the dietary study, *Peucedanum sowa* (Indian dill) was used as a source of lutein (42 mg/Kg diet). Fresh leaves were dried in a cross-flow hot air oven (for 10 hours 42 $\pm$ 2°C) and powdered by milling. The carotenoid composition of the dill leaf powder was determined by HPLC as per the method described elsewhere. Based on the results obtained, the dill leaf powder was added to the diet and thus the concentration of lutein per Kg of diet was 42 mg. The dill leaf powder was mixed along with other ingredients (Table 2.4) to obtain homogenous distribution of the powder in the diet. The diet prepared was stored in airtight opaque containers at 4°C. Prior to feeding, the lutein content of the diet was checked by HPLC by the method described elsewhere.

Extraction of carotenoids from plant materials and diets

All procedures and analyses were conducted under a dim yellow light and on ice to prevent photo-isomerization and degradation of carotenoids. Edible portions of vegetables, leaves of medicinal plants and leafy greens were washed separately with deionized water and dried on blotting paper at room temperature. The method of Raju et al. (2007) was employed with slight modification for the extraction of carotenoids. Edible portion of plant materials (20-30 g) or diet (10-15 g) was ground well using mortar and pestle, along with 2-3 grams of anhydrous sodium sulphate and 2mM α-tocopherol (antioxidant). Carotenoids (total) were extracted using ice-cold acetone (50 ml). The extraction was repeated three times, or until the residue was rendered colourless, indicating complete extraction of pigments (crude extract). The pooled crude extract (150-200 ml) was filtered through Whatman No. 1 filter paper containing 10 g of anhydrous sodium sulphate. Various steps involved in extraction and purification of carotenoids from the plant materials is given in Figure 2.1.

HPLC analysis

An aliquot of crude extract was evaporated under a stream of nitrogen and redissolved (100 μl) in acetonitrile: dichloromethane: methanol, 60:20:20 (v/v/v) containing 0.1% ammonium acetate (mobile phase) for the analysis of β-carotene, α-carotene, lutein, zeaxanthin, neoxanthin, and violaxanthin. The extract (20 μl) was injected to HPLC system (LC-10Avp; Shimadzu,

Kyoto, Japan) equipped with Shimadzu photodiode array (PDA) detector (SPD-M20A). All the carotenoids were separated on a Phenomenex RP-18 column (250 mm × 4.6 mm; 5 μm) isocratically eluting with 1ml/min. of mobile phase. The carotenoids were monitored at 450 nm using Shimadzu Class-VP version 6.14SP1 software. The peak identity of each carotenoid was confirmed by their UV-Vis spectra recorded with the PDA detector.

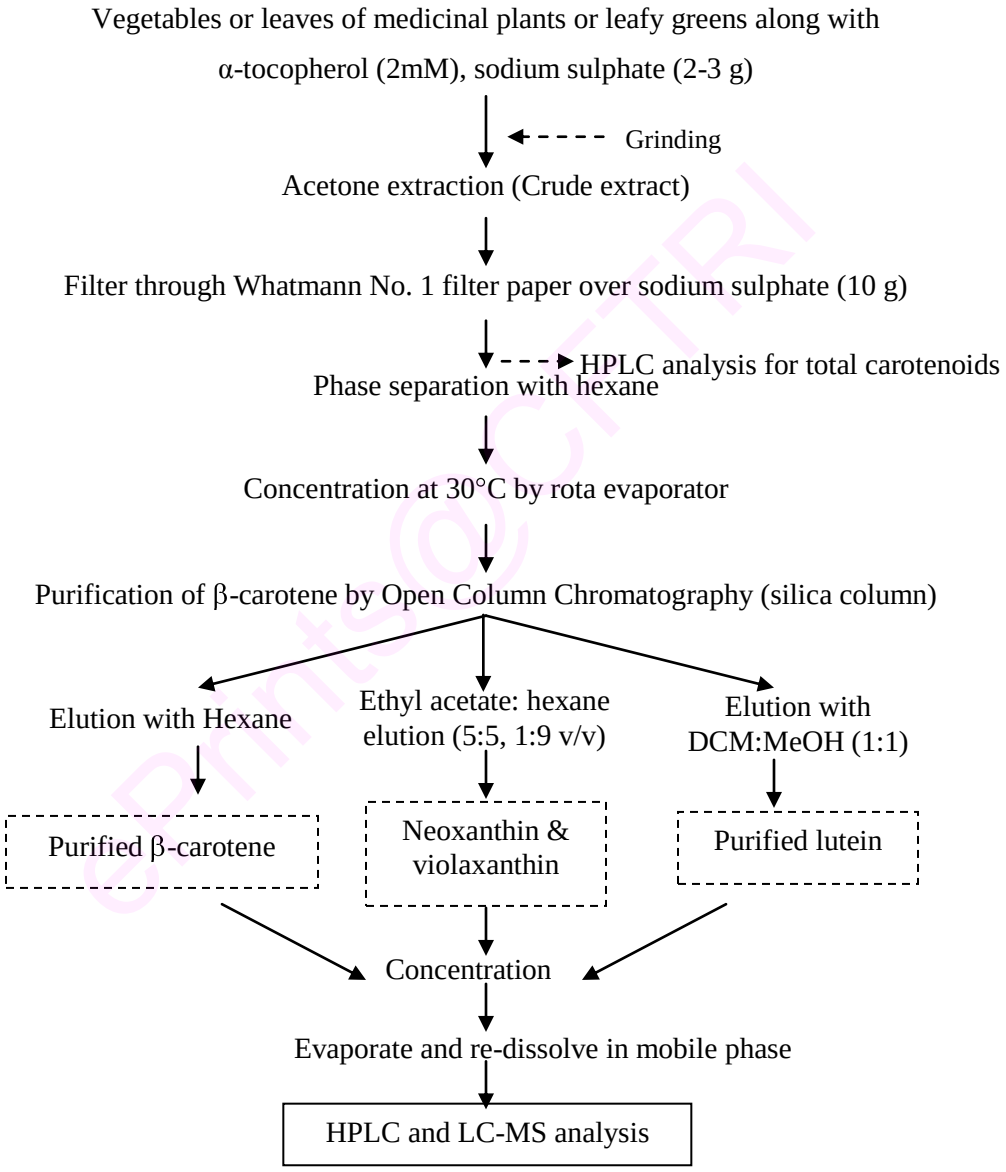


Figure 2.1: Flow diagram showing the various steps involved in extraction and purification of carotenoids from plant materials.

Purification of β-carotene and lutein by open column chromatography

The flow chart and schematic representation for the purification of β-carotene and lutein by open column chromatography is depicted in Figures 2.1 and 2.2. The crude carotenoid extract (100 ml) of *C album* was evaporated using a flash evaporator (Buchi, Switzerland) and the residue was re-dissolved in hexane (1 ml). Pre-activated silica gel (60-120 mesh size) was made into a slurry using chloroform and packed into a column (30 cm length x 3 cm diameter). The column was equilibrated with hexane (2 ml/min) until all the chloroform was eluted. The hexane extract containing carotenoids was applied on to the column. The β-carotene was eluted with hexane. After the complete elution of the β-carotene, the column was eluted with ethyl acetate: hexane (5:5, v/v) followed by ethyl acetate: hexane (1:9, v/v) for separation of neoxanthin and violaxanthin respectively. The purified β-carotene thus obtained was used for animal feeding studies

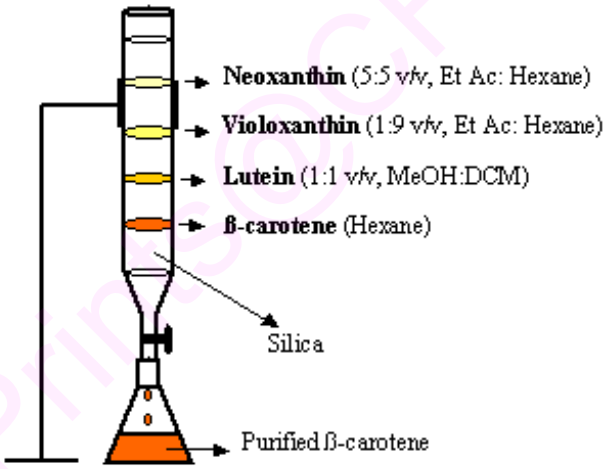


Figure 2.2: Purification of carotenoids by Open Column Chromatography.

To purify lutein from the crude extract of *C.album*, the crude acetone extract was subjected to saponification with 30% methanolic KOH (¼th volume of crude extract) for 3 hours in the dark, following which, phase separation was done with hexane. The lower aqueous layer containing chlorophylls was discarded and the hexane layer containing the carotenoids was collected and evaporated to dryness using a flash evaporator. Thereafter, the residue was dissolved in hexane (1 ml) and applied on to a column. The lutein was eluted with dichloromethane: methanol (1:1, v/v). The purity of the β-carotene and lutein thus obtained was

checked by HPLC using the conditions described earlier. The purified lutein thus obtained was used for animal feeding studies

Thin Layer chromatographic separation of carotenoids

The purity of the carotenoids purified by open column chromatography was further confirmed using thin layer chromatography (TLC). Pre-coated aluminum TLC plates (20 x 20 cm) with silica gel (mesh size 60) were used for separating the purified carotenoids. Along with the samples, corresponding standards were also spotted for comparison and confirmation. Hexane: acetone (70:30, v/v) and dichloromethane: methanol (1:1, v/v) was used as mobile phase for the separation of β-carotene and lutein respectively. The distance traveled by the solvent and carotenoids was measured and used for calculation of R_f value. R_f value was calculated by using the following formula:

$$R_f = \frac{\text{Distance traveled by compound}}{\text{Distance traveled by mobile phase}}$$

Liquid chromatography-mass spectrophotometric analysis of carotenoids (LC-MS, APCI)

LC-MS was used to confirm the identity of carotenoids. The positive ions of the carotenoids were recorded with the HPLC system (Alliance 2695, Waters, USA) connected to a LC-Q mass spectrometer (Waters 2996 modular HPLC system, UK) equipped with atmospheric pressure chemical ionization (APCI) module. The APCI source was heated at 130ºC and the probe was kept at 500ºC. 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 ion mode, $(M+H)^+$ ion signals were recorded and confirmed with respective standards. Quantification of individual compounds was evaluated by comparing their peak area with the authentic standards

Extraction and purification of Fucoxanthin

Fucoxanthin (FUCO) was extracted and purified from the Indian brown seaweed, *Padina tetrastromatica* by the procedure previously described by Haugan et al. (1992) with slight modification. In brief, fresh *P. tetrastromatica* (100 g) was washed with freshwater and dried at 38 ± 2°C in a drier (Kilburns-024 E, Mumbai, India). The dried seaweed was ground to a fine powder using a mixer grinder. FUCO was extracted by homogenization with cold acetone (4

times). For each extraction, the conical flask containing the seaweed was shaken at 100 strokes/min at 4°C for 2 hours. The pooled extract (400 mL) was filtered using glass filter paper, evaporated to dryness using a flash evaporator (Buchi, Switzerland) at 30°C and re-dissolved in methanol (100 mL). The extract was partitioned in methanol: water: hexane (10: 1: 10, v/v/v) and washed several times with hexane. The lower methanol/water phase was subjected to extraction thrice by diethyl ether (200 mL). The diethyl ether phase containing FUCO was evaporated to dryness by flash evaporation at 30 °C and re-dissolved in 5 ml of hexane.

Purification of FUCO by open column chromatography and preparatory HPLC

FUCO was purified by open column chromatography (45 cm length, 3 cm diameter), on a silica gel (mesh size 60-120) column equilibrated with hexane (bed length 30 cm). Chlorophylls and carotenoids other than FUCO were eluted with hexane (300 mL) followed by 250 mL of hexane: acetone (9:1, v/v). The FUCO rich fraction was eluted with 200 mL of hexane: acetone (4:1, v/v). The extract was evaporated to dryness using a flash evaporator at 30 °C. The residue was re-dissolved in methanol (3 ml) and used for purification. The extract was purified by preparatory HPLC (LC-8A, Shimadzu, Japan) using TSK-gel ODS 80Ts column (10 x 250mm, Tosoh) with methanol (100%) as mobile phase (4 ml/min). The volume of extract injected to HPLC was 3 ml. The purity of FUCO was checked by HPLC. The purified FUCO thus obtained was used for animal feeding studies. In addition, the purified FUCO was used for preparation of fucoxanthinol (FUOH) and amarouciaxanthin (AAx) as described by Asai et al. (2004).

Preparation of FUOH from FUCO

FUOH was prepared from FUCO by enzymatic hydrolysis (Asai et al. 2004). FUCO (5μmol) and taurocholate (100 mg) were dissolved together in dichloromethane: methanol (1 ml, 2:1 v/v) and evaporated under a stream of nitrogen gas. Potassium phosphate buffer (10 mL, 0.1 M pH 7.0) containing cholesterol esterase (10 units) was added to the residue, mixed well and incubated at 37°C for 2 h. Dichloromethane/methanol (4 volumes, 2:1, v/v) was added to the reaction mixture and FUOH was extracted into the dichloromethane phase. The extracted FUOH was then purified by preparatory HPLC on a TSK-gel ODS 80Ts column (10 x 250 mm) with acetonitrile/methanol/water (67.5:13.5:19, v/v/v) containing 0.1% (w/v) ammonium acetate as a mobile phase (4 ml/min). The purified FUOH was confirmed by UV- Vis spectra, HPLC and LC-

MS. Conditions for HPLC and LC-MS were same as those mentioned earlier for analysis of carotenoids extracted from vegetables, medicinal plants and leafy greens.

Purification of AAx from FUOH

AAx was prepared from FUOH as described previously by Asai et al. (2004). Fresh liver samples from mice were homogenized with 4 volumes of ice-cold 0.154 M KCl containing 50 mM Hepes-KOH (pH 7.4), 1.0 mM EDTA, and 0.1 mM dithiothreitrol. The reaction mixture was centrifuged at 10,000g for 10 min at 4°C. The supernatant (7 ml) was incubated at 37°C for 3 h with 50 mM fucoxanthinol, 2.4 mM NAD^+ and 0.2% Tween 20 (final concentrations) in 0.1 M glycine-KOH buffer (pH 10.0) in a total volume of 20 ml. Then the reaction mixture was mixed with 30 ml of dichloromethane/methanol (2:1, v/v). AAx was extracted into the dichloromethane phase and evaporated under a stream of nitrogen gas. The residue was suspended in 1 ml of hexane, applied to an alumina column (40 cm length, 3 cm diameter, 60-120 pore size) and eluted with increasing amounts of diethyl ether in hexane (2:8 to 5:5, v/v). The AAx was eluted with diethyl ether and further purified by preparatory HPLC using ODS, TSK gel 80Ts, column (10 x 250mm) with acetonitrile: methanol: water (50:25:25, v/v/v) containing 0.1% ammonium acetate as a mobile phase (4 ml/min). The purity of the AAx was determined by HPLC, LC-MS analysis and UV-Vis spectra. Methodology adopted for HPLC and LC-MS for separation of AAx was the same as given for FUOH.

Calculation of vitamin A activity

Vitamin A activity of β- and α-carotene extracted from vegetables, medicinal plants, leafy greens and algae was calculated in terms of retinol equivalents (RE) based on the *in vivo* conversion factor proposed by WHO and NRC (NRC, 1989; WHO, 1982), where 1RE = 1µg of retinol = 6µg of β-carotene or 12 µg of α-carotene. All the values presented are means of duplicate analyses.

Induction of retinol deficiency

Retinol deficiency was induced in rats by feeding retinol deficient (RD) diet for 8 weeks while a separate group received diet-containing retinol (control group). Composition of the experimental and control diets are given in Table 2.3. During the induction of retinol deficiency, rats received diet and water *ad libitum* and the feed intake and gain in body weight of animals

were recorded. Retinol deficiency was confirmed by estimating plasma retinol level (<0.7 nmol/ml) (Olson, 1982).

Animal feeding studies with carotenoids

Single dose study

The experimental plan adopted for the single dose study is shown in Figure 2.3. RD rats (n=65) were divided into 5 groups and gavaged with equimolar (0.8 μmol/rat) concentration of β-carotene, astaxanthin, lutein or fucoxanthin dispersed in peanut oil (0.5 ml) or peanut oil alone (baseline, RD). A separate group was fed on diet with retinol throughout the experiment was considered as control. RD group (n=5) was sacrificed at 0 h for baseline data while groups administered with either β-carotene, astaxanthin or lutein were sacrificed at 2, 4 and 8 h after gavage (n=5/time point) while animals in fucoxanthin fed group were sacrificed at 2, 4, 6 and 8h after gavage (n=5/time point).

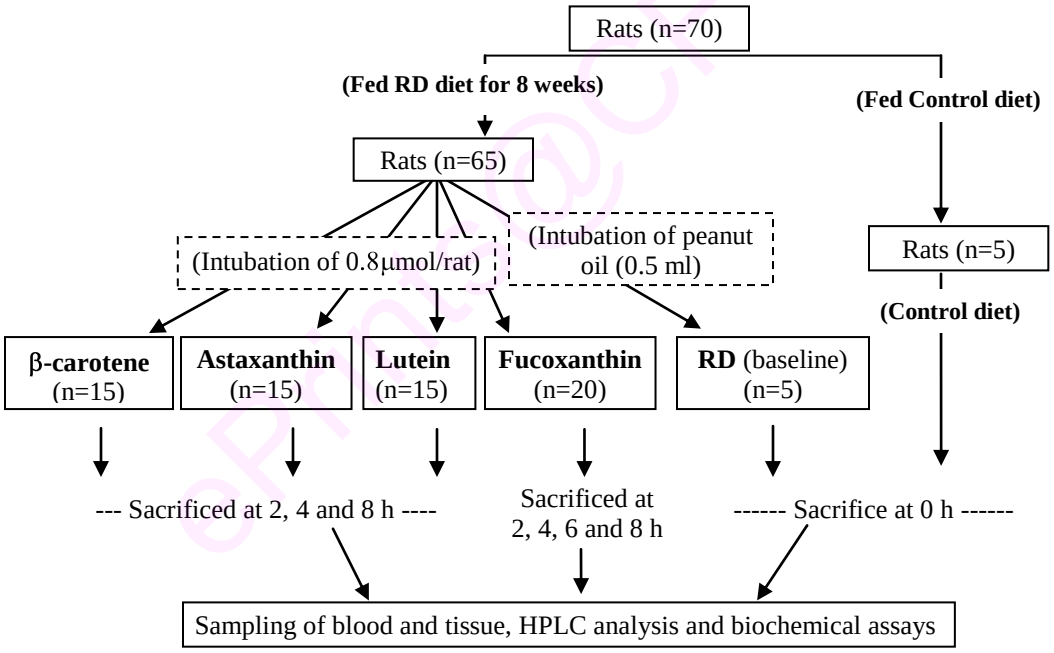


Figure 2.3 Schematic representation of experimental plan showing the induction of retinol deficiency and gavage with a single dose of β-carotene, astaxanthin, lutein or fucoxanthin to retinol deficient (RD) rats.

Blood was collected in non-heparinized tubes for serum separation and in heparinized tubes for plasma separation as per the method described elsewhere. A portion of the serum and

plasma were used immediately for separation of HDL/LDL+VLDL and antioxidant enzyme assays respectively while the remaining serum/plasma was stored at -80°C until analyzed for other parameters. Liver, intestine and brain were sampled and analyzed immediately or stored at -80°C. Carotenoids and their metabolites were extracted from the plasma, tissue homogenate and liver microsomes respectively and analyzed by HPLC.

Repeated dose study

The experimental plan adopted for the repeated dose study is shown in Figure 2.4. RD rats (n=40) were divided into 2 groups. Group 1 (n=40/group) received RD diet while group 2 (n=5) received control diet (Table 2.3). Group 1 was further divided into 4 subgroups. Rats (n=5/subgroup) in each subgroup were gavaged (0.5 ml) with equimolar concentration (0.1 mg/rat) of β-carotene, astaxanthin or lutein dispersed in peanut oil or peanut oil alone (RD group) for 7-days. Group 2 was divided into 4 subgroups (n=5/subgroup) and each subgroup was gavaged (0.5 ml) with equal concentration (0.1 mg/rat) of β-carotene, astaxanthin or lutein dispersed in peanut oil or peanut oil alone (RD group) for 15-days.

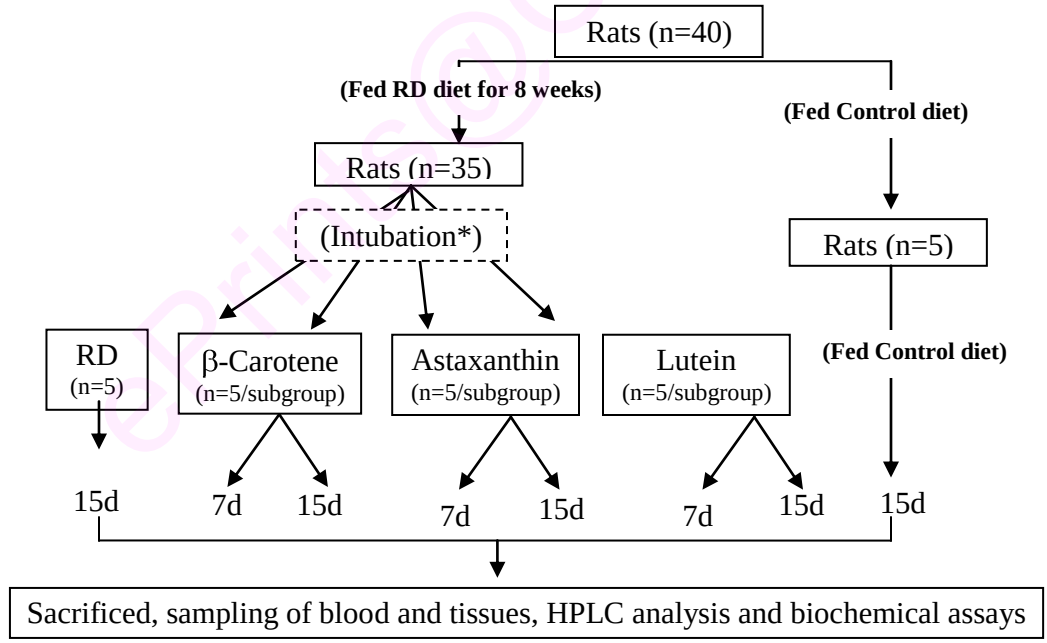


Figure 2.4 Schematic representation of experimental plan showing the induction of retinol deficiency and repeated gavage of retinol deficient (RD) rats with β-carotene, astaxanthin or lutein for 7- and 15-days. d = days.* Intubation of carotenoids separately for 7d and 15 d (0.1 mg/rat/day). RD rats were gavaged with equal volume of peanut oil (0.5 mL).

A separate group (n=5) was fed on diet with retinol throughout the experiment (control). Blood was collected in non-heparinized tubes for serum separation and in heparinized tubes for plasma separation as per the method described elsewhere. A portion of the serum and plasma were used immediately for separation of HDL/LDL+VLDL and antioxidant enzyme assays respectively. The remaining serum/plasma was stored at -80°C until analyzed for other parameters. Liver, intestine and brain tissues were sampled and analyzed immediately or stored at -80°C. Carotenoids and their metabolites were extracted from the plasma, tissue homogenate and liver microsomes respectively and analyzed by HPLC.

Dietary study

The experimental plan adopted for the dietary study is shown in Figure 2.5. RD rats (n=20) were divided into 4 groups. Groups 1, 2 and 3 were fed diet supplemented with either *D.carota* (carrot, as source of β-carotene), *P.sowa* (dill leaves, as source of lutein) or purified astaxanthin for 20 days while group 4 continued on the RD diet (RD group). A separate group was fed on diet with retinol throughout the experiment (control, n=5). Since astaxanthin was not detected in the seaweeds screened for carotenoids, standard astaxanthin was supplemented in the diet for the dietary study so that its effect could be compared with the sources of β-carotene and lutein. *D. carota* was selected as β-carotene source as it contained higher levels of β-and α-carotene. *P. sowa* was selected as a lutein source since it is rich in lutein. *D. carota* and *P. sowa* were chosen because they are locally available and consumed. Composition of the diet used for the dietary study is given in Table 2.4, while the composition of the RD and control diets is given in Table 2.3. Blood was drawn from the orbital plexus after 10 days of feeding experimental diet. At the end of 20-days of feeding, animals were sacrificed; blood and tissues were sampled and analyzed immediately or stored at -80°C. Carotenoids in plasma were analyzed by HPLC.

Preparation of plasma, serum, liver homogenate and microsomes

At the termination of experiment, rats in each group were sacrificed under ether anesthesia and blood was drawn from heart into heparinized (0.2 mg/mL blood) tubes for plasma separation and non-heparinized tubes for serum separation. Liver and intestine were sampled and washed with ice-cold saline (0.9%), while the brain was sampled and blotted for removal of blood. Samples were processed under a dim yellow light, on ice (<5°C) to minimize isomerization and oxidation of carotenoids. The plasma was separated from blood into clean tubes by centrifugation (Remi India Ltd., Mumbai) at 2000 g for 15 min at 4°C. Blood collected

in non-heparinized tubes for serum collection was allowed to stand at 4-8°C for 2 hours to allow RBC to clot. The tubes were then centrifuged at 2000 g for the separation of serum. The serum thus collected was stored in clean tubes and used for HDL/LDL analysis and quantification of retinol binding protein (RBP).

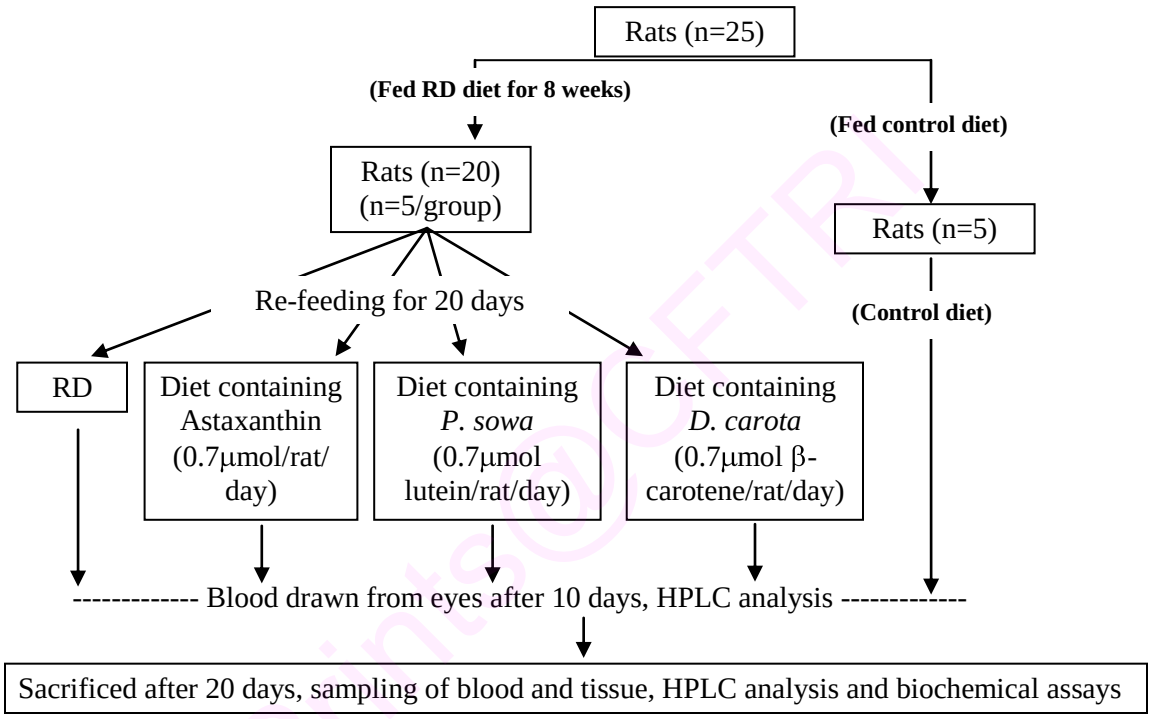


Figure 2.5 Schematic representation of experimental plan of dietary study showing the induction of retinol deficiency and feeding of astaxanthin, *P.sowa* (source of lutein) and *D.carota* (source of β-carotene). RD = retinol deficient.

Liver (1g) was homogenized (Potter Elvehjem homogenizer) at 4°C in buffered saline (0.9%) for retinol assay or in 120mM KCl and 30mM phosphate buffer (pH 7.2) for lipid peroxidation assay. In the case of liver samples stored at -80°C, sample were thawed to room temperature and processed. The suspension was centrifuged (Hermle, Germany) at 10000 g for 10 min at 4°C to remove nuclei and cell debris. The supernatant was used immediately for assay of antioxidant enzymes (Gonzalez-Fletcha et al., 1991) such as catalase, superoxide dismutase, glutathione-S-transferase and estimation of glutathione levels.

Liver microsomes (hereafter referred as microsomes) and brain microsomes were prepared according to Gutman and Katzper-Shamir (1971) from a 10% homogenate in 0.25M sucrose, 0.03M histidine and 0.001M EDTA (pH 7.4). The homogenate was centrifuged at 10000 g for 30 min in a refrigerated centrifuge (Hermle, Germany) at 4°C. The pellet was discarded and the supernatant was subjected to ultracentrifugation (L-65, Beckman, USA) at 105,000 g for 1 hour at 4°C. The supernatant was discarded and the pellet formed was re-suspended in the sucrose buffer (0.2 times the volume of the homogenate taken) and subjected to analysis or immediately stored at -80°C. Brain microsomes were prepared according to the same method used for preparation of liver microsomes.

Protein analysis

Protein content in plasma, liver homogenate and microsomes was measured by the method of Lowry et al. (1951). Freshly prepared Lowry's reagent (0.98 ml of 2% sodium carbonate, 0.01 ml of 2% copper sulphate and 0.01 ml of 2% sodium potassium tartarate) was vortexed (1 ml) with the diluted sample (1 ml) and allowed to stand for 10 min, followed by the addition of 100 µl of 1N Folin Ciocalteu's phenol reagent and immediately vortexed. The samples were allowed to stand for 30 min at room temperature and the absorbance was read at 690 nm. The protein in the samples was quantified based on the absorbance obtained in a linear graph obtained for known concentrations of standard (BSA).

Extraction of carotenoids from plasma and tissue samples

Astaxanthin, β-carotene, lutein, fucoxanthin, retinol and retinyl palmitate were extracted from the plasma and liver homogenates as per Raju et al. (2009) and Lakshminarayana et al. (2008). Plasma preparation, tissue homogenization and microsomes preparation was the same as described earlier. Plasma (0.8 ml) was diluted with 3 ml of dichloromethane: methanol (1:2; v/v) containing 2mM α-tocopherol, mixed for 1 min using a vortex mixer, followed by the addition of 1.5 ml hexane. The mixture was centrifuged at 5000 g for 15 min at 4°C and the upper hexane-dichloromethane layer was withdrawn. The extraction was repeated for the lower phase twice using dichloromethane: hexane (1:1.5; v/v). The pooled extract was evaporated to dryness under a stream of nitrogen, re-dissolved in dichloromethane: methanol (2:1; v/v) and analyzed by HPLC. Retinol and carotenoids were extracted from liver homogenate and liver microsomes (0.8 ml) by the procedure described for plasma.

HPLC analysis of retinol and carotenoids

Retinol, retinyl palmitate, astaxanthin, β-carotene, lutein and fucoxanthin in plasma, liver homogenate and microsomal extracts were quantified by HPLC (LC-10Avp; Shimadzu, Kyoto, Japan) equipped with photodiode array detector (SPD-M20A Shimadzu). All the components were separated on a Phenomenex C18-RP column (250 mm × 4.6 mm; 5 μm), isocratically eluting (1ml/min) with acetonitrile: dichloromethane: methanol (60:20:20; v/v/v) containing 0.1% ammonium acetate as mobile phase. Retinol and retinyl palmitate were monitored at 325 nm, β-carotene, lutein and fucoxanthin were monitored at 450 nm, while astaxanthin was monitored at 478 nm using Shimadzu Class-VP version 6.14SP1 software. The peak identities were confirmed by comparing their characteristic UV-Vis spectra with authentic standards and quantified by comparing their peak areas.

LC-MS analysis of carotenoids

LC-MS was used to confirm the identity of carotenoids and their metabolites, for example, retinol from β-carotene, fucoxanthinol and amarouciaxanthin from fucoxanthin, zeaxanthin from lutein, etc. The positive ions of the carotenoids were recorded with the HPLC system (Alliance 2695, Waters, USA) connected to a LC-Q mass spectrometer (Waters 2996 modular HPLC system, UK) equipped with atmospheric pressure chemical ionization (APCI) module. The APCI source was heated at 130ºC and the probe was kept at 500ºC. 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 ion mode and $(M+H)^+$ ion signals were recorded and confirmed with respective standards. Individual compounds were quantified by comparing their peak area with the authentic standards.

Assay for β-Carotene, 15-15'-monooxygenase enzyme

β-Carotene, 15-15'-monooxygenase (E.C. 14.99.36) (previously known as β-Carotene, 15-15'-dioxygenase) activity was measured as per Nagao et al. (1996) with slight modification. The upper portion of the small intestine of control and experimental animals was rinsed with saline and the mucosal layer (jejunum) was scraped off (15 cm) using a cover glass and homogenized with five volumes (5 ml) of 50mM HEPES-KOH buffer (pH 7.4) containing 0.54M KCl, 1mM EDTA and 0.1mM dithiothreitrol (DTT). The homogenate was centrifuged at 10,000 g for 30 min. The supernatant was applied to a sephadex G-25 column (1.5 × 5.5 cm),

equilibrated with 10mM HEPES-KOH buffer (pH 7.4) containing 0.1 mM EDTA, 0.05M KCl and 0.1mM DTT and the elute was used as enzyme source. The reaction mixture (0.4 ml) contained tricine-KOH buffer (pH 8.0), 15µM β-carotene, 0.5mM DTT, 0.1mM α-tocopherol, 0.15% Tween-40 and enzyme (~1.5mg protein) after pre-incubation at 37°C for 5 min. The reaction was initiated by adding 1.2nmol (80µL) of β-carotene solubilized in α-tocopherol and aqueous Tween-40 and 0.32 ml of reaction mixture and incubated at 37°C for 60 min in the presence of atmospheric oxygen under dim yellow light. The reaction was terminated by addition of 0.1 ml 37% formaldehyde, incubated again at 37°C for 10 min followed by addition of 0.5 ml acetonitrile and cooled on ice for 5 min. The insoluble matter in the reaction mixture was removed by centrifugation at 10,000 g at 4°C for 10 min. The supernatant (20µL) was subjected to HPLC analysis to determine the amount of retinal formed.

Lipid peroxidation Assay

Lipid peroxides level in plasma, liver homogenate and microsomes was estimated using thiobarbituric acid as described previously (Okhawa et al., 1979). Acetic acid (20%, 1.5 ml), 8% sodium dodecyl sulphate (0.2 ml) and 0.8% thiobarbituric acid (1.5 ml) were added to the sample in that order, incubated for 1 hour in a boiling water bath, cooled at room temperature followed by the addition of n-butanol (5 ml) and centrifuged at 3000 g for 15 min. The upper n-butanol phase containing thiobarbituric acid-reactive substances was read spectro-fluorometrically (Hitachi, Japan) with an excitation at 515 nm and emission at 553 nm. Tetramethoxypropane (TMP) was used as a standard to estimate the thiobarbituric acid -reactive substances. Absorbance of known concentrations of TMP were plotted to obtain a standard linear graph and this was used to quantify the concentration in the samples.

Assays for antioxidant molecules

Assay for catalase

Activity of catalase (CAT, E.C. 1.11.1.6) in plasma and liver homogenates was determined by measuring the decrease in absorption at 240 nm using spectrophotometer (Shimadzu- Japan, 1601) in a reaction mixture containing 950 µL phosphate buffer (0.1 mM, pH 7.0), 50 µL of sample containing the enzyme (approx. 10 µg) and 50 µL of hydrogen peroxide (8.8 mM) according to Aebi (1984). One CAT unit is defined as the amount of enzyme required to decompose 1 µM of H_2O_2/min. Reaction is initiated by addition of H_2O_2 and the initial

absorbance reads 0.5. Reading at every 60 seconds for a period of 300 seconds was noted. Specific activity was calculated using the molar coefficient for H_2O_2.

Assay for superoxide dismutase

Superoxide dismutase activity (SOD, E.C. 1.15.1.1) in plasma, liver homogenate and microsomes was measured by the inhibition of cytochrome - C reduction mediated via superoxide ions generated by xanthine-xanthine oxidase system (Flohe and Otting, 1984) and measured using spectrophotometer at 550nm. One SOD unit is defined as the amount-required to inhibit the cytochrome-C reduction by 50%. Solution A was prepared by admixing xanthine (5 μmol) in 0.001N NaOH and cytochrome C (2 μmol) with 100 ml 50 mM phosphate buffer (containing 0.1mM disodium EDTA). The solution is stable for 3 days at 4°C and is used at 25°C. Solution B containing xanthine oxidase in 0.01M disodium EDTA (approximately 0.2units/ml) is freshly prepared and kept on ice. Sufficient enzyme to produce a rate of 0.025 units/min of cytochrome C without SOD is used. For the assay, Solution A (2.9 mL) and enzyme source (approx. 10 μg) are taken in a cuvette and reaction is started by addition of Solution B (50 μl). The contents are mixed well and absorbance was read at every 60 seconds for a period of 300 seconds at 550 nm.

Assay for glutathione-S-transferase

Glutathione-S-transferase (GST, E.C. 2.5.1.18) activity in liver homogenates and microsomes was determined following the formation of conjugate of GSH and CDNB at 340 nm in a reaction mixture containing 50 μL GSH (20mM), 50 μL CDNB (20mM), enzyme source (1-20 μg) and 850-895 μl phosphate buffer (0.1mM, pH 6.5) containing 1mM EDTA, at 30°C (Gluthenberg et al., 1985). The increase in absorbance was measured every 60 seconds for a period of 5 min for the enzyme assay. Specific activity was calculated using the molar coefficient for CDNB.

Estimation of Glutathione

Glutathione (GSH) levels in plasma, liver homogenates and microsomes were measured by monitoring the rate of 5-thio-2-nitrobenzoic acid formations at 412 nm (Owens and Belcher 1965). The samples were centrifuged at 2000 rpm for 10 min at room temperature with trichloroacetic acid solution (5%). The deproteinized supernatant (200 μl) was mixed with 4.75 ml of sodium phosphate buffer (0.1M, pH 8.0) and 50 μl of 10mM dithionitrobenzoic acid

(DTNB) in buffer (pH 8.0) and allowed to stand for 5 min and absorbance was read at 412 nm. Absorbance of known concentrations of GSH were plotted to obtain a standard linear graph and this was used to quantify the concentration in the samples.

Assays for membrane bound enzymes

Assay for Na^+K^+-ATPase in microsomes

Activity of the ATPases in microsomes was estimated by the method of Kaplay (1978) with slight modification. Buffer composition used for the Na^+K^+-ATPase assay was as follows - $MgCl_2$ (3 mM), KCl (14 mM), NaCl (140 mM), EDTA (0.2 mM) and Tris-HCl (20 mM, pH 7.4). Samples were run in two batches, one containing Na^+K^+-ATPase inhibitor ouabain (2 mM) and the other without. ATP (3 mM) was used as substrate. The sample blank contained no assay standard and microsomes and was run simultaneously. The reaction was stopped by the addition of trichloroacetic acid (10%). Inorganic phosphate (P_i) liberated was determined in aliquots (0.7 ml) of incubated mixtures by the addition of ascorbic acid-ammonium molybdate solution (0.3 ml) prepared according to the method of Ames (1966). The reaction mixture was mixed well and incubated at 45°C for 20 min. Extinction at 820 nm was measured in by UV-Visible spectrophotometer. Specific activity was expressed as μmol P_i/h/mg protein. Samples that contained ouabain were measured for the Mg^{2+}-ATPase activity and this was subtracted from the total ATPase activity to determine the Na^+K^+-ATPase activity.

Assay for Mg^{2+}-ATPase and Ca^{2+}-ATPase in microsomes

Assay for Mg^{2+}-ATPase and Ca^{2+}-ATPase assay was measured as per Kaplay (1978) with slight modification. Buffer composition was as follows - $MgCl_2$ (3mM), KCl (14mM), $CaCl_2$ (140mM), EDTA (0.2mM) and Tris-HCl (20mM, pH 7.4). ATP (3 mM) was used as substrate. Samples were run in two batches, one containing Ca^{2+}-ATPase inhibitor ethylene glycol tetra-acetic acid (EGTA, 0.5 mM) and the other without. The sample blank contained no assay standard and microsomes and was run simultaneously. The reaction was stopped by the addition of trichloroacetic acid (10%). Inorganic phosphate (P_i) liberated was determined as described for the Na^+K^+-ATPase assay. Specific activity was expressed as μmol P_i/h/mg protein. Samples that contained EGTA were measured for the Mg^{2+}-ATPase activity and this was subtracted from the total ATPase activity to determine the Ca^{2+}-ATPase activity.

Assay for Acetylcholine esterase activity in brain microsomes

Acetylcholine esterase (AchE) activity in brain microsomes was determined according to the method described by Ellman et al. (1961). A cocktail containing 13 ml of 1M NaCl, 2 ml of 1M $MgCl_2$, 10 ml of 0.5M Tris-HCl (pH 7.5) and 10 ml of 0.2M EDTA was prepared. Other reagents included 1mM DTNB and the substrate 0.1M acetylthiocholine chloride. The reaction mixture which contained 10.5 ml of the cocktail, 3 ml DTNB and 6.5 ml double distilled water. 2 ml of the reaction mixture, 30 µl of 0.1 M acetylthiocholine chloride and brain microsomes containing the enzyme (10 µg) were taken and the change in optical density was measured at 412 nm over a period of 5 min. Specific activity was calculated using the molar coefficient for –SH group of DTNB.

Extraction of lipids from plasma, tissue homogenate and microsomes

Total lipid in plasma, tissue homogenates (in 0.74% KCl) and microsomes was extracted by the method of Folch et al. (1957) by adding 10 ml chloroform: methanol (2:1) and left overnight. Thereafter, it was filtered through Whatman No. 1 filter paper and washed with 2 ml 0.74% KCl, followed by washing with 1.5 ml chloroform: methanol: water (3: 48: 47) two times. The filtrate containing the lipid was made to a single phase with methanol and then made up to a known volume with chloroform: methanol (2:1) and considered as crude or total lipid extract.

Fatty acid analysis

Fatty acid methyl esters were prepared from the lipid extracted from plasma and microsomes using boron triflouride in methanol (Morrison and Smith, 1965) and analyzed by gas chromatography (Shimadzu 14B, Shimadzu, Kyoto, Japan) fitted with flame ionization detector and a fused silica capillary column (25 m × 0.25 mm; Konik Tech, Barcelona, Spain). The lipid extracted from tissue samples was incubated at 65°C for 1 hour with 1 ml of 0.5 M methanolic KOH. Thereafter, the unsaponified layer matter was drawn by addition of 2 ml hexane, which was allowed to stand for 15 min, followed by centrifugation for 15 min. The upper layer was discarded. To the lower phase, 1 ml of 0.7 N HCl and 2 ml hexane was added and allowed to stand for 15 min and centrifuged for 15 min. The hexane layer was collected. This process was repeated twice with addition of 2 ml hexane. All the collected hexane layers were pooled and evaporated under a stream of nitrogen gas. Benzene (0.2 ml) and boron trifluoride in methanol (0.5 ml) were then added to it and incubated at 65°C for 45 min. After cooling, 1 ml water was

added and extraction performed thrice with 2 ml hexane. Double distilled water (5 ml) was added to the pooled hexane layers and centrifuged. The hexane layer was carefully separated, evaporated under a stream of nitrogen and re-dissolved in 25-100 µl of hexane. The hexane containing the fatty acid methyl was esters injected to the gas chromatograph. The injector, column and detector temperatures were 220, 230 and 240°C respectively with nitrogen as carrier gas at 1 ml/min. Individual fatty acids were identified by comparing their retention time with those of standards and were quantified using Clarity Lite 420 integrator.

Estimation of triglycerides

Triglycerides were measured by the method of Fletcher (1968). Isopropanol (3 ml) and alumina (2 g) were added to the sample, vortexed and centrifuged at 4000 g for 5 min. To the supernatant (2 ml), 5% KOH (0.6 ml) was added and incubated at 60-70°C for 15 min. Working solution (12 ml stock and 20 ml isopropanol made to 100 ml with 1N acetic acid) of sodium metaperiodate stock (0.025M in 1N acetic acid) was prepared fresh. After cooling samples, 1 ml of working sodium metaperiodate was added, mixed well followed by addition of 0.5 ml of acetyl acetone reagent (0.75 ml acetyl acetone and 2.5 ml isopropanol made to 100 ml with 2M ammonium acetate (pH 6.0), mixing and incubation at 50°C for thirty min. The colour that developed was read at 405nm. Assay with standard triolein was simultaneously run for plotting of standard graph and calculations for samples.

Estimation of phospholipids

Phospholipids were determined by the method of Stewart (1980). To the dry sample, 2 ml chloroform and 2 ml ammonium ferrothiocyanate reagent (2.7 g hexahydrate ferric chloride and 3.24 g ammonium thiocyanate in 100 ml deionized water) were added, gently mixed, allowed to stand for 5 min and centrifuged at 3000 rpm. The absorbance of the lower chloroform layer was read at 488 nm. Assay with standard dipalmitoyl phosphatidylcholine (DPPC) was run simultaneously for plotting of standard graph and calculations for samples.

Estimation of cholesterol

Cholesterol in plasma, liver and microsomes was estimated according to the method of Zlatkis et al. (1963). The ferric chloride acetic acid reagent stock was prepared by dissolving 840 mg of hexahydrate ferric chloride in 10 ml of acetic acid. Lipid previously extracted from tissue

was taken, evaporated and 1.5 ml of working solution of ferric chloride acetic acid (1 ml stock made to 100 ml with glacial acetic acid) was added to it. The mixture was mixed well by vortexing and allowed to stand for 10 min, thereafter, 1 ml sulphuric acid was added, mixed well, and allowed to stand for 45 min in the dark. The absorbance of the colour developed was read at 540 nm in a spectrophotometer. Assay with standard cholesterol was simultaneously run for plotting of standard graph and calculations for samples.

Estimation of LDL and HDL-Cholesterol

HDL and LDL were separated by the method of Warnick and Albers (1978). Serum (0.5 ml), heparin (5000 U/ml, 25 µl) and 2M manganese chloride (25 µl) were mixed well by vortexing and allowed to stand for 30 min at 4°C. The mixture was then centrifuged at 1600 g for 20 min. HDL in the supernatant was extracted using 3 ml acetone: ethanol (1:1 v/v) while the LDL and VLDL rich precipitate was dissolved in 0.5 ml saline and the cholesterol in both fractions was estimated by the method of Zlatkis et al. (1963), as described earlier.

Separation of Retinol Binding Protein by gel electrophoresis

Vertical gel electrophoresis was used to separate retinol-binding protein (RBP) in blood. Two millimeter of polyacrylamide (29.2 g acrylamide and 0.8g bisacrylamide) was used as supporting media along with 2.4 ml water, 1.5 ml running gel buffer (18.6 g tris in 100 ml, pH 8.8), 60 µl of 15% ammonium per sulphate (APS) and 15 µl TEMED to form the gel. Followed by, the stacking gel [0.35 polyacrylamide, 0.5 ml stacking gel buffer (3.06 Tris in 50 ml, pH 6.8), APS and TEMED] was poured to the plates. Plasma samples dissolved in sample buffer (5 ml stacking gel buffer, 4 ml glycerol and a pinch of bromophenol blue, made to 10 ml) were loaded in the stacking gel and subjected to 50V electrical power supply till they reached running gel after which 100V supply was given. After the completion of the run, the gel is stained in 0.1% Brilliant blue R-250 (with 50:10 methanol: acetic acid made to 100 ml) and further de-stained in a solution containing 20:10 methanol: acetic acid made to 100 ml.

Enzyme Linked Immunosorbent Assay for quantification of RBP

The ELISA kit obtained for the quantification of RBP in serum contained a standard, wash solution, diluent, secondary antibody, detector substrate-1, substrate-2, a quality control (QC) sample and a stop solution. All kit components and samples were brought to room temperature (20-25°C) prior to reagent preparation. The wash solution was diluted 1: 5 with triple

distilled water to prepare the working wash solution. Similarly, the working diluent was prepared by diluting 1: 5 with triple distilled water. The detector was freshly diluted 1:100 with working diluent to obtain working detector and used within one hour of preparation. Just before the use, equal volumes of substate-1 and substrate-2 were added to obtain the working substrate. The QC sample was reconstituted in 1 ml of triple distilled water. Serial dilution of the standard was performed by first adding 1 ml triple distilled water to the vial containing the standard (24 ng) to obtain the stock standard. An aliquot of 500 µl of the stock standard was drawn and diluted with an equal volume of working diluent to obtain a concentration of 12 ng. This step was repeated 5 times to obtain serially diluted concentrations of the standard (6, 3, 1.5, 0.75, 0.375 and 0.188 ng). A separate vial containing no standard and with working diluent was considered as 'zero concentration'. The serial dilution of the standard is depicted in Figure 2.6. Serum samples were diluted 10000 times before using for the assay. To obtain 10000 dilutions, samples were diluted 1: 100 with working diluent and from this dilution an aliquot was drawn and it was further diluted 1: 100 with the working diluent.

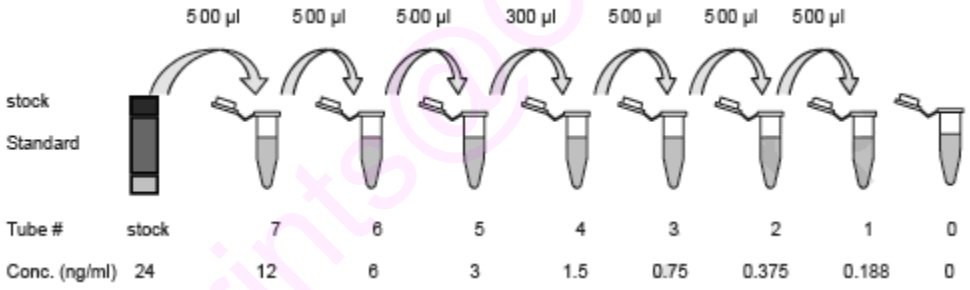


Figure 2.6 Serial dilution of the RBP standard for ELISA.

A schematic representation of the steps involved in the ELISA is depicted in Figure 2.7. Prior to starting the assay, the plate configuration was planned and a plate map was created. Standards and sample were run in duplicate. The appropriate numbers of microwell strips were removed from the sealed foil pouch. 100 µl of standard/QC sample/diluted serum samples were pipetted into the antibody-coated plate, according to the plate configuration and incubated at 37°C for 1 hour. The solution was removed and each well was washed thrice with 250 µl of the working wash solution. Secondary antibody was added (100 µl) to each well and incubated at 37°C for 1 hour. The solution was aspirated and washed thrice with 250 µl of the working wash solution to each well. Freshly prepared (within one hour) working detector solution was added

(100 µl) to each well and incubated at 37°C for 1 hour. The solution was discarded and washed five times with 250 µl of the working wash solution to each well. Very freshly prepared working substrate solution was added (100 µl) to each well and incubated at room temperature for 20 mins in dark, protected from light. Using the multi-channel pipette, 100 µl of stop solution was added to each well and absorbance was read at 450 nm within 30 mins. A standard graph was plotted based on the absorbance values obtained for different concentration of standards and used for the calculation of RBP in samples. Reading of the blank is subtracted from each standard and sample prior to graph plotting and calculations.

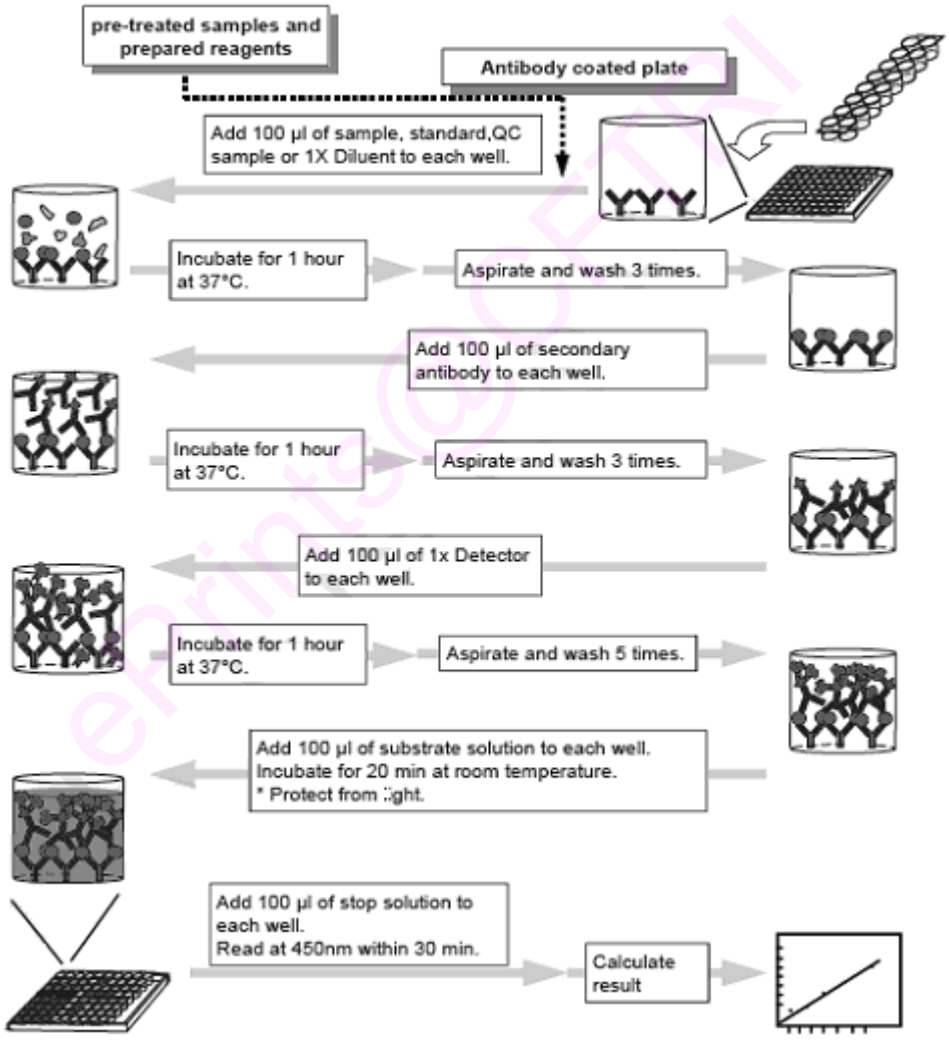


Figure 2.7 Schematic representation (flow chart) of ELISA for Retinol Binding Protein in serum of rats.

Statistical analysis

The experimental data obtained for different parameters were subjected to analysis of variance (ANOVA). In case of significant difference, mean separation was accomplished by Tukey's test using Origin software (ver. 5.1). The percent differences between groups were calculated and considered significant at $p<0.05$ level.

CHAPTER 3: Isolation and purification of carotenoids (β-carotene, lutein, astaxanthin and fucoxanthin) from leafy greens, medicinal plants, vegetables and algae.

Introduction

The twenty first century is the age of awareness, especially with regard to health and nutrition. Abundant consumption of fruits, vegetables and leafy greens to receive maximum health benefits is recommended. Studies suggest that vegetables and fruit intake must be increased to improve protection from various physiological disorders and diseases (Pajk et al., 2006; Kesari et al., 2005; Niizu and Rodriguez-Amaya, 2005). With this awakening, an interest has risen- the need for determining the bioactive component (s) in fruits and vegetables that have this protective effect. The association of fruit and vegetable consumption and the delay or prevention of chronic disease appears to be attributable to the biological activity of constituent nutrients.

Vitamin A deficiency (VAD) is an established public health problem (WHO 2009) in the Indian subcontinent, other parts of Asia and Africa. Clinical and sub-clinical manifestations are prevalent and thus, alternate sources for vitamin A have become necessary. In developed countries, VAD has been largely eliminated, except in people who suffer from lipid malabsorption which interferes with the absorption of fat-soluble vitamins such as vitamin A. The absence of VAD is largely due to the consumption of vitamin A-fortified foods. There is additional growing interest in fruits and vegetables because of a negative association between their consumption and the incidence of certain chronic diseases (Table 3.1). This negative association could be due to the phytochemicals like carotenoids and/or other antioxidants, both of which normally exist in fruits, vegetables and leafy greens. Thus, carotenoids are important dietary components because of their provitamin A activity (Olson, 1982) as well as their possible roles in the prevention of degenerative diseases (Maeda et al., 2005; Shiratori et al., 2005; Kotake-Nara et al., 2001).

Mammalian species, including humans, do not have the ability to synthesize carotenoids. Although it is recognized that some bacteria can synthesize carotenoids, the microflora of most animals (except ruminants) occurs in the distal part of the digestive tract where carotenoid absorption is almost nonexistent. Carotenoids found in mammalian tissues are therefore derived from dietary sources. Apart from major nutrients such as proteins, fat, carbohydrates, vitamins

and minerals, over 10,000 bioactive phytonutrients have been identified in fruits, vegetables and leafy greens and the major classes include carotenoids, polyphenols, anthocyanins, flavonoids, isothiocyanates, sulfides and phytosterols. These are an integral part of the human diet.

Table 3.1. Chronic degenerative diseases/disorders that have been reported to be negatively associated with increased consumption of fruits and vegetables

Disease	Model System	Study
Cancer	Human, Rat	Giovannucci et al., 1998; Fahey et al., 1997
Cardio-vascular	Human	Menotti et al., 1999; Yochum et al., 1999
Stroke	Human	Joshipura, 1999; Yochum et al., 1999
Macular Degeneration	Human	O'Connell et al., 2008; Seddon, 2007
Cataracts	Human	Brown et al., 1999; Jacques and Chylack, 1991
Neurodegeneration	Human	Floyd, 1999; Behl, 1999
Diabetes	Human	Williams et al., 2002

Vitamin A is present in our diet in its preformed state (retinyl ester, retinol, retinal, 3-dehydroretinol and retinoic acid) from foods of animal origin such as liver, milk and milk products, fish and meat (liver and organelles), or as carotenoids, generally from plant sources, that can be biologically converted to vitamin A. Globally, about 60% of dietary vitamin A is estimated to come from plant foods. However, due to the prohibitive cost of animal foods, the dietary contribution of provitamin A could rise to 80 to 90% in developing countries (Hickenbottom et al., 2002). In many countries, dark green leafy vegetables are the most common and relatively abundant sources of provitamin A carotenoids. Due to their relative ease of cultivation and their availability the year-around, they are an inexpensive and accessible source of provitamin A carotenoids. For most of the developing countries that lie in subtropical and tropical areas, β-carotene is essentially the most important source of vitamin A, with α-carotene and α- or β-cryptoxanthin reported occasionally at relatively low levels. Thus, greens are an important source of carotenoids, especially lutein and β-carotene. At the same time, even though fruits generally have lower levels of provitamin A carotenoids than leafy vegetables, they are usually more readily accepted by both children and adults and their provitamin A content is believed to be more bioavailable. Tropical and subtropical fruits may be more advantageous as they generally contain carotenoids predominate and are in higher amounts as compared to temperate fruits in which the anthocyanin pigments (noncarotenogenic) predominate.

Provitamin A carotenoids have the advantage of being converted to vitamin A only when vitamin A is needed in the body, thus avoiding potential toxicity due to excesses. On the other hand, many factors influence the absorption and utilization of provitamin A carotenoids, thus the bioavailability of carotenoids is variable and difficult to appraise. The activity of the provitamin A carotenoids (Table 3.2) are ranked on the basis of retinol formation (Combs, 1992).

Table 3.2. Relative biological activity of carotenoids

Carotenoid	Relative Activity (%)*
β-Carotene	50
α-Carotene	26
γ-Carotene	21
β-Cryptoxanthin	28
Zeaxanthin	0
Lycopene	0

* Activity is on the basis of retinol formation

There are methods available for the extraction and separation of carotenoids. Kimura and Rodriguez-Amaya (2003) studied separation of carotenoids from lettuce by HPLC under gradient elution, with a runtime of 50 min, whereas, Bhaskarachary et al. (2008) achieved separation of carotenoids from leafy vegetables in 25 mins with acetonitrile: dichloromethane: methanol (70:10:20 v/v/v). Aman et al. (2005) have used a gradient elution for the separation of lutein and zeaxanthin isomers in a runtime of 75 mins. Different HPLC methods (gradient) was proposed for separation of natural pigments including carotenoids by Airs et al. (2001) with a run time ranging from 85 to 115 mins. Seo et al. (2005) achieved separation of carotenoids in pumpkin by gradient elution with a runtime of 48 mins. Raju et al. (2007) and Lakshminarayana et al. (2005) used an isocratic elution method and achieved separation of the carotenoids within 20 mins with acetonitrile: dichloromethane: methanol (60:20:20 v/v/v).

Thus, with the information already known about carotenoids and their beneficial effects as provitamin A and strong antioxidant agents, it is essential to establish the food sources that are rich in carotenoids. Though carotenoids are pigments and impart bright hues to fruits and vegetables (yellow, orange to red), they may be masked as in the case of greens. Hence, it is necessary to evaluate and establish the carotenoid profile of the variety of natural produce

consumed. Over and above this, there are various plant foods that are not commonly consumed. They are often used in folk medicine or discarded as weeds. However, these are edible and may be consumed by some specific populations such as tribal groups and used in Ayurvedic (Indian folk medicine) preparations. Similarly, although algae are popular as delicacies in far-east Asian and European countries, they are rarely consumed in the Southeast Asian Subcontinent. Moreover, it is now known that carotenoids and other components of algae can have health benefits such as anti-inflammatory (Shiratori et al, 2005), anti-obesity (Maeda et al., 2005) and anti-oxidant (Sachindra et al, 2007) effects. It is therefore important to promote these natural produce that are rich but under-exploited sources of carotenoids for human consumption. Knowledge of the carotenoid profile of these vegetables, plants and algae will help to establish the beneficial effects afforded and also may help in better understanding the vitamin A activity of these food materials. Hence, the objective of this chapter was to isolate, purify and quantify carotenoids from leafy greens, vegetables and algae that are commonly consumed, underexploited as well as plants used in Indian folk medicine.

Results

Purification of carotenoids by open column chromatography

The schematic representation of the column purification for β-carotene and lutein (used for animal feeding studies) from plant materials is shown in Figure 3.1. Carotenoids were extracted from plant materials such as leafy greens, vegetables and macro algae (seaweeds) using ice-cold acetone (crude extract). The carotenoids in the crude extract were separated on a silica column by eluting with respective solvent systems described in Chapter 2. The purity of the β-carotene, lutein + zeaxanthin, neoxanthin and violaxanthin were calculated as 91 $\pm$ 3%, 94 $\pm$ 2%, 93 $\pm$ 2% and 90 $\pm$ 4% respectively (Table 3.3). Fucoxanthin was separated from the crude acetone extract obtained from brown algae and purified through the silica column. The purity of the fucoxanthin was estimated as 85 ± 3%. The column-purified fucoxanthin was further purified (96 ± 2 %) with preparative HPLC before it was used for animal feeding trials. The fucoxanthin thus obtained was used to prepare fucoxanthinol and amarouciaxanthin. Their purity was 99 and 90% respectively. The purity of the β-carotene and lutein was confirmed by TLC and compared with the respective standards and further confirmed by HPLC.

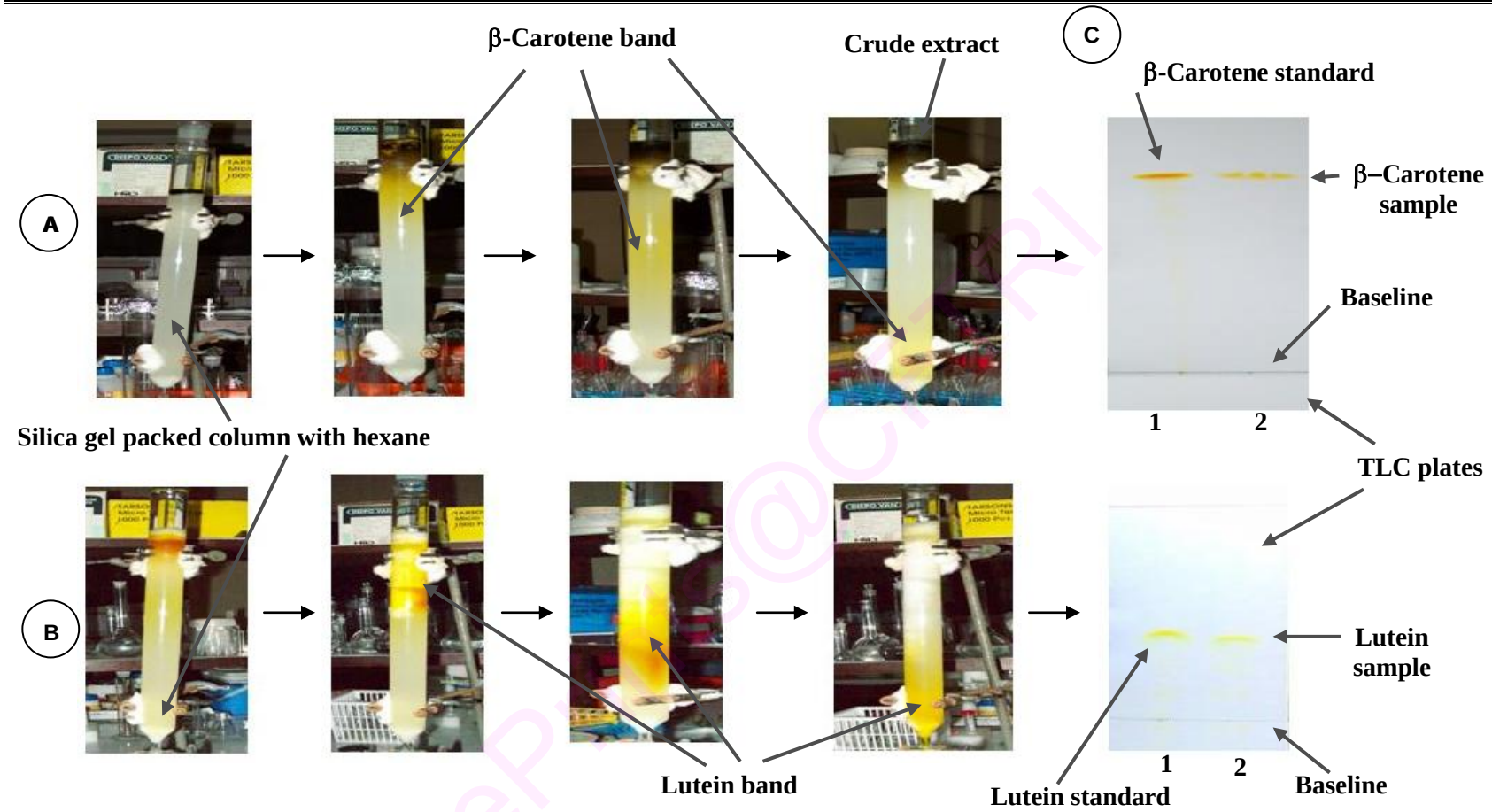


Figure 3.1. Purification of β-carotene (A) and lutein (B) from *P. sowa* by open column chromatography and confirmation by thin layer chromatography (C). Silica size= 60-120 mesh, column size = 30 cm length x 3 cm diameter, column packed in chloroform, equilibrated with hexane. Mobile phase for β-carotene = hexane, mobile phase for lutein = dichloromethane/ methanol (1:1, v/v). TLC mobile phase = hexane: acetone (70:30, v/v) for β-carotene and dichloromethane: methanol (1:1, v/v) for lutein.

Table 3.3. Purity of carotenoids eluted by open column chromatography or preparatory HPLC.

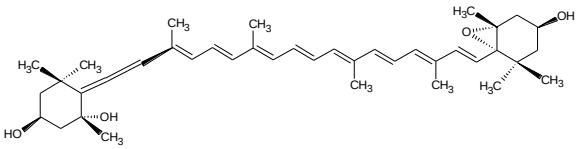
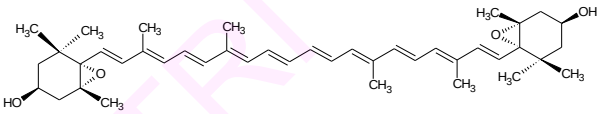
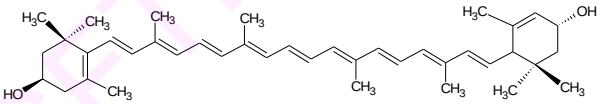
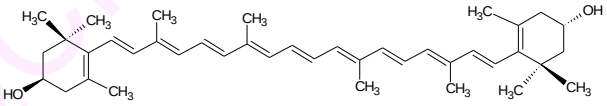
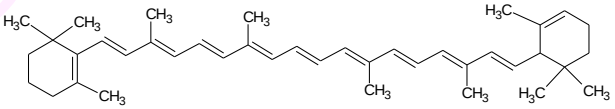
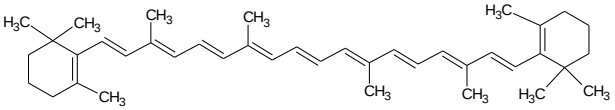
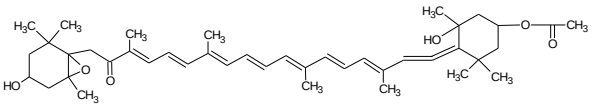
Carotenoid	Mobile phase	Purity	R_f by TLC
β-Carotene	Hexane	91 ± 3%,	0.9471
Lutein + Zeaxanthin	Dichloromethane/Methanol (1:1)	94 ± 2%	0.1545
Neoxanthin	Ethyl acetate/Hexane (5:5)	93 ± 2%	-
Violaxanthin	Ethyl acetate/Hexane (1:9)	90 ± 4%	-
Fucoxanthin*	ACN:MeOH: H_2O (67.5:13.5:19)	96 ± 2%	-
Fucoxanthinol*	ACN:MeOH: H_2O (67.5:13.5:19)	99 ± 2%	-
Amarouciaxanthin*	ACN:MeOH: H_2O (67.5:13.5:19)	90 ± 2%	-

* Purified by preparatory HPLC, ACN=Acetonitrile, MeOH=Methanol.

HPLC and LC-MS analysis of carotenoids

Under the HPLC conditions adopted (Chapter 2), carotenoids from leafy greens, vegetables and medicinal plants' extracts were well separated (Figure 3.5) and the peaks eluted in the following order: neoxanthin (peak 1), violaxanthin (peak 2), lutein (peak 3), zeaxanthin (peak 4), chlorophylls a and b (peaks 5 and 6), α-tocopherol (peak 7), α-carotene (peak 8) and β-carotene (peak 9). Fucoxanthin was detected only in marine algae. Since cis-β-carotene did not separate well, β-carotene was calculated as sum of cis-β-carotene and trans-β-carotene. Xanthophylls were eluted first (within 4.5 mins), followed by the chlorophylls (6.5-7.5 mins) and then the carotenes (17-20 mins). Carotenoids in this study were separated within 20 mins. The absorption maxima (λ_{max}) of the carotenoids eluted were 426, 454, 480 (β-carotene), 421, 448, 475 (α-carotene), 421, 447, 475 (lutein), 415, 440, 468 (neoxanthin), 425, 449, 476 (violaxanthin) and 432, 454, 480 (zeaxanthin) and 447, 470 (fucoxanthin). The chemical structures of the carotenoids and their λ_{max} in comparison with reported values are given in Table 3.4. The HPLC chromatogram and characteristic spectra of the standards are shown in Figure 3.2. The LC-MS profile of carotenoids extracted from the samples was shown in Figure 3.3.

Table 3.4. UV-Visible absorption maxima (λ_{max}) for carotenoids isolated from leafy greens, vegetables, medicinal plants and marine algae and their chemical structures.

Carotenoid	λ_{max} [a]	λ_{max} [b]	λ_{max} [c]	λ_{max} [d]	Chemical structure of the carotenoid
Neoxanthin	440	439	438-440	441	
Violaxanthin	449	443	440-442	439	
Lutein	447	445	446	-	
Zeaxanthin	454	452	-	450	
α-Carotene	448	444	448-450	-	
β-Carotene	454	453	452-454	450	
Fucoxanthin [d]	447	-	-	447	

[a]Present study; [b]Eitenmiller and Lander (1999), [c]Khachik et al. (1992), [d]Haugan and Jensen (1994)

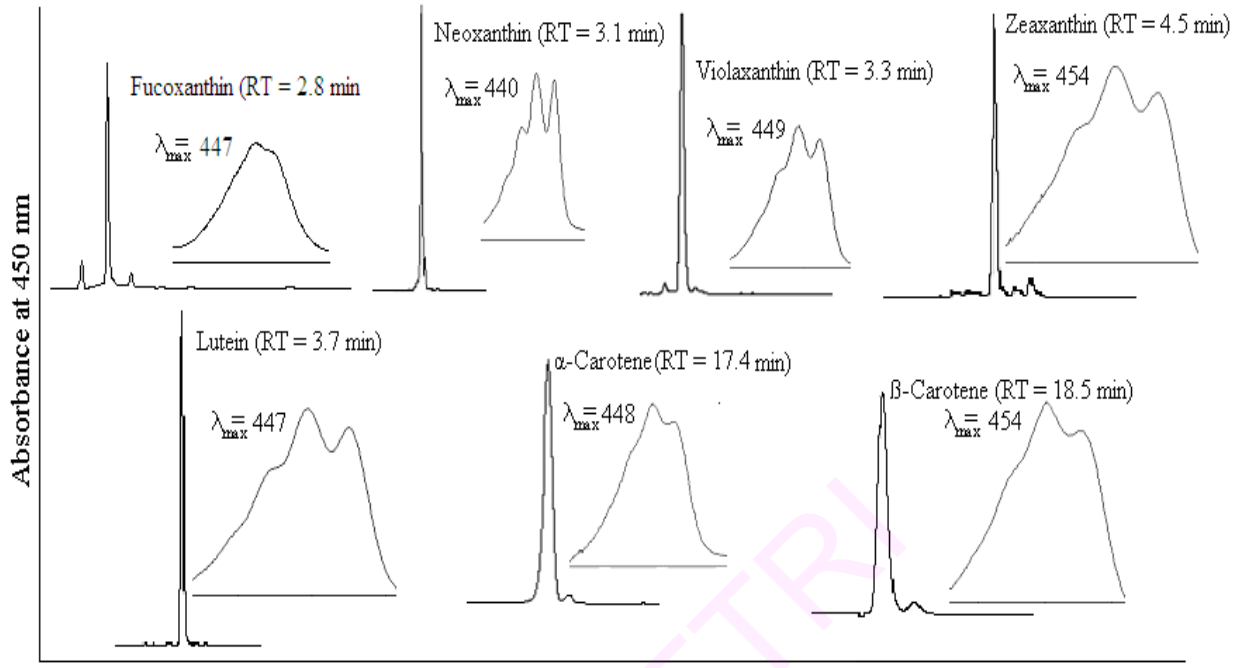


Figure 3.2. HPLC chromatogram of carotenoid standards along with their retention time (RT) and characteristic spectra with absorption maxima (λ_{max}). HPLC conditions are described in Chapter 2 in methods section.

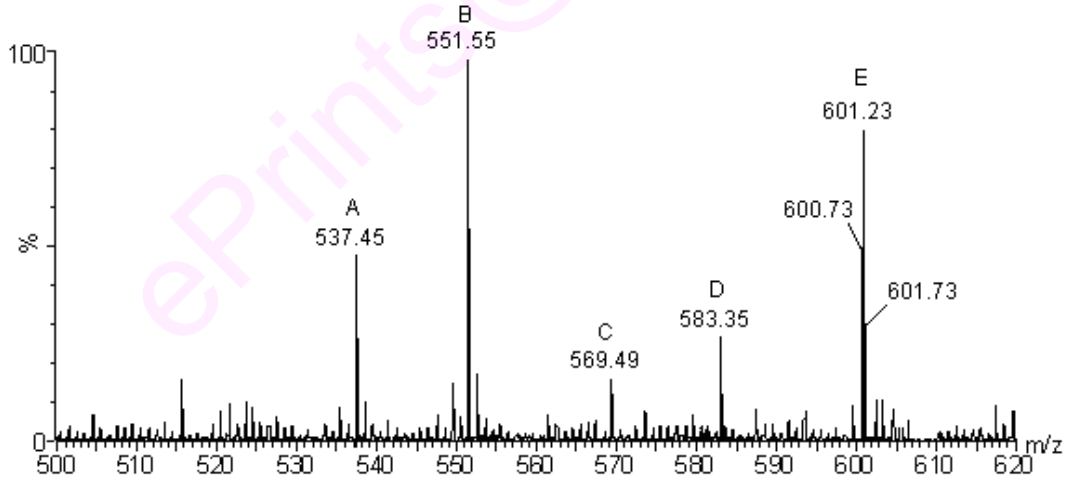


Figure 3.3. Representative LC-MS (APCI) profile of carotenoids extracted from *C. auriculata*. A = β-carotene (537 $[M+H]^+$), B = Lutein (551 $[M+H-H2O]^+$), C = Zeaxanthin (569 $[M+H]^+$), D = Neoxanthin, violaxanthin (583 $[M+H-H2O]^+$), E = Neoxanthin, violaxanthin (601 $[M+H]^+$). LC-MS conditions are described in Chapter 2, methods section.

Carotenoid composition in green leafy vegetables

Green leafy vegetables (n = 24) analyzed in this study are given in Figure 3.4 while their botanical, family, common, local names and moisture content are given in Table 3.5. The HPLC chromatograms of carotenoids obtained from the acetone extracts are given in Figure 3.5. The highest values for β-carotene were for lamb's quarters (*C. album*), prickly amaranth (*A. spinosus*), jio (*C. benghalensis*), Indian dill leaf (*P. sowa*), colocasia leaves (*C. anti-quorum*) and amaranth leaves (*Amaranthus sp.,* keerai). The highest values for lutein were found in lamb's quarters, jio, dill leaf, chilli leaves (*C. annuum*), *I. pestigridis*, prickly amaranth and colocasia leaves. The lamb's quarters, jio, dill leaf, prickly amaranth and colocasia leaves are rich sources of both β-carotene and lutein. Concentration (mg/100g dry weight) of β-carotene and lutein in green leafy vegetables ranged between 1.5 to 120.2 and 11.7 to 185.2 respectively. Neoxanthin levels ranged from 0.03 to 54.1 while violaxanthin levels were 0.03 to 140.5 (Table 3.6). α-Carotene (mg/100g dry weight) was detected in 8 leafy greens (0.3-35.6) and was highest in jio (35.6) demonstrating that among the leafy greens analyzed, jio possesses β-carotene, lutein and α-carotene.

The total carotenoids in the leafy greens and the percentage of lutein, β-carotene, α-carotene and vitamin A activity as retinol equivalent (RE) is given in Table 3.7. Total carotenoids (xanthophyll + hydrocarbon carotenoids) ranged from 0.62 to 450.93 mg/100g dry weight with lamb's quarters having higher concentration (450.9) and green cabbage (*B. oleracea var capitata*) having minimum level (0.62). Percentage of lutein in total carotenoids ranged from 34-95% while that of β-carotene and α-carotene were in the range of 2-36% and 0-18% respectively. RE values (in mg) were highest for Lamb's quarters (20) followed by jio (19), prickly amaranth (16), amaranth (keerai, 11), dill leaf (10), colocasia leaves (10) and lowest for green cabbage (0.01) and red cabbage (0.03).

Figure 3.4. Green leafy vegetables analyzed in this study for their carotenoid composition.

Table 3.5. Common, botanical, family, local names, medicinal applications and moisture content of green leafy vegetables analyzed for carotenoid composition.

Common name	Botanical name	Family name	Local name	Medicinal applications/ Health benefits[#]	Moisture Content (%)
Amaranth leaf (sirukeerai)	*Amaranthus polygonoides*	Amaranthacae	Sirukeerai	Source of vitamins, antioxidant	88.7
Amaranth leaf (keerai)	*Amaranthus sp.*	Amaranthacae	Keerai	Source of vitamins, antioxidant	86.2
Amaranth leaf (yelavare)	*Amaranthus sp.*	Amaranthacae	Yelavare	Source of vitamins, antioxidant	89.9
Amaranth prickly	*Amaranthus spinosus*	Amaranthacae	Mullu harive soppu	Sudorific, febrifuge, used for eruptive fevers, lactagogue, emollient	90.3
Cabbage (Chinese)	*Brassica chinesis*	Cruciferae	Chini kosu	Antioxidant	91.1
Cabbage (green)	*Brassica oleracea var. Capitata*	Cruciferae	Ele kosu	Antioxidant	91.9
Cabbage (red)	*Brassica oleracea var. Capitata*	Cruciferae	Kempu ele kosu	Antioxidant	92.2
Celery	*Apium graveolus var. dulce*	Umbelliferae	Ajmoda	Stimulant, carminative, sedative, anti-rheumatic	82.0
Chilli leaf	*Capsicum annuum*	Solanaceae	Mensenkai soppu	Carminative, rubefacient, anti-rheumatic	83.3
Chives	*Allium schoenoprasum*	Liliacae	Not available	Anti-cancer, beneficial for circulatory, digestive and respiratory disorders	88.9
Colocasia leaf	*Colocasia anti-quorum*	Araceae	Shamangade soppu	Astringent and styptic	83.1

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Common purslane	*Portulacae oleracae*	Portulaceae	Doddagoni soppu	Vulnerary, antiscorbutic, aperient, diuretic	92.3
Hog Weed	*Boerhavia diffusa*	Nyctaginacae	Nelabasale	Cures *asthma*	92.8
Indian Dill	*Peucedanum sowa*	Apiaceae	Sabsige	Carminative, diuretic	90.2
Jio	*Commelina benghalensis*	Commelinaceae	Kanuraka soppu	Demulcent, emollient, laxative, anti-leprosy	88.5
Knol khol greens	*Brassica oleracea var. caulorapa*	Cruciferae	Gedde kosu soppu	Rich in calcium	87.2
Lamb's quarters	*Chenopodium album*	Chenopodiaceae	Sakothina soppu	laxative, antihelminthic	91.8
Lettuce (green)	*Lactuca sativa*	Compositae	Lettuce	Hypnotic in bronchitis, asthma	94.1
Lettuce (iceberg)	*Lactuca sativa*	Asteraceae	Lettuce	Antioxidant	88.6
Lettuce (red)	*Lactuca sativa*	Compositae	Lettuce	Hypnotic in bronchitis, asthma	92.2
Parsley	*Petroselinum crispum*	Umbelliferae	Achumooda	Diuretic, ecbolic and emmenagogue	75.0
Tamarind	*Tamarindus indica*	Caesalpiniaceae	Uli soppu, Chinch	Carminative, laxative	70.9
Turnip Greens	*Brassica rapa*	Cruciferae	Turnip soppu	Calcium and vitamin C source, stomachic	82.2
- (*I.pestigridis)*	*Ipimoea pes-tigridis*	Convolvulaceae	Punaikkirai	Cures boils, sores, pimples and carbuncles	74.2

[#] National Institute of Science Communication, CSIR (1986) unless otherwise mentioned

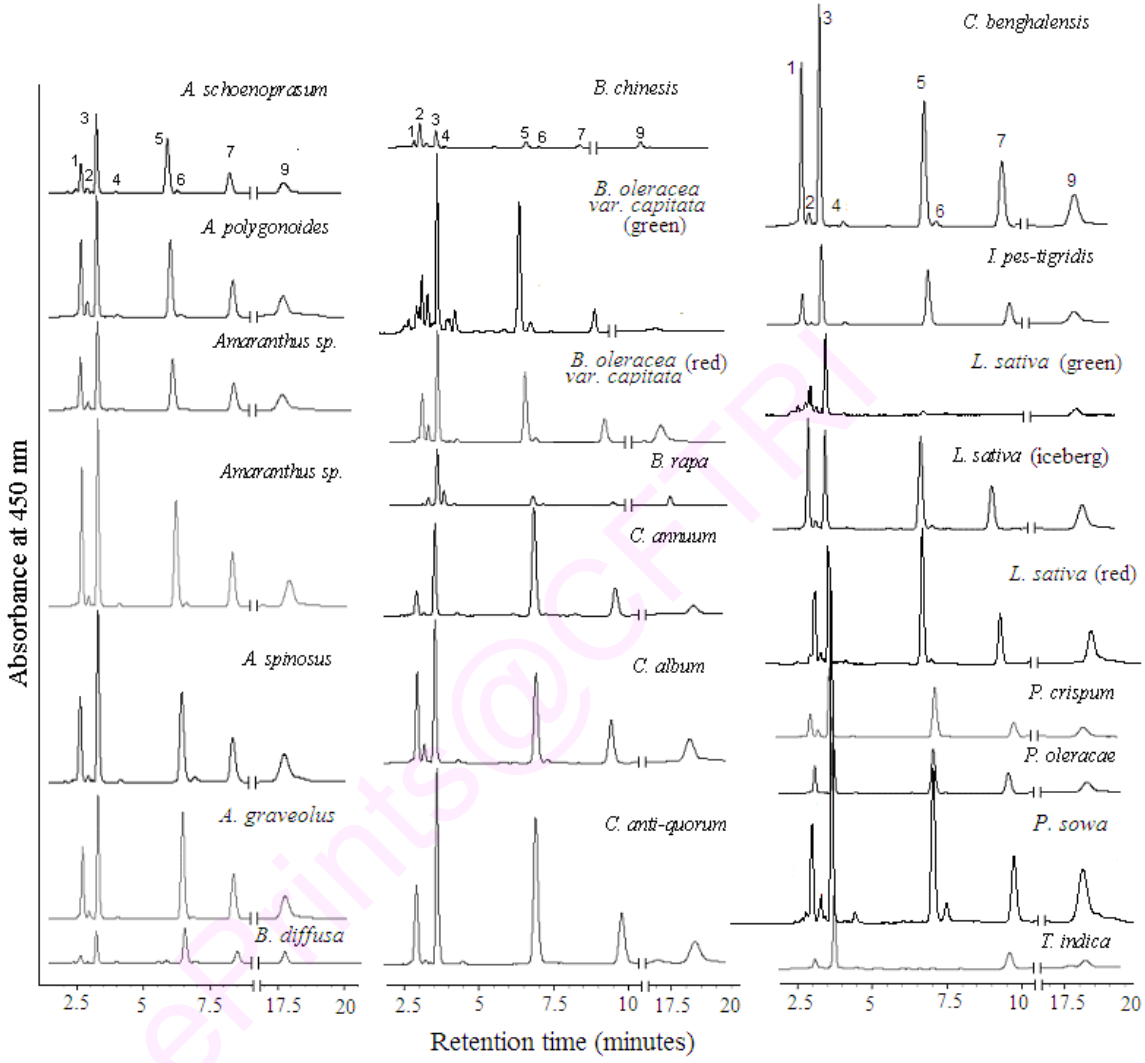

Figure 3.5. HPLC profile of carotenoids extracted from leafy greens. 1 = neoxanthin, 2 = violaxanthin, 3 = Lutein, 4 = zeaxanthin, 5 = chlorophyll b, 6 = chlorophyll a, 7 = α-tocopherol, 8 = α-carotene, 9 = β-carotene. HPLC conditions are described in Chapter 2, methods section.

Table 3.6. Carotenoid composition (mg/100g dry weight) of green leafy vegetables[a].

Green leafy vegetable	Xanthophylls				Total Xanthophylls[b]	Provitamin-A carotenoids		Total provitamin-A carotenoids[c]
	Neoxanthin	Violaxanthin	Lutein	Zeaxanthin		α-Carotene	β-Carotene	
Amaranth leaves	12.51	2.41	25.73	1.19	41.84	ND	20.61	20.61
Amaranth leaves	38.08	4.92	71.92	1.30	116.21	1.03	66.39	67.42
Amaranth leaves	16.42	2.74	29.19	0.95	49.30	1.38	23.20	24.57
Amaranth Prickly	54.07	3.27	116.86	2.78	176.98	ND	97.88	97.88
Celery	30.79	2.87	56.52	1.45	91.63	ND	49.93	49.93
Chilli leaf	2.73	0.27	136.24	0.39	139.63	0.31	3.48	3.79
Cabbage, Chinese	0.69	3.07	22.42	0.63	26.24	0.00	1.5	1.5
Cabbage, green	0.09	0.06	0.37	0.06	0.58	ND	0.04	0.04
Cabbage, red	0.30	ND	1.34	ND	1.64	0.36	ND	0.36
Chives	23.53	3.15	73.40	44.40	144.47	ND	48.14	48.14
Colocasia Leaves	44.14	1.33	104.68	2.43	152.58	6.70	57.28	63.98
Common purslane	2.37	0.09	36.19	0.20	38.84	ND	4.20	4.20
Hog Weed	1.64	0.16	54.13	0.32	56.24	0.31	2.43	2.75
Indian Dill	13.5	7.3	170.1	3.1	194.0	ND	61.5	61.5
Jio	0.03	100.30	175.60	2.06	277.99	35.60	95.70	131.30
Knol khol greens	18.50	4.73	42.97	1.57	67.76	0.67	27.11	27.78

Contd.

Green leafy vegetable	Xanthophylls				Total Xanthophylls[b]	Provitamin-A carotenoids		Total provitamin-A carotenoids[c]
	Neoxanthin	Violaxanthin	Lutein	Zeaxanthin		α-Carotene	β-Carotene	
Lamb's Quarters	0.03	140.50	185.20	5.00	330.73	ND	120.20	120.20
Lettuce, green	1.39	0.19	2.40	0.05	4.03	ND	1.57	1.57
Lettuce iceberg	2.53	0.15	26.48	0.04	29.19	ND	2.87	2.87
Lettuce, red	2.19	0.17	2.58	0.05	4.99	ND	2.48	2.48
Parsley	10.40	2.56	29.71	0.81	43.48	ND	18.43	18.43
Tamarind	2.43	0.03	16.40	0.55	19.41	0.76	4.77	5.53
Turnip Greens	0.54	5.41	11.73	1.54	19.21	ND	8.98	8.98
- (*I.pestigridis)*	2.71	0.13	134.78	0.31	137.93	ND	4.31	4.31

[a]Values are mean of duplicate analysis; [b]Total xanthophylls = neoxanthin + violaxanthin + lutein + zeaxanthin ; [c]Total provitamin-A carotenoids = α-carotene + β-carotene, ND = Not Detected.

Table 3.7. Total carotenoids (TC), percent lutein, β-carotene and α-carotene in TC and vitamin A activity (RE) of α- and β-carotene in green leafy vegetables.

Leafy vegetable	TC[*1]	% Lutein in TC	% α-Carotene in TC	% β-Carotene in TC	RE[2]
Amaranth leaves	62.45	41.20	0.00	33.00	3.43
Amaranth leaves	183.64	39.16	0.56	36.15	11.15
Amaranth leaves	73.87	39.51	1.86	31.40	3.98
Amaranth prickly	274.86	42.52	0.00	35.61	16.13
Celery	141.56	39.92	0.00	35.27	8.32
Chilli leaf	143.42	94.99	0.21	2.43	0.61
Chinese cabbage	27.75	80.80	0.00	5.40	0.25
Cabbage, green	0.62	61.67	0.00	6.67	0.01
Cabbage, red	2.00	67.00	18.00	0.00	0.03
Chives	192.62	38.11	0.00	24.99	8.02
Colocasia leaves	216.56	48.34	3.09	26.45	10.11
Common purslane	43.04	84.09	0.00	9.75	0.70
Hog weed	58.99	91.75	0.53	4.13	0.43
Indian dill	255.50	66.57	0.00	24.07	10.25
Jio	409.29	42.90	8.70	23.38	18.92
Knol khol greens	95.54	44.97	0.70	28.37	4.57
Lamb's quarters	450.93	41.07	0.00	26.66	20.03
Lettuce green	5.60	42.86	0.00	28.04	0.26
Lettuce iceberg	32.07	82.58	0.00	8.95	0.48
Lettuce red	7.47	34.40	0.00	33.07	0.41
Parsley	61.91	47.99	0.00	29.77	3.07
Tamarind	24.94	65.74	3.03	19.14	0.86
Turnip greens	28.19	41.59	0.00	31.85	1.50
I.pestigridis	142.24	94.76	0.00	3.03	0.72

Values are mean of duplicate analysis, [*]mg/100g dry weight, [1]TC = Total xanthophylls + provitamin A carotenoids, [2]RE (retinol equivalent) = 1 RE= 6mg β-carotene or 12 mg α-carotene.

Carotenoid composition in medicinal plants

Medicinal plants (n = 33) analyzed in this study for carotenoid composition are given in Figure 3.6 and their botanical, family, local names and moisture content are given in Table 3.8. The HPLC elution profiles of carotenoids in medicinal plants were almost similar to that of green leafy vegetables and are shown in Figure 3.7 while their composition is given in Table 3.9. The highest levels of β-carotene (mg/100 g dry weight) were recorded in butterfly pea (*C. ternatea*), thyme leaved gratiola (*B. monnieri*) and holy basil (*O. sanctum*). The highest values for lutein content were found in butterfly pea, conch grass (*C. dactylon*) and hoary basil (*O. canum*). Thus, butterfly pea is the richest source of both β-carotene and lutein among the medicinal plants analyzed. Concentration (mg/100g dry weight) of β-carotene and lutein in medicinal plants ranged between 0.4 to 34.7 and 11.8 to 679 respectively. Neoxanthin levels ranged from 1.8 to 18.5 while violaxanthin levels were in the range of 0.1 to 6.4. α-Carotene mg/100g dry weight) was detected in 25 medicinal plants (0.1-15.7) and was highest in Indian borage (*C.aromaticus*, 15.73).

The total carotenoids in the leaves of medicinal plants and the percentage of lutein, β-carotene, α-carotene and vitamin A activity as retinol equivalent (RE) are given in Table 3.10. Total carotenoids (xanthophyll + hydrocarbon carotenoids) ranged from 23 to 731 mg/100g dry weight with butterfly pea having maximum concentration (730.8) and margosa (*Azadirachta indica*) having minimum concentration (23.2). Percentage of lutein in total carotenoids ranged from 50-97% while that of β-carotene and α-carotene ranged from 0.7-33% and 0-30% respectively. RE values (in mg) were highest for thyme-leaved gratiola (5.7) followed by butterfly pea (5.3) and holy basil (5.3) and lowest for ginger leaf (*Z. officianale*, 0.4), bitter gourd leaf (*M. charantia*, 0.5) and Chinese lantern (*P. alkekengi*, 0.7).

Figure 3.6. Medicinal plants analyzed in this study for carotenoid composition.

Table 3.8. Common, botanical, family, local names, medicinal uses and moisture content of medicinal plants screened for carotenoids.

Common name	Botanical name	Family name	Local name	Medicinal applications/Health benefits[#]	Moisture Content (%)
Acalypha	*Acalypha indica*	Euphorbiaceae	Kuppigida	Laxative, emetic, cures cough	71.2
Allheal	*Prunella vulgaris*	Labiatae	NA	Astringent, cures sore throat	79.0
Aloe	*Aloe vera*	Liliaceae	Kathalae	Cathartic, refrigerant, cures liver spleen, eye ailments, skin disorders	98.8
Bel	*Aegle marmelos Correa*	Rutaceae	Bilpatre	Antidiabetic*, astringent, digestive, stomachic	60.7
Bindweed	*Convolvulus sepium*	Convolvulaceae	NA	Laxative, gall bladder problems.	85.0
Bittergourd leaf	*Momordica charantia*	Cucurbitaceae	Hagalekai soppu	Antidiabetic*, stomachic, cures cold, headache, menstrual cramps, insect bites	78.2
Bittersweet Nightshade	*Solanum dulcamara*	Solanaceae	Blood berry	Treatment for tumours, warts, chronic rheumatism, skin afflictions	73.8
Butterfly pea	*Clitoria ternatea*	Papilionaceae	Shankha-pushpa	Catharrtic, diuretic	83.0
Castor	*Ricinus communis*	Euphorbiaceae	Haralu	Poultice applied to boils and sores	76.9
Chinese lantern	*Physalis alkekengi*	Solanaceae	NA	Diuretic, hydragogue, febrifuge, vermifuge	87.1
Conch/dog grass	*Cynodon dactylon*	Poaceae	Garikaihallu	Diuretic, used for anasarca	55.1
Country fig	*Ficus glomerata*	Moraceae	Athi soppu	Used in bilious infections	76.0
Gigantic swallow wort	*Calotropis gigantean*	Asclepiadaceae	Ekka	Treatment of kesarayer disease	91.4
Ginger leaf	*Zingiber officianale*	Zingiberaceae	Shunti elai	Carminative, stimulant, cures flatulence, colic	80.1
Henna	*Lawsonia inermis.*	Lythraceae	Goranti	Prophylactic against skin problems	64.3
Hoary Basil	*Ocimum canum*	Labiatae	Vishnu tulsi	Diuretic	74.2
Holy Basil	*Ocimum sanctum*	Labiatae	Krishna tulsi	Antidiabetic*, Stimulant, stomachic, diaphoretic, antiperiodic, cures bronchitis	68.0

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Indian birthwort	*Aristolochia indica*	Aristolochiaceae	Ishwari beru	Used for cough	82.0
Indian Borage	*Coleus aromaticus*	Labiatae	Dodpatra	Cures urinary diseases, chronic cough, asthma	80.0
Indian pennywort	*Centella asiatica*	Umbelliferae	Vondelaga	Diuretic, anti-leprosy	80.8
Indian privet	*Vitex negundo*	Verbenaceae	Lakkigidda	Vermifuge, anti-rheumatic, anticancer, tranquilizer	69.3
Jimson weed	*Datura stramonium*	Solanaceae	Dattura, umathe	Narcotic, antispasmodic, mydriatic, anodyne, relieves asthma	77.9
Lemon balm	*Melissa officinalis*	Labiatae	NA	Headache and toothache	88.0
Lemongrass	*Cymbopogon citratus*	Gramineae	Purvali hullu	Carminative	61.6
Margosa, Neem	*Azadirachta indica*	Meliaceae	Bevu	Antidiabetic*, antiseptic, cures ulcers, eczema	60.0
Mexican daisy	*Tridax procumbens*	Compostitae	Gabbu sanna savanthi	Used in bronchial catarrh, dysentery, diarrhoea, hemorrhage of wounds	65.0
Sage	*Salvia officinalis*	Labiatae	NA	Diaphoretic, carminative, relieves sore throat	63.2
Tanner's Cassia	*Cassia auriculata*	Caesalpiniaceae	Aavarai	Antihelmenthic	61.8
Thyme leaved gratiola	*Bacopa monnieri*	Scrophularia-ceae	Nirubrahmi	Diuretic and aperient. Beneficial for epilepsy, insanity, nervous diseases.	84.0
Turmeric leaf	*Curcuma domestica (longa)*	Zingiberaceae	Harishina ele	Stimulant, tonic, stomachic, depurative	81.0
Wild brinjal	*Solanum surattense*	Solanaceae	Nella gula	Carminative, antiviral, beneficial for rheumatism, dengue fever, bronchitis, fever	66.6
- *(L. aspera)*	*Leucas aspera*	Labiatae	Thumbe	Antipyretic, cures psoriasis, chronic skin erruptions	71.6
- *(P. zeylanica)*		Plumbaginecae	Chitramula	Vesicant, diuretic	69.0

NA = Not available, [#] National Institute of Science Communication, CSIR (1986) unless otherwise mentioned, Grover et al. (2002).

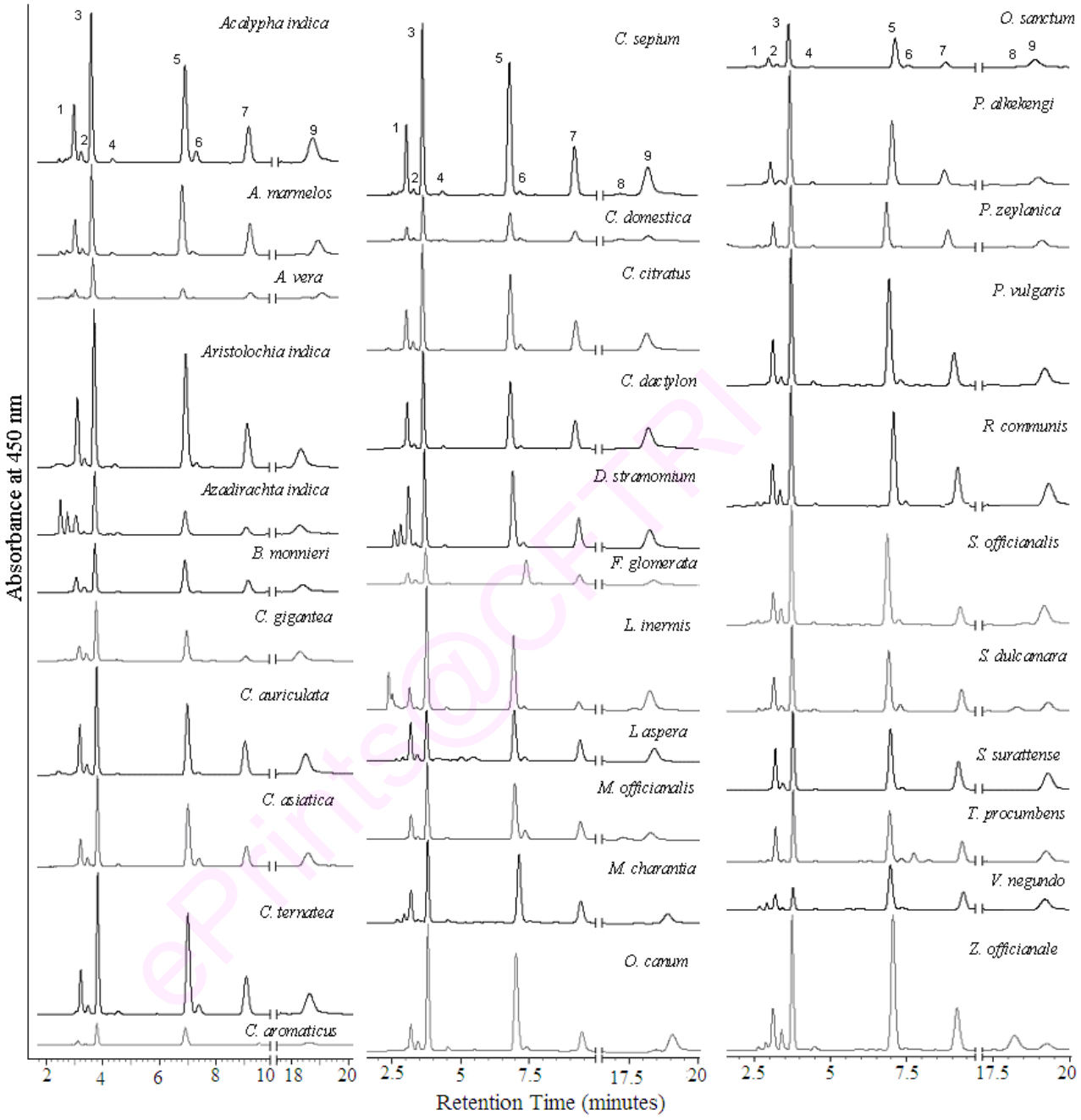


Figure 3.7. HPLC profile of carotenoids extracted from medicinal plants. 1 = neoxanthin, 2 = violaxanthin, 3 = Lutein, 4 = zeaxanthin, 5 = chlorophyll b, 6 = chlorophyll a, 7 = α-tocopherol, 8 = α-carotene, 9 = β-carotene. HPLC conditions are described in Chapter 2, methods section.

Table 3.9. Carotenoid composition (mg/100g dry weight) of medicinal plants[a].

Medicinal Plant	Xanthophylls				Total	Provitamin A carotenoids		Total provitamin-A
	Neoxanthin	Violaxanthin	Lutein	Zeaxanthin	Xanthophylls[b]	α-Carotene	β-Carotene	carotenoids[c]
Acalypha	7.57	1.28	419.80	0.75	429.40	0.33	14.31	14.64
Allheal	5.61	0.92	349.28	0.72	356.53	0.19	8.67	8.86
Aloe	2.27	0.15	14.85	0.39	17.67	0.87	6.59	7.46
Bel	2.89	0.42	193.17	0.27	196.75	0.60	5.28	5.88
Bindweed	11.83	0.92	394.78	1.07	408.60	0.86	20.22	21.08
Bittergourd leaf	2.15	0.10	108.49	0.23	110.98	0.09	3.03	3.13
Bittersweet Nightshade	6.27	0.90	325.37	0.59	333.12	3.11	7.18	10.29
Butterfly pea	15.33	2.39	678.99	1.44	698.15	1.50	31.16	32.66
Castor	7.84	2.46	431.81	0.61	442.73	ND	20.83	20.83
Chinese lantern	3.49	0.68	184.65	0.48	189.29	ND	4.47	4.47
Conch/dog grass	8.79	0.69	484.08	0.70	494.26	0.22	16.48	16.70
Country fig	3.85	0.21	184.94	0.25	189.25	4.42	3.27	7.69
Gigantic swallow wort	4.47	1.75	134.66	0.48	141.36	0.57	11.16	11.73
Ginger leaf	1.84	0.75	100.98	0.22	103.79	2.59	1.21	3.80
Henna	2.52	0.11	360.67	0.38	363.68	0.67	8.93	9.60

Contd.

Hoary Basil	4.66	1.29	458.30	0.92	465.18	0.87	12.72	13.59
Holy Basil	11.36	2.75	48.22	1.58	63.91	0.72	31.23	31.96
Indian birthwort	11.05	1.19	391.53	0.79	404.57	0.69	14.23	14.91
Indian Borage	4.85	1.08	28.64	1.01	35.59	15.73	0.35	16.08
Indian pennywort	4.83	1.22	234.88	0.55	241.48	ND	8.88	8.88
Indian privet	3.00	0.27	100.55	0.16	103.98	ND	9.33	9.33
Jimson weed	2.83	9.70	307.07	0.62	320.23	0.16	13.79	13.95
Lemon balm	6.81	0.46	225.18	0.64	233.08	3.32	9.44	12.75
Lemongrass	4.55	0.76	291.98	0.26	297.55	ND	7.85	7.85
Margosa,Neem	3.33	0.40	11.75	0.48	15.96	0.27	6.93	7.19
Mexican daisy	5.48	0.26	284.95	0.26	290.94	ND	8.72	8.72
Sage	3.18	1.34	300.46	0.35	305.33	0.43	7.85	8.28
Tanner's Cassia	3.54	0.63	195.84	0.19	200.20	0.05	6.96	7.01
Thyme leaved gratiola	18.47	6.37	54.69	1.52	81.05	ND	34.70	34.70
Turmeric leaf	5.29	0.96	292.88	0.77	299.91	3.06	7.64	10.70
Wild brinjal	5.58	0.87	260.48	0.27	267.20	ND	11.56	11.56
- (*L. aspera*)	4.31	0.68	134.41	0.12	139.52	0.07	8.11	8.17
- (*P. zeylanica*)	4.46	0.14	244.53	0.46	249.60	0.79	5.38	6.17

[a]Values are mean of duplicate analysis, [b]Total xanthophylls = neoxanthin + violaxanthin + lutein + zeaxanthin,

[c]Total provitamin-A carotenoids = α-carotene + β-carotene. ND = Not Detected

Table 3.10. Total carotenoids (TC), percent lutein and β-carotene in TC and vitamin A activity (RE) of α-carotene and β-carotene in medicinal plants.

Medicinal Plant	TC[*1]	% Lutein in TC	% α-Carotene in TC	% β-Carotene in TC	RE[2]
Acalypha	444.04	94.54	0.08	3.22	2.41
Allheal	365.39	95.59	0.05	2.37	1.46
Aloe	25.13	59.10	3.45	26.23	1.17
Bel	202.63	95.33	0.29	2.61	0.93
Bindweed	429.67	91.88	0.20	4.70	3.44
Bittergourd leaf	114.11	95.08	0.08	2.66	0.51
Bittersweet Nightshade	343.41	94.75	0.91	2.09	1.46
Butterfly pea	730.81	92.91	0.21	4.26	5.32
Castor	463.56	93.15	0.00	4.49	3.47
Chinese lantern	193.76	95.30	0.00	2.31	0.74
Conch/dog grass	510.96	94.74	0.04	3.22	2.77
Country fig	196.95	93.90	2.25	1.66	0.91
Gigantic swallow wort	153.09	87.97	0.37	7.29	1.91
Ginger leaf	107.59	93.85	2.41	1.12	0.42
Henna	373.28	96.62	0.18	2.39	1.54
Hoary Basil	478.77	95.72	0.18	2.66	2.19
Holy Basil	95.87	50.30	0.75	32.58	5.27
Indian birthwort	419.48	93.34	0.16	3.39	2.43
Indian Borage	51.66	55.44	30.44	0.67	1.37
Indian pennywort	250.36	93.82	0.00	3.55	1.48
Indian privet	113.31	88.74	0.00	8.24	1.56
Jimson weed	334.18	91.89	0.05	4.13	2.31
Lemon balm	245.84	91.60	1.35	3.84	1.85

Contd.

Lemongrass	305.40	95.60	0.00	2.57	1.31
Margosa,Neem	23.16	50.73	1.15	29.92	1.18
Mexican daisy	299.67	95.09	0.00	2.91	1.45
Sage	313.62	95.81	0.14	2.50	1.35
Tanner's Cassia	207.21	94.51	0.02	3.36	1.17
Thyme leaved gratiola	115.75	47.25	0.00	29.98	5.78
Turmeric leaf	310.61	94.29	0.99	2.46	1.53
Wild brinjal	278.76	93.44	0.00	4.15	1.93
- (*L. aspera*)	147.70	91.00	0.04	5.49	1.36
- (*P. zeylanica*)	255.77	95.61	0.31	2.10	0.96

Values are mean of duplicate analysis, [*]mg/100g dry weight, [1]TC = Total xanthophylls + provitamin A carotenoids, [2]RE (retinol equivalent) = 1 RE= 6mg β-carotene or 12 mg α-carotene

Carotenoid composition in vegetables

Vegetables (n = 25) analyzed in this study are shown in Figure 3.8 and their botanical, family, common, local names and moisture content are given in Table 3. 11. The HPLC elution profile of the carotenoids was almost similar to those of green leafy vegetables and medicinal plants. The HPLC chromatograms of carotenoids obtained from the extracts of vegetables are given in Figure 3.9A, B and C. β-Carotene and α-carotene were the predominant provitamin-A carotenoids detected in vegetables except for β-cryptoxanthin, a provitamin-A xanthophyll that was detected only in bitter orange (*C. aurantium*). The carotenoid composition of the vegetables is given in Table 3.12. In general, kenaf (*H. cannabius*), red/green lettuce (*L. sativa*), carrot (*D. carota*) and yellow zuchchini (*C. pepo*) were found to be better sources (mg/100 g dry weight) of β-carotene (> 1 mg/100g dry weight) while carrot, yellow zuchchini, tomato (*L. esculentum*), and kenaf contain appreciable amounts of α-carotene (> 1). Yellow zuchchini, kenaf and carrot contain higher levels (mg/100g dry weight) of lutein (>10). Kenaf contained highest levels of zeaxanthin (> 0.1). Neoxanthin and violaxanthin were highest in yellow zucchini (29.9, 8.7). As mentioned earlier, β-cryptoxanthin was detected only in bitter orange while lycopene was

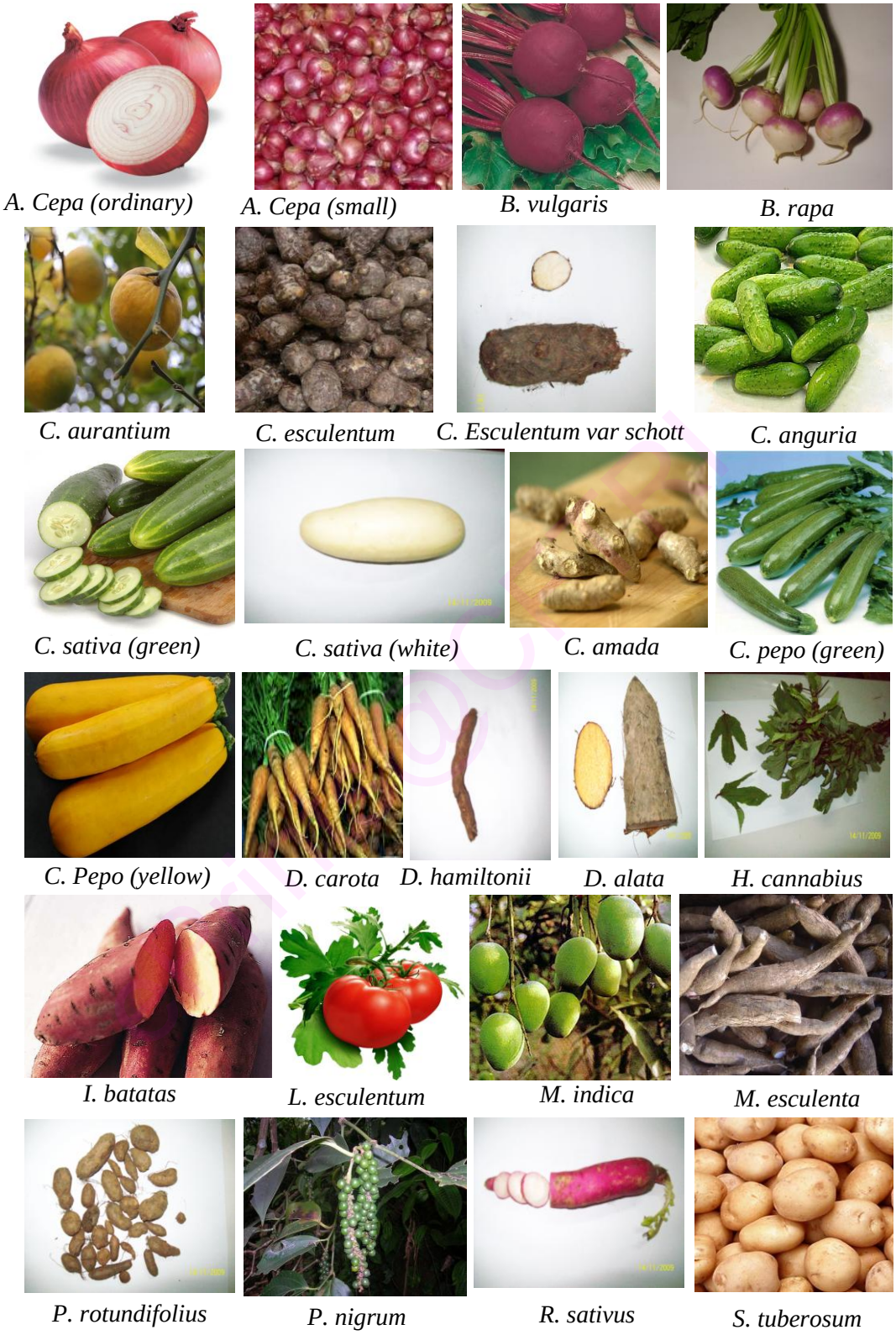


Figure 3.8. Vegetables analyzed in this study for their carotenoid composition.

Table 3.11. Common, botanical, family, local names and moisture content of vegetables screened for carotenoids.

Common name	Botanical name	Family name	Local name	Moisture content (%)
Beetroot	*Beta vulgaris*	Chenopodiacae	Beetroot	87.7
Bitter orange	*Citrus aurantium*	Rutaceae	Irlekai	93.0
Carrot	*Daucus carota var sativa*	Umbelliferae	Carrot	85.9
Colocasia	*Colocasia esculenta*	Araceae	Shamane gadde	77.9
Colocasia white	*Colocasia esculenta var. schott*	Araceae	Chembu	74.5
Cucumber green	*Cucumis sativa*	Cucurbitacae	Southekai	98.1
Cucumber white	*Cucumis sativa*	Cucurbitacae	Southekai	95.5
Decalepis	*Decalepis hamiltonii*	Asclepiadaceae	Magadiberu	78.8
Gerkhin	*Cucumis anguria var. anguria*	Cucurbitacae	Southekai	95.0
Greater yam	*Dioscorea alata*	Papilionaceae	Huttari genusu	66.9
Green pepper	*Piper nigrum*	Piperaceae	Hasi menasu	71.9
Kenaf	*Hibiscus cannabius*	Malvaceae	Pundi	86
Mango ginger	*Curcuma amada*	Zingiberaceae	Mavinahasi shunti	88.3
Mango raw	*Mangifera indica*	Anacardiaceae	Mavinakai	85.2
Onion, ordinary	*Allium cepa*	Liliaceae	Iruli	91.3
Onion, small	*Allium cepa*	Liliaceae	Sambar iruli	84.6
Plectranthus	*Plectranthus rotundifolius*	Labiatae	Sambrani gedde	74.0
Potato	*Solanum tuberosum*	Solanaceae	Alu gedde	79.7
Radish, red	*Raphanus sativus*	Cruciferae	Mullangi	94.1
Sweet potato	*Ipomoea batatas*	Convolvulaceae	Genasu	69.9
Tapioca	*Manihot esculenta*	Euphorbiaceae	Maragenasu	59.8
Tomato	*Lycopersicon esculentum*	Solanaceae	Tomato	95.1
Turnip	*Brassica rapa*	Cruciferae	Turnip	92.6
Zucchini, green	*Cucurbita pepo*	Cucurbitacae	Zucchini	94.5
Zucchini, yellow	*Cucurbita pepo*	Cucurbitacae	Zucchini	95.3

detected only in tomato (Figure 3.9). The total carotenoids, percentage of lutein, β-carotene, α-carotene and vitamin A activity as retinol equivalent (RE) of the vegetables is given in Table 3.13. Total carotenoids (xanthophyll + hydrocarbon carotenoids) ranged from 0.3 to 260 mg/100g dry weight with yellow zucchini having higher level (260.2) and turnip (*B. rapa*) having minimum level (23.2). Percentage of lutein in total carotenoids ranged from 0-96% while that of β-carotene and α-carotene ranged from 0-100% and 0-69% respectively. RE values (in mg) were highest for carrot (26.8) followed by kenaf (8.6), yellow zucchini (2.0) and tomato (1.5) and lowest (<0.01) for beetroot (*B. vulgaris*), white colocasia (*C. esculanta var. schott*), gerkhin (*C. anguria*), onion (*A. cepa*) and turnip.

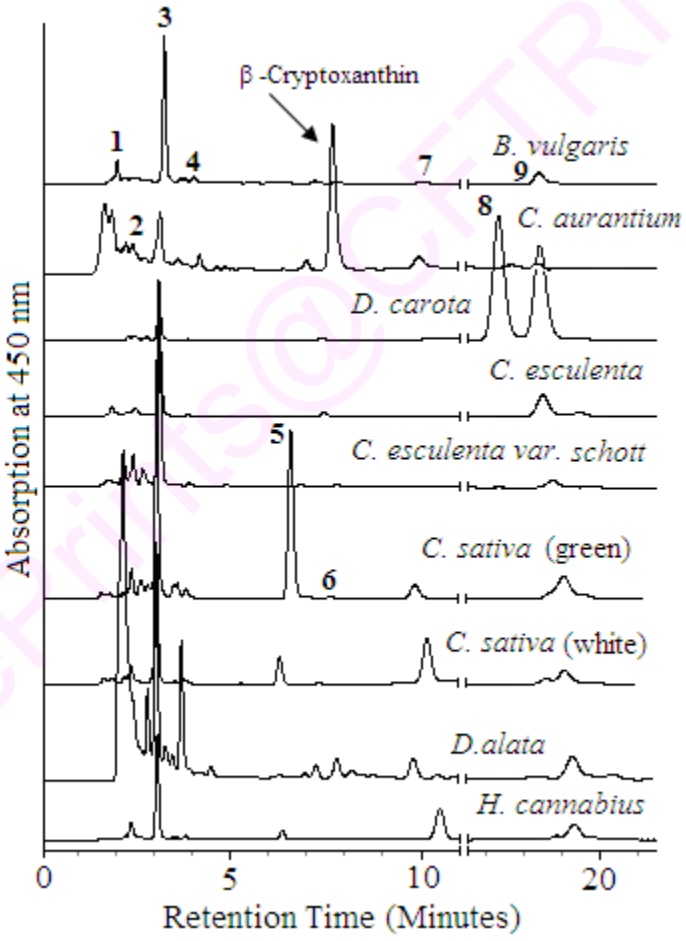


Figure 3.9A. HPLC profile of carotenoids extracted from vegetables. 1=neoxanthin, 2=violaxanthin, 3=lutein, 4=zeaxanthin, 5=chlorophyll b, 6=chlorophyll a, 7=α-tocopherol, 8=α-carotene, 9= β-carotene. HPLC conditions are described in Chapter 2, methods section.

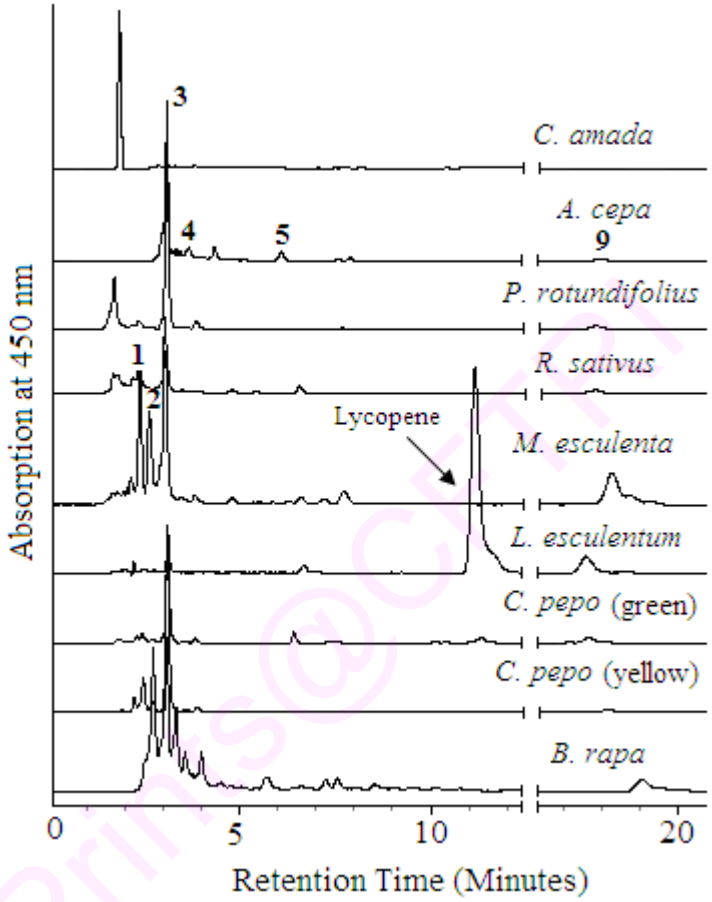


Figure 3.9B. HPLC profile of carotenoids extracted from vegetables. 1=neoxanthin, 2=violaxanthin, 3=Lutein, 4=zeaxanthin, 5=chlorophyll b, 6=chlorophyll a, 7=α-tocopherol, 8=α-carotene, 9= β-carotene. HPLC conditions are described in Chapter 2, methods section.

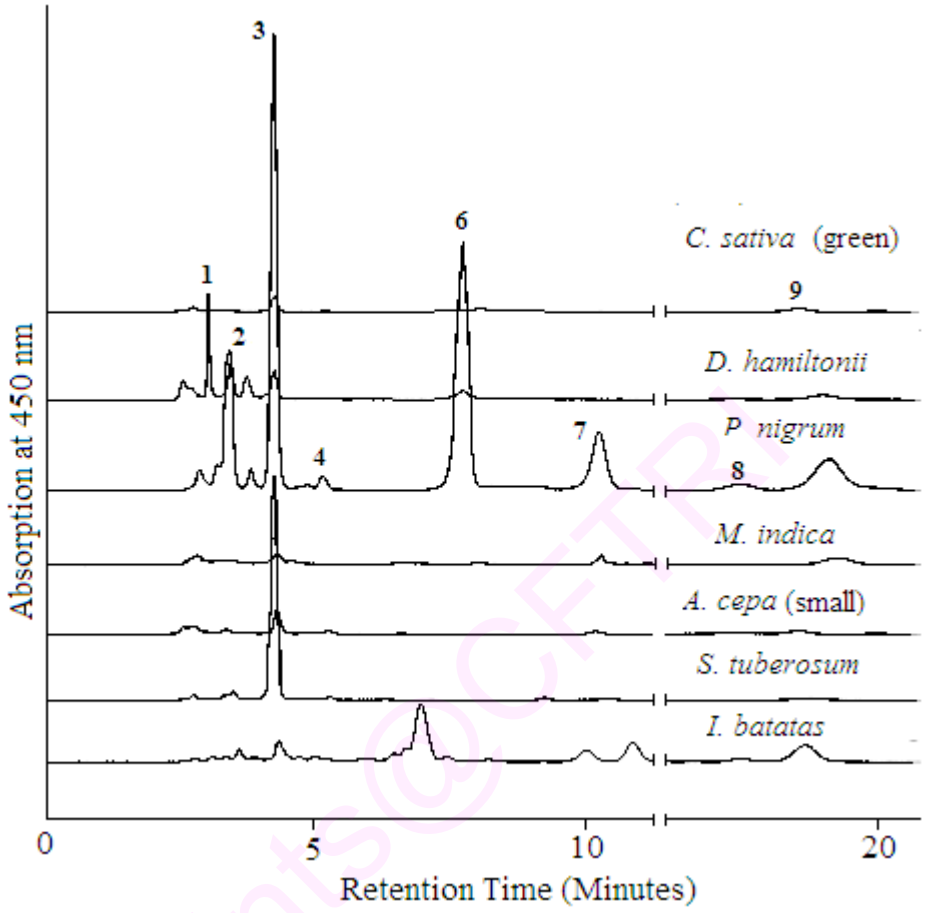


Figure 3.9C. HPLC profile of carotenoids extracted from vegetables. 1=neoxanthin, 2=violaxanthin, 3=Lutein, 4=zeaxanthin, 5=chlorophyll b, 6=chlorophyll a, 7=α-tocopherol, 8=α-carotene, 9= β-carotene. HPLC conditions are described in Chapter 2, methods section.

Table 3.12. Carotenoid composition (mg/100g dry weight) in vegetables[a].

Vegetable	Xanthophylls				Total Xanthophylls[b]	Provitamin A carotenoids		Total provitamin-A carotenoids[c]
	Neoxanthin	Violaxanthin	Lutein	Zeaxanthin		α-Carotene	β-Carotene	
Beetroot	ND	ND	0.31	0.02	0.33	ND	0.02	0.02
Bitter orange	<0.01	ND	0.02	<0.01	0.03	<0.01	0.01	0.02
Carrot	ND	ND	14.46	ND	14.46	110.41	50.31	160.72
Colocasia	<0.01	ND	0.08	<0.01	0.09	ND	0.08	0.08
Colocasia (white)	0.01	<0.01	0.04	<0.01	0.06	<0.01	0.01	0.02
Cucumber (green)	ND	0.29	1.74	<0.01	2.04	ND	0.87	0.87
Cucumber (white)	0.03	0.02	0.31	0.01	0.37	<0.01	0.12	0.13
Decalpis	0.09	0.07	0.08	<0.01	0.25	0.01	0.05	0.06
Gerkhin	0.01	ND	0.08	<0.01	0.10	<0.01	0.02	0.03
Greater yam	0.40	0.03	0.30	0.03	0.76	ND	0.26	0.26
Green pepper	0.01	0.13	0.51	<0.01	0.66	0.04	0.23	0.27
Kenaf	11.43	0.84	104.24	4.59	121.10	3.33	48.21	51.54

Contd.

Mango ginger	ND	ND	ND	ND	0.00	ND	0.08	0.08
Mango (raw)	0.01	0.01	0.08	<0.01	0.11	ND	0.13	0.13
Onion (ordinary)	0.03	0.02	0.02	ND	0.07	ND	0.01	0.01
Onion (small)	ND	0.03	0.20	<0.01	0.24	ND	0.13	0.13
Plectranthus	0.04	ND	1.48	0.07	1.59	0.08	0.01	0.09
Potato	0.01	0.03	0.38	<0.01	0.43	ND	0.04	0.04
Radish (red)	0.01	ND	0.05	<0.01	0.62	ND	0.01	0.01
Sweet potato	<0.01	0.01	0.02	<0.01	0.04	ND	0.10	0.10
Tapioca	0.01	ND	0.06	<0.01	0.08	ND	0.03	0.03
Tomato	ND	ND	3.89	ND	3.89	8.80	ND	8.80
Turnip	0.01	<0.01	0.01	ND	0.03	ND	0.01	0.01
Zucchini (green)	0.01	0.01	3.27	0.02	3.31	<0.01	0.05	0.06
Zucchini (yellow)	29.87	8.66	209.79	ND	248.32	10.90	1.02	11.92

[a]Values are mean of duplicate analysis; [b]Total xanthophylls = neoxanthin + violaxanthin + lutein + zeaxanthin ; [c]Total provitamin-A carotenoids = α-carotene + β-carotene. ND = Not Detected.

Table 3.13. Total carotenoids (TC), percent lutein β-carotene and α-carotene in TC and vitamin A activity (RE) of α-carotene and β-carotene in vegetables

Vegetables	TC[*1]	% Lutein in TC	% α-Carotene in TC	% β-Carotene in TC	RE[2]
Beetroot	0.30	89.57	0.00	5.71	0.00
Bitter orange	0.04	50.00	22.50	25.00	0.00
Carrot	175.20	8.25	63.02	28.72	17.59
Colocasia	0.20	40.00	0.00	40.00	0.01
Colocasia (white)	0.10	40.00	9.00	10.00	0.00
Cucumber (green)	2.90	60.00	0.00	30.00	0.15
Cucumber (white)	0.50	62.00	1.80	24.00	0.02
Decalpis	0.30	26.67	3.33	16.67	0.01
Gerkhin	0.10	80.00	9.00	20.00	0.00
Greater yam	1.00	30.00	0.00	26.00	0.04
Green pepper	0.90	56.67	4.44	25.56	0.04
Kenaf	172.60	60.39	1.93	27.93	8.31
Mango ginger	0.10	0.00	0.00	100.00	0.01
Mango (raw)	0.20	40.00	0.00	65.00	0.02
Onion (ordinary)	0.10	20.00	0.00	10.00	0.00
Onion (small)	0.40	50.00	0.00	32.50	0.02
Plectranthus	1.70	87.06	4.71	0.59	0.01
Potato	0.50	76.00	0.00	8.00	0.01
Radish (red)	0.10	50.00	0.00	10.00	0.00
Sweet potato	0.10	40.00	0.00	20.00	0.02
Tapioca	0.10	60.00	0.00	30.00	0.01
Tomato	12.70	30.63	69.29	0.00	0.73
Turnip	0.03	33.33	0.00	33.33	0.00
Zucchini (green)	3.40	96.18	0.26	1.47	0.01
Zucchini (yellow)	260.20	80.63	4.19	0.39	1.08

Values are mean of duplicate analysis, [*]mg/100g dry weight, [1]TC = Total xanthophyll and provitamin A carotenoids, [2]RE (retinol equivalent) = 1 RE= 6mg β-carotene or 12 mg α-carotene

Carotenoid composition in marine algae (seaweeds)

The macro algae (n = 18) analyzed in this study along with their botanical and common names are listed in Table 3.14. The HPLC elution profile of the carotenoids was different from that of green leafy vegetables, medicinal plants and vegetables. The HPLC chromatograms of carotenoids obtained from the extracts of carotenoids of the algae are shown in Figure 3.10 while the carotenoid composition is given in Table 3.15. The highest level of β-carotene (mg/100 g dry weight) was found in *Enteromorpha* (18.9), *C. racemosa* (16.9), *T. connoides* (15.5) and Palmaria (15.3). The highest values for lutein (mg/100 g dry weight) were found in *S. cristaefolium* (2.9), *H. stipulacia* (1.42) and *Enteromorpha* (1.33) while *D. dichromata, S. tenerrium, S. cristaefolim* and *P. tetrastromatica* are better sources of fucoxanthin (3.6-9.2 mg/100g dry weight). *Enteromorpha* and *H. pinifoliata* contained highest levels of zeaxanthin (17.1 and 2.6 mg/100g dry weight). Thus, *Enteromorpha* is a good source of lutein, zeaxanthin and β-carotene among the algae analyzed. Concentration (mg/100g dry weight) of β-carotene and lutein in the seaweeds ranged between 0 to 18.9 and 0 to 2.9 respectively. Neoxanthin and violaxanthin levels ranged from 0 to 1.38 and 0 to 4.0, while fucoxanthin levels were 0 to 9.2. α-Carotene mg/100g dry weight) was detected in 6 algae (0.04 to 7.1) and was highest in *C.sertularciocles* (7.07).

The total carotenoids in the algae and the percentage of lutein, β-carotene, α-carotene and vitamin A activity as retinol equivalent (RE) is given in Table 3.16. Total carotenoids (xanthophyll + hydrocarbon carotenoids) ranged from 0.2 to 42.4 mg/100g dry weight with *Enteromorpha* having maximum level (730.8) and *G. corticata* having minimum level (0.2). Percentage of lutein in total carotenoids ranged from 0-58% while that of β-carotene and α-carotene ranged from 0-99% and 0-76% respectively. RE values (in mg) were highest for *Enteromorpha* (3.2) followed by *C. racemosa* (2.9), *T. connoides* (2.6) and *Palmaria* (2.6) and lowest for *H.* pinifoliata (0.01), *Acanthophora* (0.02), *E. kappaphycus* (0.3), *G. corticata* (0.3), *Ulva* (0.04) and *P. tetrastromatica* (0.07).

Table 3.14. Common and botanical names of marine algae screened for carotenoids.

Common name	Botanical name
Acanthophora	*Acanthophora Sp.*
Caulerpa	*Caulerpa racemosa*
Caulerpa	*Caulerpa sertularciocles*
Dictyota	*Dictyota dichromata*
Enteromorpha	*Enteromorpha Sp.*
Euchema	*Euchema kappaphycus*
Gracilaria	*Gracilaria corticata*
Gracilaria	*Gracilaria edulis*
Gracilaria	*Gracilaria verucossa*
Halophila	*Halophila pinifoliata*
Halophila	*Halophila stipulacia*
Padina	*Padina tetrastomatica*
Palmaria	*Palmaria Sp.*
Sargassum	*Sargassum cristaefolium*
Sargassum	*Sargassum tenerrium*
Turbinaria	*Turbinaria connoides*
Ulva	*Ulva Sp.*
Undaria	*Undaria pinaifida*

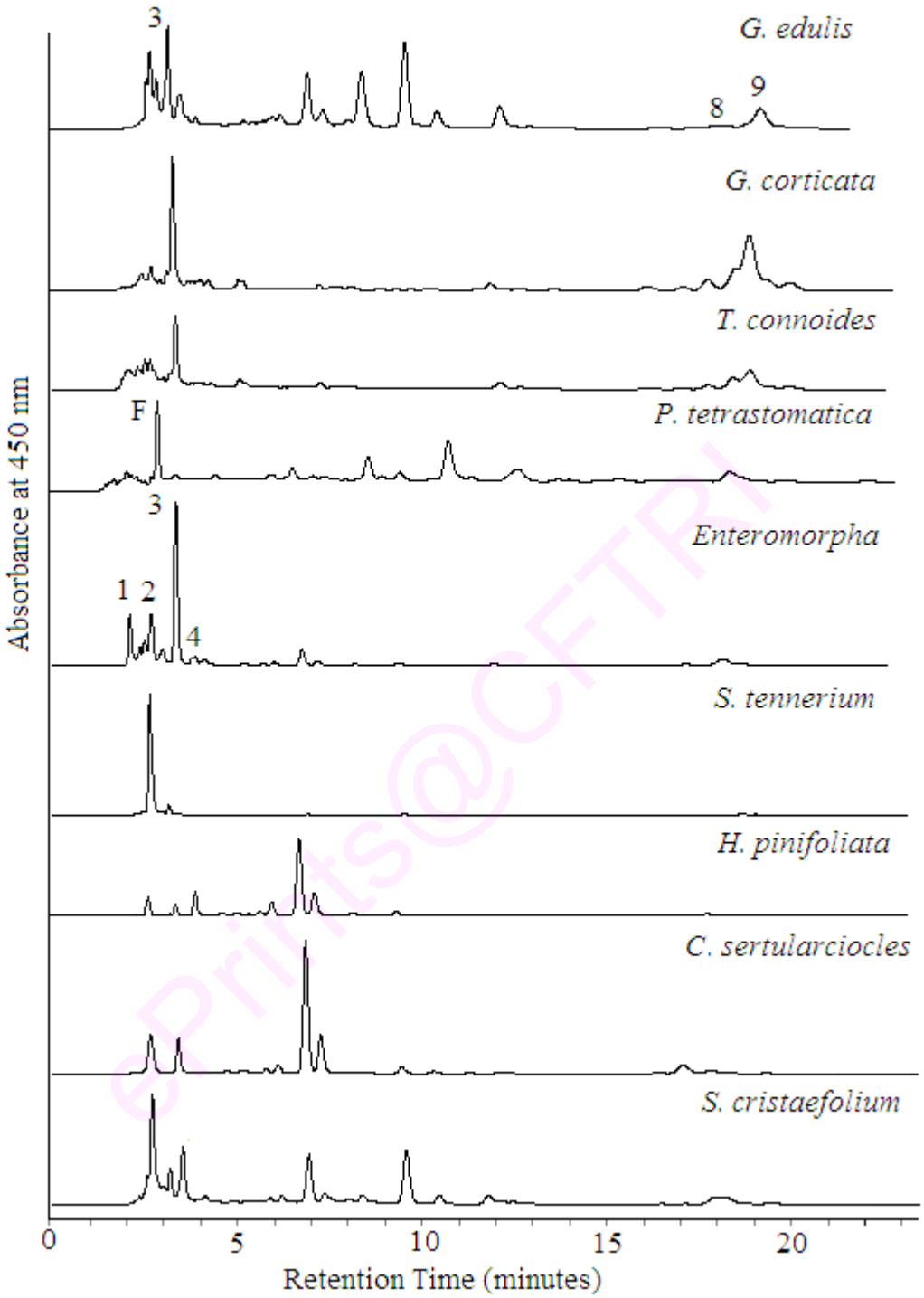


Figure 3.10A. HPLC profile of carotenoids extracted from marine algae. 1=neoxanthin, 2=violaxanthin, 3=Lutein, 4=zeaxanthin, 5=chlorophyll b, 6=chlorophyll a, 7=α-tocopherol, 8=α-carotene, 9= β-carotene, F=fucoxanthin. HPLC conditions are described in Chapter 2, methods section.

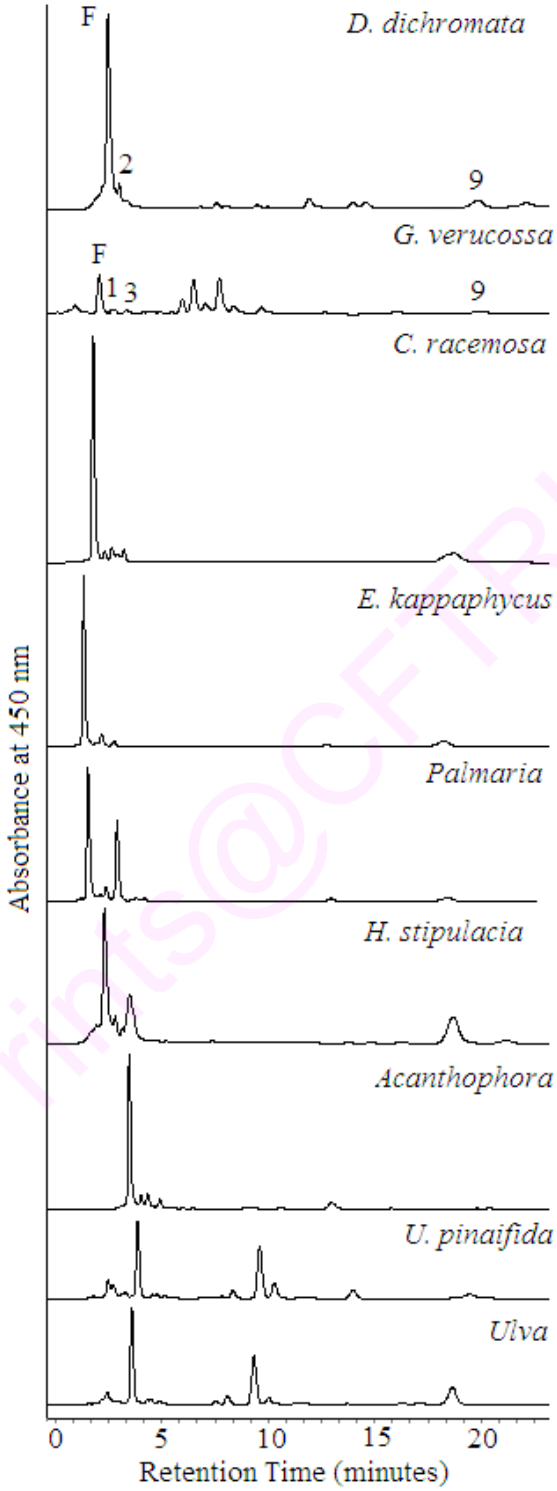


Figure 3.10B. HPLC profile of carotenoids extracted from marine algae. . 1=neoxanthin, 2=violaxanthin, 3=Lutein, 4=zeaxanthin, 5=chlorophyll b, 6=chlorophyll a, 7=α-tocopherol, 8=α-carotene, 9= β-carotene, F=fucoxanthin. HPLC conditions are described in Chapter 2, methods section.

Table 3.15. Carotenoid composition (mg/100g dry weight) in marine algae[a].

Algae	Xanthophylls					Total Xantho-phylls[b]	Provitamin A carotenoids		Total provitamin-A carotenoids[c]
	Fucoxanthin	Neoxanthin	Violaxanthin	Lutein	Zeaxanthin		α-Carotene	β-Carotene	
Acanthophora	0.17	ND	ND	0.03	0.10	0.30	0.04	0.09	0.13
Caulerpa racemosa	0.70	1.38	0.80	0.88	ND	3.76	1.07	16.92	17.99
Caulerpa sertularciocles	2.00	ND	ND	0.25	ND	2.25	7.07	ND	7.07
Dictyota dichromata	9.22	ND	0.84	ND	ND	10.06	ND	1.35	1.35
Enteromorpha	ND	0.78	4.04	1.33	17.12	23.27	0.30	18.86	19.16
Euchema kappaphycus	ND	ND	ND	0.02	ND	0.02	ND	0.19	0.19
Gracilaria corticata	ND	ND	ND	0.02	ND	0.02	0.04	0.14	0.18
Gracilaria edulis	ND	ND	ND	0.03	ND	0.03	0.09	0.81	0.90
Gracilaria verucossa	0.12	0.04	ND	0.20	ND	0.36	ND	2.00	2.00
Halophila pinifoliata	ND	ND	0.48	0.05	2.55	3.08	ND	0.04	0.04
Halophila stipulacia	0.14	0.09	0.09	1.42	ND	1.74	ND	3.68	3.68
Padina tetrastomatica	3.60	ND	ND	0.24	ND	3.84	ND	0.40	0.40
Palmaria	1.29	ND	ND	0.85	ND	1.29	ND	15.32	15.32
Sargassum cristaefolium	3.62	ND	0.04	2.87	0.14	6.67	ND	7.34	7.34
Sargassum tenerrium	4.87	ND	ND	ND	0.21	5.08	ND	9.60	9.60
Turbinaria connoides	ND	ND	ND	0.16	ND	0.16	ND	15.52	15.52
Ulva	ND	0.07	0.004	0.39	0.004	0.46	ND	0.21	0.21
Undaria pinaifida	1.45	ND	ND	ND	ND	1.45	ND	1.96	1.96

[a]Values are mean of duplicate analysis; [b]Total xanthophylls = neoxanthin + violaxanthin + lutein + zeaxanthin ; [c]Total provitamin-A carotenoids = α-carotene + β-carotene. ND = Not Detected.

Table 3.16. Total carotenoids (TC), percent lutein, β-carotene and α-carotene in TC and vitamin A activity (RE) of α-carotene and β-carotene in marine algae.

Algae	TC[*1]	% Lutein in TC	% α-Carotene in TC	% β-Carotene in TC	RE[2]
Acanthophora	0.43	6.98	9.30	20.93	0.02
Caulerpa racemosa	21.75	4.05	4.92	77.79	2.91
Caulerpa sertularciocles	9.32	2.68	75.86	0.00	0.59
Dictyota dichromata	11.41	0.00	0.00	11.83	0.23
Enteromorpha	42.43	3.13	0.71	44.45	3.17
Euchema kappaphycus	0.21	9.52	0.00	90.48	0.03
Gracilaria corticata	0.2	10.00	20.00	70.00	0.03
Gracilaria edulis	0.93	3.23	9.68	87.10	0.14
Gracilaria verucossa	2.36	8.47	0.00	84.75	0.33
Halophila pinifoliata	3.12	1.60	0.00	1.28	0.01
Halophila stipulacia	5.42	26.20	0.00	67.90	0.61
Padina tetrastomatica	4.24	5.66	0.00	9.43	0.07
Palmaria	16.61	5.12	0.00	92.23	2.55
Sargassum cristaefolium	14.01	20.49	0.00	52.39	1.22
Sargassum tenerrium	14.68	0.00	0.00	65.40	1.60
Turbinaria connoides	15.68	1.02	0.00	98.98	2.59
Ulva	0.67	58.21	0.00	31.34	0.04
Undaria pinaifida	3.41	0.00	0.00	57.48	0.33

Values are mean of duplicate analysis, [*]mg/100g dry weight, [1]TC = Total xanthophyll and provitamin A carotenoids, [2]RE (retinol equivalent) = 1 RE= 6mg β-carotene or 12 mg α-carotene.

Discussion

Extraction and purification of carotenoids

The purity of column-purified individual carotenoids on neutral alumina ranged between 90-94 % (91% for β-carotene, 94% for lutein and zeaxanthin, 93% for neoxanthin, 90 for violaxanthin) These values were almost consistent with the results of Kimura and Rodriguez-Amaya (2005), who have purified carotenoids from leafy vegetable using OCC on MgO: hyflosupercel (1:1). They have reported the purity as 91- 97% for neoxanthin, 95-98% for violaxanthin, 92-96% for lutein, and 90-97% for β-carotene.

HPLC and LC-MS analysis of carotenoids extracted

Under the HPLC conditions employed for the separation of the carotenoids from the leafy greens, vegetables and medicinal plants, xanthophylls were eluted within 4.5 mins followed by the chlorophylls at 6.5-7.5 mins and lastly the carotenes at 17-20 mins. Kimura and Rodriguez Amaya (2003) reported separation of carotenoids from lettuce by HPLC under gradient elution, with a runtime of 50 mins, whereas, Bhaskarachary et al. (2008) achieved separation of carotenoids from leafy vegetables in 25 mins with acetonitrile: dichloromethane: methanol (70:10:20 v/v/v). In contrast, carotenoids in this study were well separated within 20 mins. The absorption maxima (λ_{max}) of β-carotene (426, 454, 480), α-carotene (421, 448, 475), lutein (421, 447, 475), neoxanthin (415, 440, 468) and violaxanthin (425, 449, 476) were comparable with reported values (Eitenmiller and Lander, 1999; Khachik et al., 1992). They have reported λ_{max} of β-carotene (452-454, 453 nm), α-carotene (448-450, 444 nm), lutein (446, 445), neoxanthin (438-440, 439) and violaxanthin (440-442, 443). Eitenmiller and Lander Jr. (1999) have reported the λ_{max} of zeaxanthin as 452 nm and it was 454 nm in this study. Fucoxanthin was reported to have λ_{max} 448, 466 (Airs and Llewellyn, 2005) and 446, 475 (Haugan and Jensen, 1994), which is comparable with the present results where λ_{max} was 447, 470. The results demonstrate that the HPLC method standardized in this study was simple and effective for good separation of carotenoids within 20 mins.

Carotenoid composition of leafy greens, vegetables and algae

The HPLC profiles of carotenoids extracted from leafy greens, vegetables and medicinal plants (Figures 3.4-3.6), showed almost a similar elution profile, except for α-carotene, β-cryptoxanthin and lycopene that were detected in few samples. Although, the HPLC profile of all

plant sources was found to be comparatively similar, the major difference appears to be in their concentrations. For example, the chromatogram from the extract of *C. album* showed the presence of both hydrocarbon and xanthophyll carotenoids, however, their relative concentrations were found to be different compared with other greens studied. Among leafy greens, *C. album, A. benghalensis, A. spinosus* and *P.sowa* are rich sources of both lutein and β-carotene. This corroborates well with a previous study (Raju et al., 2007) that reported the levels (mg% dry weight) of β-carotene and lutein as 114.6 and 92.8, 187.6 and 181.3 in *C. album* and *C. benghalensis* respectively. The difference in the concentration of carotenoids in greens is most likely related to difference in species and variety. Differences in carotenoid levels have been reported even within the species and these can be attributed to cultivar, climate, growing conditions, seasonal changes (Aizawa and Inakuma, 2007), variety and stage of maturity of the samples used for analysis (Kimura and Rodriguez-Amaya, 2003).

Concentration (mg/100g dry weight) of β-carotene and lutein in green leafy vegetables ranged between 1.5 to 120.2 and 11.7 to 185.2 respectively. Neoxanthin and violaxanthin levels ranged from 0.03 to 54.1 and 0.03 to 140.5. α-Carotene was detected in 8 leafy greens (0.3-35.6) and was highest in *C. benghalensis* (35.6). For comparison with previously reported values, the present results were calculated on fresh weight basis, based on the moisture content. Aizawa and Inakuma (2007) have previously reported the β-carotene content (3.64 mg/100g fresh weight) of *A. tuberosum.* In this study, higher content of lutein and β-carotene was recorded in *A. schoenoprasum* (8.2 and 5.4 mg/100g fresh weight). β-Carotene content of amaranth leaves ranged from 2.3-14.7 mg/100g fresh weight in the present study and is almost similar to reports of Rajyalakshmi et al. (2001) and Singh et al. (2001) who reported 10.1 and 5.4 mg/100g fresh weight respectively. β-Carotene content of *C. album* (120.2 mg) and *C. benghalensis* (95.7) were comparable with those reported by Raju et al. (2007). Lutein content of *C. album* and *C. benghalensis* recorded in this study were comparable with those reported by Raju et al. (2007) whereas it was higher (54.13 mg/100g dry weight) in *B. diffusa.* Lutein content (7.4 mg/100g fresh weight) in *P. crispum* was higher than β-carotene (4.6 mg/100g fresh weight). Total provitamin A carotenoids and total xanthophylls were higher than those reported by Chanwitheesuk et al. (2005) for *A. graveolens* and *T. indicus.*

Among medicinal plants, concentration (mg/100g dry weight) of β-carotene and lutein ranged between 0.4 to 34.7 and 11.8 to 679 respectively. Neoxanthin and violaxanthin levels ranged from 1.8 to 18.5 and 0.1 to 6.4 respectively. α-Carotene was detected in 25 medicinal plants (0.1-15.7) and was highest in *C. aromaticus* (15.73). There are no reports available on the

carotenoid profile for many of the medicinal plants covered in this study. There is very limited literature on the carotenoid composition of plants used in Ayurvedic medicine. β-Carotene content in *C. asiatica* corroborates well with Raju et al. (2007), while the lutein value reported by them was lower (15.9 mg/100g dry weight) compared to the present result. β-Carotene level in *L. aspera* (2.3 mg/100g fresh weight) found in the present study is in agreement with values (2.3 mg/100g fresh weight) reported by Rajyalakshmi et al. (2001). β-Carotene level in *O. sanctum* in the present study (10 mg/100g fresh weight) was similar to those reported by Aizawa and Inakuma (2007) and Bhaskarachary et al. (1995) (6.83 and 8.2 mg/100g fresh weight). Lutein level of *O. sanctum* (15.4 mg/100g fresh weight) was almost twice the amount reported by Aizawa and Inakuma (2007). The difference in carotenoid levels may be due to the difference in extractability of carotenoids using different solvent system (hexane:ethanol:acetone:toluene, 10:7:6:7, v/v/v) used for extraction. Chanwitheesuk et al. (2005) reported total xanthophyll (4.24 mg%) and total provitamin A carotenoid (2.54 mg%) levels in *Coleus Amboinicus,* whereas, it was higher (35.59, 16.08 mg%) in *C. aromaticus.*

There are reports available on the carotenoid composition of some of the vegetables covered in this study. El-Qudah (2009) and the USDA (2007) reported higher concentration (>14 fold) of β-carotene (0.12 and 0.15 mg/100 g fresh weight) in zucchini as compared to the present result. However, they do not report α-carotene as found in this study (<0.01 mg/100 g dry weight). They have also reported higher (>14 fold) concentrations (mg/100 g fresh weight) of lutein (2.13, 2.34) and zeaxanthin (0.0, 4.1) in green zucchini as compared to the present study. Aizawa and Inakuma (2007) have reported 4 fold higher levels of β-carotene and 5 fold lower lutein content (0.2, 2.1 mg/100 g fresh weight) in yellow zucchini, than the present study (1.02, 3.27 mg/100g dry weight). Moreover, a higher level of α-carotene (10.9 mg/100 g dry weight) was detected in yellow zucchini in the present study. Contrasting reports on provitamin-A carotenes in carrot are available. USDA (2007) reported double the amount (mg/100 g fresh weight) of β-carotene (6.4) than α-carotene (3.8) while equal concentrations of both (4.8, 4.7) and no lutein were reported by El-Qudah (2009) in carrot. Whereas, the present study shows almost twice the concentration (mg/100g dry weight) of α-carotene (110.4) in carrot than β-carotene (50.3) and the values are higher than those reported by the above mentioned researchers. Niizu and Rodriguez-Amaya (2005) have reported lower level of β-carotene and lutein (510, 6.15 μg/100 g fresh weight) in carrots while Singh et al. (2001) have reported comparable concentration of β-carotene (47.8 mg/100g dry weight) in carrot. β-Carotene levels (6.5 mg/ 100 g fresh weight) reported by Bhaskarachary et al. (1995) for carrot corroborates well with the

present study while the total carotenoids observed in this study are 2 fold higher. Aizawa and Inakuma (2007) have reported β-carotene level as 0.18 mg/100g fresh weight in green cabbage. The level (mg/100g fresh weight) of β-carotene (2.34, 1.77) and α-carotene (0.1, 0.06) reported by them for green and red cabbage was 20 fold higher than the values found in this study. They have also reported lutein content as 0.23, 0.04, 1.87 and 1.2 mg/100g fresh weight in green and red cabbage and red lettuce respectively. In comparison, lutein level was higher in the present study for red cabbage, whereas, it was lower for green cabbage, green and red lettuce along with the presence of zeaxanthin in both lettuce varieties. El-Qudah (2009) has reported lower level of (>4 fold) β-carotene and lutein in tomato (79, 0.39 μg/100g fresh weight) while no β-carotene was detected in the present study. On the other hand, Aizawa and Inakuma (2007) have reported α-, β-carotene and lutein in tomato (0.01, 0.45 and 0.12 mg/100g fresh weight), whereas, α-carotene level was lower by 44 fold than the present study (98.8 mg/100g dry weight). Niizu and Rodriguez-Amaya (2005) have also reported the presence of β-carotene and >190 fold lower values for lutein in tomato (320, 100 μg/100 mg fresh weight). Lutein values (0.13 mg/100g fresh weight) reported by the USDA (2007) for tomato are comparable with the results of the present study.

Aizawa and Inakuma (2007) have reported 3.3 mg/100g fresh weight of β-carotene and higher lutein levels (3.11 mg/100g fresh weight) in turnip while this study reports lower β-carotene level (0.01 mg/100g dry weight). Niizu and Rodriguez-Amaya (2005) have reported lower β-carotene and lutein levels (270 and 770 μg/100 g fresh weight) and no α-carotene and zeaxanthin in green pepper. Bhaskarachary et al. (1995) have analyzed the carotenoids in *Hibiscus sabdariffa* and reported (mg/100g fresh weight) β-carotene (5.8) and total carotenoids (15.8). Moreover, we have also found carotenoids other than β-, α-carotene and lutein in vegetables screened. Similar to the present results, only lutein was detected in onion (0.17) by Muller (1997). Thus, he has reported comparable values for lutein in potato and higher lutein concentration in onion (9 fold) and zeaxanthin in potato (>240 fold) as compared to the present results. Bhaskarachary et al. (1995) have reported higher values for β-carotene and total carotenoids (1.87 and 2.23 mg/100 g fresh weight) in sweet potato as compared to the present study, however they have not reported the presence of other carotenoids. No report is available on the level of carotenoids in kenaf. In this study, the level (mg/100 g dry weight) of β- (48.21) and α-carotene (3.33) in kenaf was considerable.

Among the 18 Indian marine algae analyzed, *D. dichromata*, *H. stipulacia* and *Enteromorpha* contained higher concentrations (mg/ 100g dry weight) of lutein while

Enteromorpha, *C. recemosa*, *T. connoides*, *Palmaria*, *S. tenerrium* and *S. cristaefolium* had higher levels of β-carotene. α-Carotene and zeaxanthin were detected in 6 algae species with *C. sertularciocles* and *Enteromorpha* having the higher levels respectively. Fucoxanthin, a marine carotenoid was found in 11 algae and was higher in *D. dichromata*, *S. tenerrium*, *S. cristaefolium*, *P. tetrastomatica*, *C. sertularciocles*, *U. pinafida* and *Palmaria*. Neoxanthin was detected in 5 algae and was highest in *C. racemosa* while violaxanthin was present in 7 algae and was highest in *Enteromorpha*. There are studies on the carotenoid composition of algae and related species. Yoshii et al. (2004) have reported siphonoxanthin, 9-cis neoxanthin, loroxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin and β-carotene in fresh water green alga *Aegagrophila linnaei*, *Ulvophyceae* and *Chlorophyta* while Schubert et al. (2006) have studied many varieties of red algae and reported violaxanthin, antheraxanthin, zeaxanthin, β-cryptoxanthin and β-carotene in *Gracilaria gracilis* and α-carotene in *G. textorii*. Esteban et al. (2009) have demonstrated the presence of lutein, β- and α-carotene in *Palmaria palmata* while Remias et al. (2005) detected astaxanthin, neoxanthin, lutein, β-carotene and pool of xanthophyll pigments (violaxanthin, antheraxanthin, zeaxanthin) in *Chlamydomonas nivalis*. Haugan and Jensen (1994) have reported β-carotene, zeaxanthin, luteoxanthin, violaxanthin, fucoxanthin, neochrome, neoxanthin and fucoxanthinol as the major carotenoids in 6 brown algae (*Fercus serratus, F. vesiculosis, Pelvetia canaliculata, Ascophyllum nodosum, Laminaria digitalia, L. saccharina*). It is therefore evident that studies on the carotenoid composition of seaweeds of the Indian sub-continent are scarce and many of the algae presently analyzed have not been studied earlier.

Vitamin A activity as retinol equivalents (RE)

Provitamin-A activity measured as RE in mg (where 1 RE = 6 mg β-carotene or 12 mg α-carotene) was found in the range of 0.4 – 20 (leafy greens), 0.42 – 5.8 (medicinal plants) and <0.01 to 17.59 (vegetables). Of the 19 greens analyzed, 7 had RE greater than 5 mg (*A. schoenoprasum, A. graveolus, C. anti-quorum, Amaranthus sp.* (keerai), *A. spinosus, C. album and C. benghalensis*). Of 33 medicinal plants studied, 3 had RE greater than 5 mg (*O. sanctum, C. ternatea* and *B. monnieri)* and 2 of the 25 vegetables studied had RE greater than 5 mg (*H. cannabius and D. carota).* Whereas, Rajyalakshmi et al. (2001) have reported lower RE value for *A. spinosus* (1.68 mg%), *C. antiquorum* (0.58 mg%), *C. benghalensis* (0.53 mg%), *L. aspera* (0.39 mg%) and *P. oleraceae* (0.07 mg%) as compared to the present study. These differences may have arisen due to geographical differences from where the plant materials were collected and the difference in extraction procedure adopted.

The present results show individual values for different carotenoids present in leafy greens, medicinal plants and vegetables. Previously, only few studies have reported values of both xanthophyll and provitamin-A carotenoids (Aizawa and Inakuma, 2007; Niizu and Rodriguez-Amaya, 2005, Raju et al., 2007; Muller, 1997). In the Southeast Asian subcontinent, only Raju et al. (2007) have reported levels of individual carotenoids in leafy greens. Information on medicinal plants and other greens is thus still very limited. Moreover, no reports are available for nearly 50% of the plants analyzed, many of which are used by the local people as food or medicine. *C. album, A. spinosus, C. benghalensis, C. anti-quorum,* and *Amaranth sp.* (keerai) were rich in β-carotene while *C. album, C. benghalensis, C. annuum, I. pes-tigridis, A. spinosus* and *C. anti-quorum* were found to have considerable amounts of lutein. Interestingly, *C. album, A. benghalensis and A. spinosus* are rich sources of both lutein and β-carotene. *C. ternatea*, a medicinal plant had highest lutein content. Lutein is predominant in the macula of the retina where it acts as an antioxidant against photo-oxidation. Thus, the lutein-rich plants can be exploited for the management of age related macular degeneration (Klein et al., 1995). Other plants like *C. dactylon, O. canum, A. indica* and *R. communis* also had very high levels of lutein. Medicinal plants such as *B. monnieri, O. sanctum* and *C. ternatea* were rich sources of β-carotene. *C. ternatea* is thus a good source of both lutein and β-carotene. From the results, it is evident that RE level of medicinal plants are lower than leafy greens, however, the total carotenoid values are significantly higher (1.5 fold). This could be one of the reasons for the medicinal properties of these plants and their use in Ayurvedic preparations. On the other hand, calculation of RE for the vegetables showed that carrot had highest RE values (>17), followed by kenaf, and yellow zucchini (1.08). Although RE of vegetables, in general, was lower than the leafy greens, consumption of vegetables may still be recommended as β-carotene source as some studies have found carotenoids to be more bioavailable from fruits as compared to leafy greens (de Pee et al, 1998). Among the 18 algae analyzed, *Enteromorpha, C. racemosa, T. connoides, Palmaria, S. tenerrium,* and *S. cristaefolium* had higher RE (> 1mg) as compared to the others.

It is evident from the above results that there is difference in the carotenoid composition of leafy greens, medicinal plants and vegetables of various geographical origins. Carotenoid content is known to vary in plant foods due to difference in characteristics of cultivars, climate and growing conditions. Differences in carotenoid levels have been reported even within the species and these can be attributed to cultivar, climate, growing conditions, seasonal changes (Aizawa and Inakuma, 2007) variety and stage of maturity of the samples used for analysis (Kimura and Rodriguez-Amaya, 2003). This could have been the reason for the difference between our results and previously reported values for the produce analyzed. Data generated in

this study on the carotenoid composition and their retinol equivalents will help to compile information on the carotenoid profile of the locally available and consumed vegetables, leafy greens and algae, which will help in creating and compiling a database on carotenoids present in agri-horticulture produce of the region. Moreover, these results will be of use to health and community workers to recommend and improve awareness about the consumption of these natural and locally available plant materials to meet the requirements for β-carotene/retinol (vitamin A) and lutein (macular pigment) for the prevention of deficiency related diseases such as night blindness and other vitamin A deficiency related disorders as well as age related macular degeneration in the populace.

CHAPTER 4: Effect of vitamin A deficiency on bioavailability of dietary carotenoids (β-carotene, astaxanthin, lutein and fucoxanthin).

Introduction

Carotenoids have several health benefits. β-Carotene and α-carotene are precursors of vitamin A (Barua and Olson, 2000). Astaxanthin, lutein, zeaxanthin, neoxanthin and fucoxanthin have been reported to have anti-proliferative, anti-inflammatory, anti-cancer, anti-oxidative and anti-obesity effects (Kotake-Nara et al., 2001; Shiratori et al., 2005; Goto et al., 2001, Maeda et al., 2005, Asai et al., 2004, Chew and Park, 2004). Astaxanthin has been shown to suppress tumour growth in mice (Jyonouchi et al., 2000), enhance innate immunity in fish (Amar et al., 2004) and decrease macrophage infiltration, apoptosis and vulnerability in atheroma of hyperlipidemic rabbits (Li et al., 2004). Lutein is thought to play a significant role in the prevention of age related macular degeneration (Barlett and Eperjesi, 2003; Wisniewska and Subczynski, 2006). Therefore, consumption of foods containing these carotenoids would be of immense benefit for good health.

Vitamin A deficiency (VAD) affects about 40% of the global population mainly children, pregnant and lactating women, because their vitamin A requirement is higher. VAD increases the susceptibility towards other diseases and can cause irreversible blindness (Lorch, 2005). VAD is recognized as a serious health problem in India. Though, Government of India has made a remarkable progress in the elimination of blindness due to VAD, a recent report demonstrated prevalence of night blindness (1.03%) among children aged 24-71 months (Feldon et al., 2005). VAD is primarily caused due to either poor diet or inadequate intake of retinol or provitamin A carotenoids or poor conversion of carotene to vitamin A. VAD is common in protein-energy malnutrition (marasmus or kwashiorkor), principally because the diet is deficient with vitamin A and also due to defective vitamin A storage and transport. It is a leading cause of childhood blindness in the developing world affecting over 120 million children worldwide. In countries where immunization programs are not widespread and VAD is common, millions of children die each year from complications of infectious disease such as measles. VAD and its related disorders are prevalent in developing nations and have been declared to be a public health problem. Globally, nearly 127 million preschool children are vitamin A deficient and 7.2 million pregnant women in the developing world suffer from VAD (West et al., 2005). Besides vitamin A

precursors, it has been reported that nonprovitamin-A carotenoids such as fucoxanthin, astaxanthin and lutein afford protection against the biochemical changes caused by VAD. However, their biological availability is very limited.

Absorption and bioavailability of carotenoids is an important aspect, as their health benefits will increase with increased absorption. In general, the bioavailability of carotenoids is very poor and only 10-20% of the total carotenoids in the food material are bioavailable. Absorption of carotenoids depends upon several dietary and non-dietary factors including level and origin of dietary fat, amount of carotenoids, digestibility of food, presence of antioxidants or dietary fibres and vitamin A status (Erdman et al., 1993). The various factors that affect the bioavailability of carotenoids have been included into a mnemonic (SLAMENGHI) and are given in Table 4.1. Some of these factors have been represented graphically in Figures 1.7 and 1.8 in Chapter 1.

Amongst the factors, the modifiable ones include dietary effectors that can promote bioavailability and bioconversion of carotenoids from food matrix. Castenmiller et al. (1999) have reported that lutein from spinach is more bioavailable than β-carotene. However, enzymatic disruption of the matrix enhanced the bioavailability of β-carotene. Roodenburg et al. (2000) and Deming et al. (2000) have studied the importance of fat in the diet that promotes the bioavailability of carotenoids. Five times higher bioavailability of lutein as compared to β-carotene, from vegetables was reported by van het Hof et al. (1999). Borel et al. (1998) on the other hand have studied role of triglyceride on the bioavailability of β-carotene and its conversion to retinyl palmitate. The type of fatty acid present may also influence the bioavailability of carotenoids as reported by Raju et al. (2006) and Lakshminarayana et al. (2006). From the limited human studies available, lutein seems to be more bioavailable than β-carotene (Zaripheh and Erdman Jr., 2002). Studies have reported that people with higher retinol status appear to absorb retinol more efficiently than people with low retinol status (Burri and Park, 1998). In contrast, it was reported that the bioconversion of carotenoids was higher in the retinol deficient than the retinol sufficient individual (Mc Larsen and Frigg, 2001).

Carotenoid bioavailability is a subject of great interest due to their multiple health benefits. Therefore, understanding the absorption and factors affecting their bioavailability are crucial for improving their bioefficacy. Although the route of absorption for all carotenoids is similar, it has been reported that the rate of absorption was different. Lakshminarayana et al. (2006) and Raju et al. (2006) reported maximum absorption of micellar lutein and β-carotene in rats 2 hours after feeding, whereas, Coral-Hinostroza et al. (2004) reported maximum absorption of astaxanthin esters in man in 11.5 h. It is evident from the literature that not much work has

been done on the bioavailability and bioefficacy (conversion of carotenoids to retinol) of the carotenoids in vitamin A deficient rodents. Hence, the present investigation was carried out to evaluate the effect of vitamin A deficiency on the bioavailability and biological conversion (bioefficacy) of β-carotene, lutein, astaxanthin and fucoxanthin in rat model.

Table 4.1. Factors affecting carotenoid bioavailability.

Factors affecting carotenoid bioavailability		
S	*S'pecies of carotenoids*	The all-*trans* form is better absorbed than the 9-*cis* form. The 9-*cis* form is converted to all-*trans* form in intestine.
L	*Molecular 'L'inkage*	Carotenoids are present in food (fruits, vegetables, seafood, etc) in complex ester forms. Lutein esters are more bioavailable.
A	*'A'mount of carotenoids consumed*	Nearly 10-20 % of carotenoids in a meal are absorbed. The duration of supplementation can affect the bioavailability.
M	*'M'atrix in which carotenoid is incorporated*	Carotenoids require disruption of chloroplasts containing pigment-protein complexes/ release from lipid droplets. Physical breakdown and processing methods assist in carotenoid release.
E	*'E'ffectors of absorption and bioconversion*	Dietary fat is essential for adequate micelle formation. Type of dietary fat, protein, zinc, α-tocopherol, fibre, chlorophyll and mixed carotenoids affect bioavailability.
N	*'N'utrient status of the host*	Vitamin A status and zinc deficiency affect bioconversion of β-carotene to retinol.
G	*'G'enetic factors*	Enzymatic failure to cleave β-carotene in the intestine, reduction of plasma RBP and mutations in the gene for RBP affect metabolism of β-carotene.
H	*'H'ost related factors*	The serum response to β-carotene in women is higher than men, while men are more susceptible to develop VAD. Interference with intestinal absorption of lipids affects bioavailability.
I	*Mathematical 'I'nteractions*	Two or more factors may have a synergistic or antagonistic effect on bioavailability when acting together.

In this study, three experiments – single dose, repeated dose and dietary feeding of carotenoids to retinol deficient rats were performed to measure the bioavailability and bioefficacy of carotenoids.

In the single dose study, rats were made retinol deficient (RD) by feeding diet devoid of retinol while a separate group received diet with retinol was treated as control. On confirmation of retinol deficiency, rats were intubated a single dose (0.8 µmol) of either astaxanthin, lutein or β-carotene dissolved in peanut oil and sacrificed at 2, 4 and 8 h after gavage (n=5/time point). In case of fucoxanthin intubated rats, they were sacrificed at 2, 4, 6 and 8 h after intubation (n=5/time point). RD rats were intubated with peanut oil alone and sacrificed at 0 h (baseline). In the repeated dose study, two sets of RD rats were gavaged a dose (0.1 mg/rat/day) of astaxanthin, lutein or β-carotene for 7-days and 15-days and sacrificed at the end of the respective gavage periods. RD rats intubated with the same volume of peanut oil alone, served as baseline, while the group that received diet with retinol throughout the experimental run served as control. In the dietary study, RD rats were fed diet containing (0.7 µmol/day) astaxanthin or *P. sowa* (lutein source) or *D. carota* (β-carotene source) for 20 days. Blood was drawn from the orbital plexus of rats 10 days after feeding the supplemented diets and they were sacrificed after 20 days. A separate group of RD rats served as baseline while another group of rats that received retinol in the diet during the experimental run served as control. At the termination of the experiments, rats were sacrificed and blood was drawn from the heart into heparinized tubes. Liver was sampled and washed with ice-cold saline, while the brain was sampled and blotted for removal of blood and stored at -80°C until analyzed. Plasma was separated immediately and liver microsomes were prepared as described in Chapter 2 (methods section). The bioavailability of carotenoids was calculated based on the level of intact carotenoids present in the plasma, liver and intestine. Bioefficacy of the β-carotene was calculated from the amount of retinol formed in the plasma, liver and intestine. Retinol formed was calculated after baseline correction and was expressed as total retinol (sum of retinol and ½ retinylpalmitate).

Results

Induction of retinol deficiency and baseline value of carotenoids

Rats were made retinol deficient (RD) by feeding diet devoid of retinol before using them for various experiments. Retinol deficiency resulted in depleted retinol levels in plasma (µmol/l) and liver (nmol/g) to 0.38 ± 0.036, 3.05 ± 0.34 (single dose), 0.30 ± 0.022, 2.0 ± 0.03 (repeated dose) and 0.40 ± 0.03, 2.96 ± 0.03 (dietary) from 50.7 $\pm$ 0.5, 295.9 $\pm$ 5.9 demonstrating that the

rats attained retinol deficient state. Blood retinol level <0.7 µmol/l is considered as deficient. No carotenoid was detected in the plasma, liver or intestine of the animals in the baseline group.

Effect of retinol deficiency on growth parameters

Growth in terms of gain-in-body weight was monitored during the experimental run. Retinol deficiency was found to affect the food intake and weight gain of the rats. The food intake pattern and the gain-in-body weight of the rats during the induction of retinol deficiency are shown in Figure 4.1. Results show that the gain-in-body weight was decreased (25%) significantly (Figure 4.1 b) with a reduction in food intake (Figure 4.1 a). Retinol deficiency was associated with decreased food intake, weight loss, fur/hair loss, and lack of activity and blood spots from the nose. The organ weights such as liver, kidneys, spleen and brain of the RD, control and experimental groups are given in Table 4.2. It is seen from the results that the difference in weight of liver of RD and control rats was not statistically significant. Similarly, administration of carotenoids resulted in increased liver weight, but this was not statistically significant ($p>0.05$).

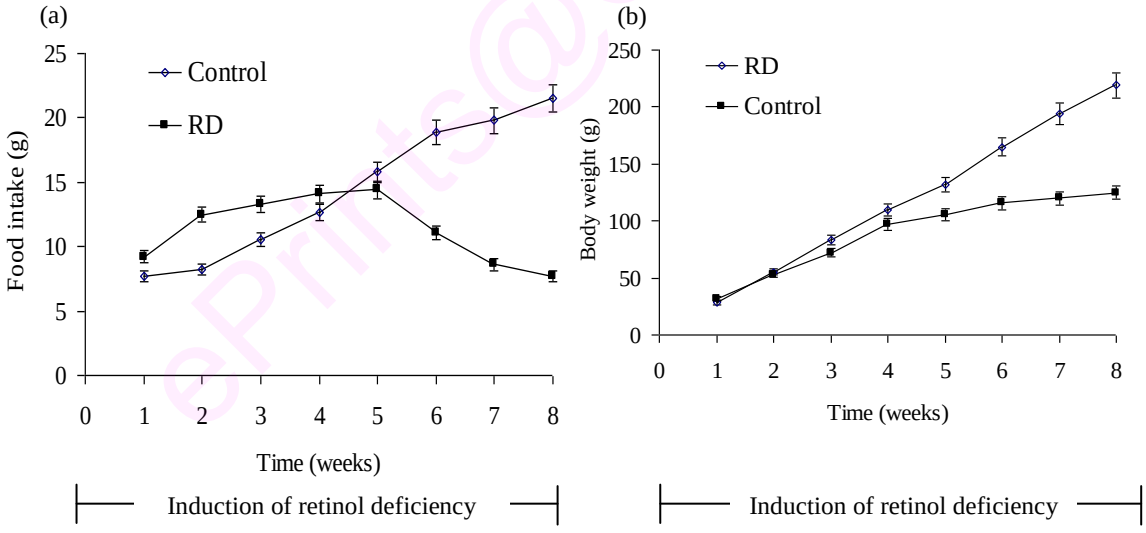


Figure 4.1. Effect of feeding vitamin A deficient diet on food intake (a) and growth (b) of rats in comparison with control group that received retinol sufficient diet. Values are mean ± SD (n=5).

Table 4.2. Effect of retinol deficiency and subsequent carotenoid feeding on organ weights of rats.

Group	Liver	Kidneys	Spleen	Brain	Body weight	Liver-somatic Index
Single dose study						
Control	7.3 ± 0.2	1.6 ± 0.1	0.8 ± 0.05	1.7 ± 0.1	219.1 ± 1.9	0.033
RD	6.1 ± 0.6	1.4 ± 0.1	0.7 ± 0.05	1.5 ± 0.1	125.2 ± 1.1	0.049
β-Carotene	6.5 ± 0.7	1.4 ± 0.1	0.7 ± 0.03	1.7 ± 0.1	126.1 ± 1.0	0.052
Astaxanthin	6.0 ± 0.4	1.3 ± 0.1	0.6 ± 0.02	1.5 ± 0.1	124.7 ± 1.2	0.048
Lutein	6.3 ± 0.4	1.4 ± 0.1	0.7 ± 0.04	1.4 ± 0.1	125.5 ± 1.5	0.051
Repeated dose study (15-days)						
Control	7.8 ± 0.7	1.7 ± 0.1	0.8 ± 0.05	1.6 ± 0.1	268.1 ± 2.2	0.029
RD	6.2 ± 0.4	1.3 ± 0.1	0.6 ± 0.04	1.4 ± 0.1	124.5 ± 1.1	0.050
β-Carotene	7.4 ± 0.5	1.5 ± 0.1	0.9 ± 0.07	1.7 ± 0.1	169.3 ± 1.5	0.044
Astaxanthin	6.9 ± 0.5	1.4 ± 0.1	0.8 ± 0.03	1.6 ± 0.1	158.7 ± 1.3	0.043
Lutein	6.5 ± 0.6	1.3 ± 0.1	0.7 ± 0.02	1.7 ± 0.1	132.0 ± 0.9	0.049
Dietary study						
Control	7.5 ± 0.4	1.7 ± 0.1	0.8 ± 0.05	1.7 ± 0.1	282.7 ± 2.1	0.027
RD	6.0 ± 0.5	1.3 ± 0.1	0.6 ± 0.02	1.4 ± 0.1	125.9 ± 1.1	0.048
β-Carotene[*]	7.2 ± 0.2	1.5 ± 0.1	0.9 ± 0.06	1.7 ± 0.1	199.1 ± 1.4	0.036
Astaxanthin[**]	6.8 ± 0.5	1.4 ± 0.1	0.7 ± 0.03	1.7 ± 0.1	171.1 ± 1.2	0.040
Lutein[***]	7.1 ± 0.5	1.5 ± 0.1	0.8 ± 0.05	1.7 ± 0.1	195.4 ± 1.3	0.036

Data represent mean ± SD (n=5). [*]Carrot powder as β-carotene source. [**]Purified astaxanthin. [***]Dill leaf powder as lutein source. RD = retinol deficient.

Single dose study: Bioavailability and bioefficacy of β-carotene, astaxanthin, lutein and fucoxanthin

RD rats were gavaged with an equimolar concentration of β-carotene, astaxanthin or lutein (0.8 μmol) and sacrificed after 2, 4 and 8 h while RD rats gavaged with fucoxanthin (0.8 μmol) were sacrificed after 2, 4, 6 and 8 h to determine carotenoids' bioavailability. No carotenoids were detected in plasma and liver of the RD and control rats. Retinol levels in plasma and liver of RD rats was 0.38 μmol/l and 3.1 nmol/g while for control rats it was 59.3 μmol/l and 306.4 nmol/g respectively. The weight gain (25%) and food intake (60%) of the rats during the induction of retinol deficiency was significantly lower ($p<0.05$) as compared to control group (Figure 4.1). Similarly, the liver somatic index of RD rats was significantly lower ($p<0.05$) than the control rats, whereas, no significant difference was found between RD and control rats with respect to various organs (Table 4.2).

Bioavailability and efficacy of β-carotene

HPLC chromatograms of β-carotene and retinol extracted from the plasma, liver and intestine of RD rats gavaged with β-carotene are depicted in Figure 4.2. The plasma and liver levels of β-carotene, plasma triglycerides (TG) levels and retinol levels in plasma, liver and intestine of RD rats after gavage with β–carotene are given in Table 4.2 and Table 4.3 respectively. β-Carotene was not detected in plasma upto 8 hours after its gavage to RD rats while it was detected in liver at 8h (10.72 pmol/ml). The corresponding TG level in plasma showed a significant rise ($p<0.05$) in β-carotene gavaged groups with maximum concentration at 4h. The AUC of plasma β-carotene was 1369 pmol/ml/8h indicating the bioavailability of β-carotene. Although intact β-carotene was not observed in plasma and liver (except in 8 h group), an increase in the retinol levels ($p<0.05$) was found over 8 h (Table 4.3). The increase in the retinol levels may explain the absence of β-carotene wherein; the β-carotene may be cleaved to yield the retinol. The absence of β-carotene was accompanied by corresponding rise in retinol levels in plasma (0.63-1.75 μmol/l), liver (6.48-20.54 nmol/g) and intestine (5.19-12.66 nmol/g) on gavage with β-carotene, indicating its conversion to retinol. The enhanced activity of intestinal monooxygenase over 2-8 h on β-carotene gavage further supports the above result (results given at the end of this chapter). The plasma level of total β-carotene (AUC of β-carotene + ½ AUC of total retinol) was calculated as 2.51 nmol/ml/8h.

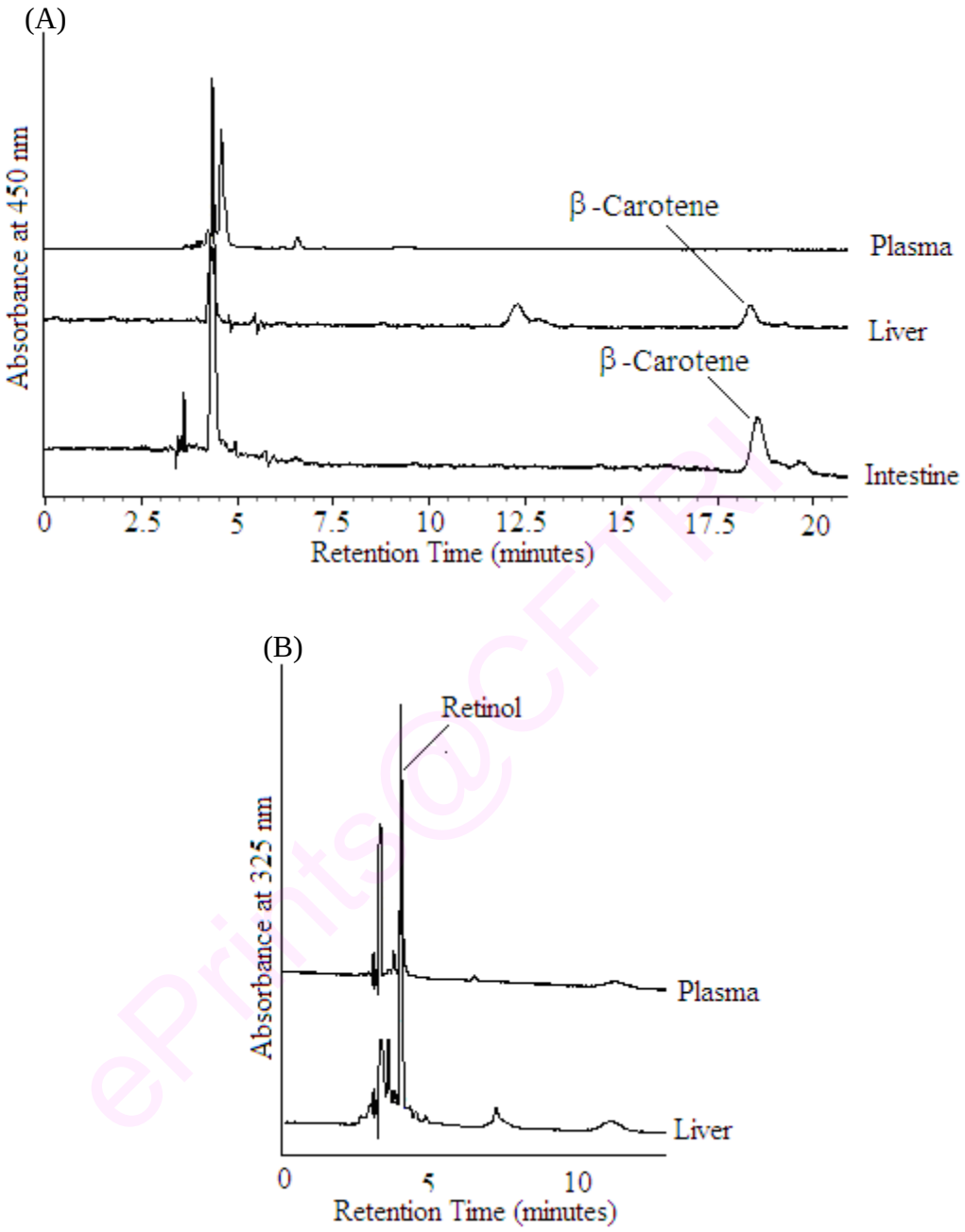


Figure 4.2. HPLC chromatogram of β-carotene (A) and retinol (B) extracted from RD rats at 8 h after gavage of a single dose of β-carotene showing the presence of β-carotene in liver and intestine.

Table 4.3. Postprandial β-carotene levels in plasma (pmol/ml), liver (pmol/g) and intestine (nmol/g) and triglycerides (TG) in plasma (mg/dl) of RD rats fed on β-carotene.

Samples	0h (RD)	2h	4h	8h	AUC[#]
Plasma	ND	ND	ND	ND	ND
Liver	ND	ND	ND	10.72 ± 1.0	21.44
Intestine	ND[a]	45.61 ± 3.9[b]	110.10 ± 12.3[c]	70.39 ± 7.9[d]	562.3
Plasma TG	120.8 ± 5.9[a]	155.6 ± 8.8[b]	205.1 ± 10.5[c]	160.9 ± 9.2[b]	1369

[#]AUC = Area under the curve. Unit for AUC, plasma = pmol/ml/8h, liver = pmol/g/8h, intestine = nmol/g/8h, TG = mg/dl/8h.

Data represent mean ± SD (n=5). Values at each time point within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. ND = Not detected, detectable limit = 1 pmol, RD = Retinol deficient.

Table 4.4. Total retinol* levels in plasma (μmol/l), liver (nmol/g) and intestine (nmol/g) of RD rats gavaged with a single dose of β-carotene.

Parameter	0h (RD)	2h	4h	8h	AUC[#]
Plasma	0.38 ± 0.036[a]	0.63 ± 0.05[b]	1.75 ± 0.20[c]	0.96 ± 0.09[d]	8.810
Liver	3.05 ± 0.34[a]	6.48 ± 0.71[b]	12.58 ± 0.85[c]	20.54 ± 0.22[d]	94.83
Intestine	2.99 ± 0.31[a]	12.66 ± 0.92[b]	9.2 ± 0.79[c]	5.19 ± 0.35[d]	66.29

*Total retinol = retinol + ½ retinyl palmitate, RD = Retinol deficient.

[#]AUC=Area under the curve, Unit for AUC, plasma = pmol/ml/8h, liver = pmol/g/8h, intestine = nmol/g/8h, TG = mg/dl/8h.

Data represent mean ± SD (n=5). Values at each time point within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

Astaxanthin

HPLC chromatograms of astaxanthin and retinol extracted from the plasma, liver and intestine of RD rats after gavage with astaxanthin is shown in Figure 4.3. The plasma and liver

levels of astaxanthin and plasma TG levels of RD rats gavaged with astaxanthin are presented in Figure 4.4 while the retinol levels in the plasma, liver and intestine are given in Table 4.4. Astaxanthin was detectable after 2h in plasma (28.8 pmol/ml) and liver (6.91 pmol/g) of RD rats gavaged with astaxanthin. The plasma astaxanthin levels were higher at 4h after gavage (81.6 pmol/ml) and remained detectable upto 8h (27.03 pmol/ml). Correspondingly, triglycerides (TG) level in plasma showed a rise ($p<0.05$) over 8h with concentration maxima at 4h (199.5 mg/dl). The AUC value for plasma astaxanthin was calculated as 356.6 pmol/ml/8h indicating bioavailability of astaxanthin. The AUC value of astaxanthin in intestine (1100 mg/dl/8h) is higher than the liver (197.1 mg/dl/8h) and plasma demonstrating the transfer of absorbed astaxanthin to other tissues (Figure 4.4). Results show no significant difference in the plasma (0.47-0.39 μmol/l), liver (3.12-2.98 nmol/g) or intestine (2.71-2.72 nmol/g) retinol levels ($p>0.05$) over a period of 8h as compared to RD group (0.38 μmol/l, 3.05, 2.99 nmol/g).

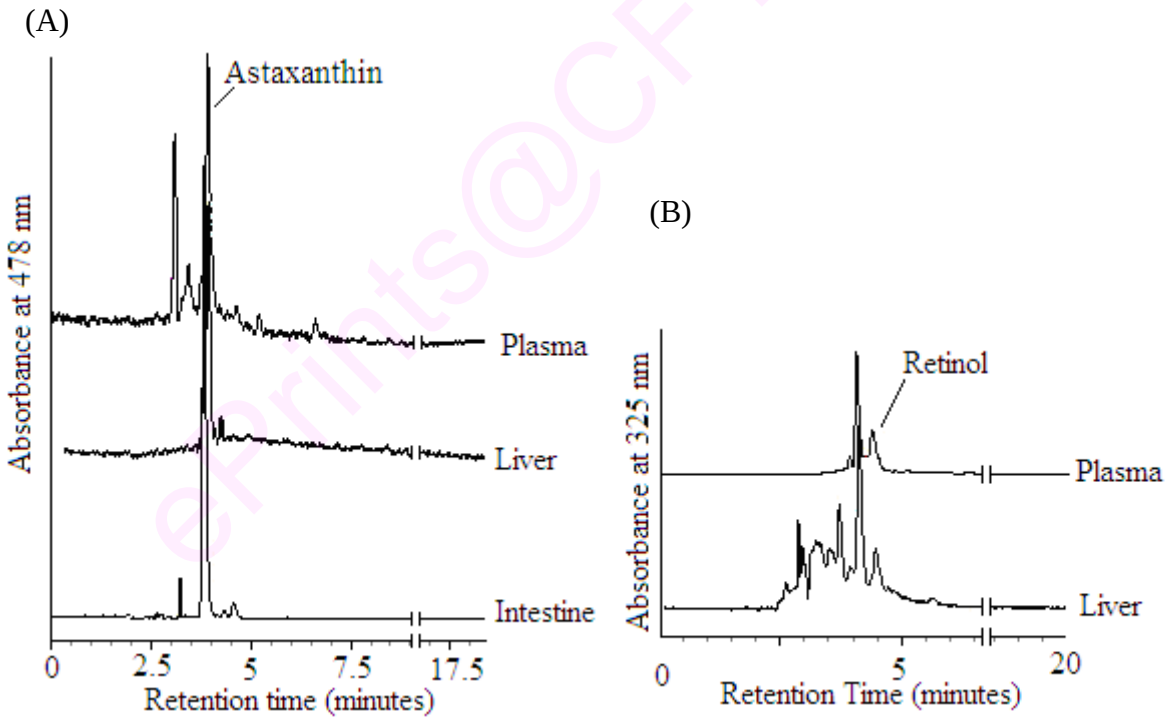


Figure 4.3. HPLC chromatogram of astaxanthin (A) and retinol (B) extracted from RD rats at 4 h after gavage of a single dose of astaxanthin.

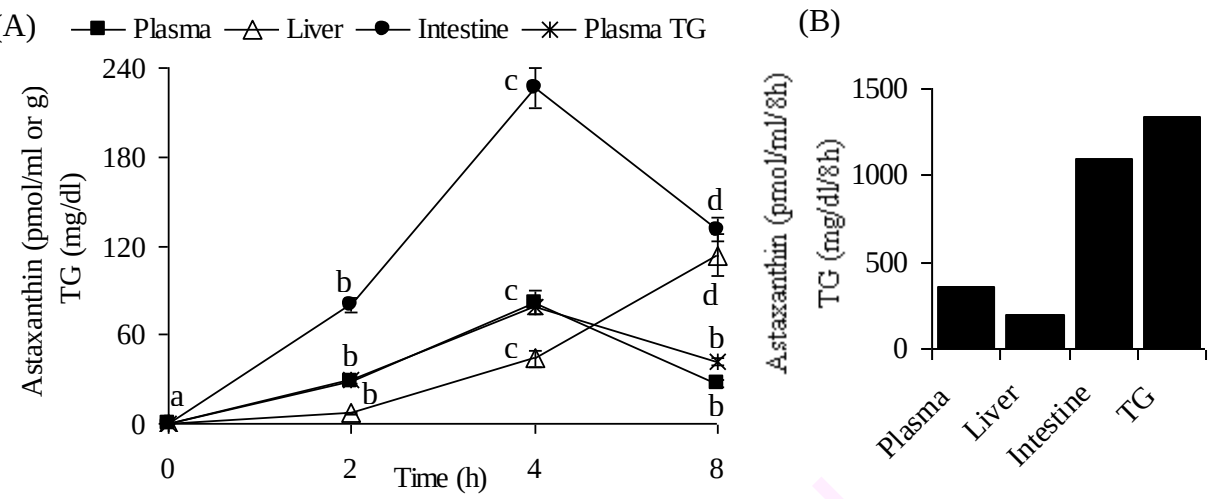


Figure 4.4. Postprandial astaxanthin levels in plasma (pmol/ml) and liver (pmol/g) and triglycerides (TG) in plasma (mg/dl) (A) and area under the curve (AUC) values for astaxanthin in plasma (pmol/ml/8h), liver, intestine (pmol/g/8h) and TG values (mg/dl/8h) in plasma over 8h (B) after a gavage of astaxanthin. TG values are baseline corrected. Data represent mean ± SD (n=5). Values at each time point not sharing a common letter within a sample are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test after log transformation. RD group (baseline) values are represented at 0h.

Table 4.5. Total retinol* levels in plasma (μmol/l), liver (nmol/g) and intestine (nmol/g) of RD rats gavaged with a single dose of astaxanthin.

Parameter	0h (RD)	2h	4h	8h	AUC[#]
Plasma	0.38 ± 0.036^a	0.47 ± 0.05^a	0.34 ± 0.04^a	0.39 ± 0.04^a	3.12
Liver	3.05 ± 0.34^a	3.12 ± 0.21^a	3.18 ± 0.3^a	2.98 ± 0.24^a	24.79
Intestine	2.99 ± 0.31^a	2.71 ± 0.23^a	2.80 ± 0.26^a	2.72 ± 0.23^a	22.25

*Total retinol=retinol + ½ retinyl palmitate, RD = retinol deficient.

[#]AUC=Area under the curve, Unit for AUC, plasma = pmol/ml/8h, liver = pmol/g/8h, intestine = nmol/g/8h, TG = mg/dl/8h.

Data represent mean ± SD (n=5). Values at each time point within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

Lutein

HPLC chromatograms of lutein and its isomer, zeaxanthin and retinol extracted from the plasma, liver and intestine of RD rats after gavage with lutein are shown in Figure 4.5 while the time course plasma and liver lutein response and plasma TG levels are shown in Figure 4.6. The time-course retinol levels in the plasma, liver and intestine of RD rats after gavage with lutein are given in Table 4.6. The results show that the plasma lutein levels were maximum 4h after gavage (36.8 pmol/ml) and remained detectable upto 8h (10.9 pmol/ml) but decreased markedly from 4th h. Corresponding to the increase in lutein levels, TG levels in plasma showed an increase ($p<0.05$) over 8h with maximum concentration at 4h (186.2 mg/dl) and AUC of 1242 pmol/ml/8h. Further, the AUC value of lutein in intestine (756.3 pmol/g/8h) is higher than liver (203.1 pmol/g/8h) and plasma (140.8 pmol/ml/8h) demonstrating the process of intestinal absorption and transfer of absorbed lutein to other tissues (Figure 4.6). It was evident that no significant change ($p>0.05$) in retinol levels was observed in plasma (0.42-0.39 μmol/l), liver (3.09-3.13 nmol/g) or intestine (3-2.7 nmol/g) over a period of 8h as compared to RD group (0.38, μmol/l, 3.1, 3 nmol/g).

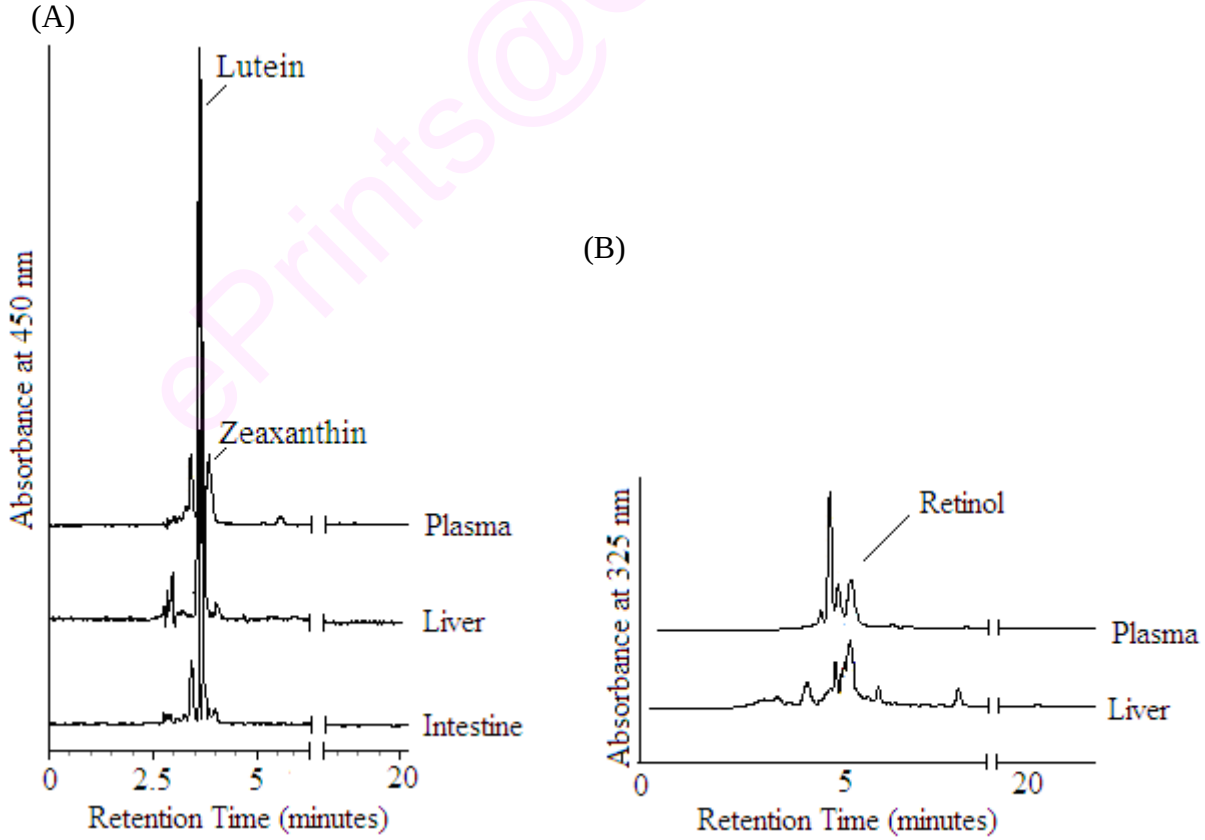


Figure 4.5. HPLC chromatogram of lutein and zeaxanthin (A) and retinol (B) extracted from rats at 4 h after gavage with a single dose of lutein.

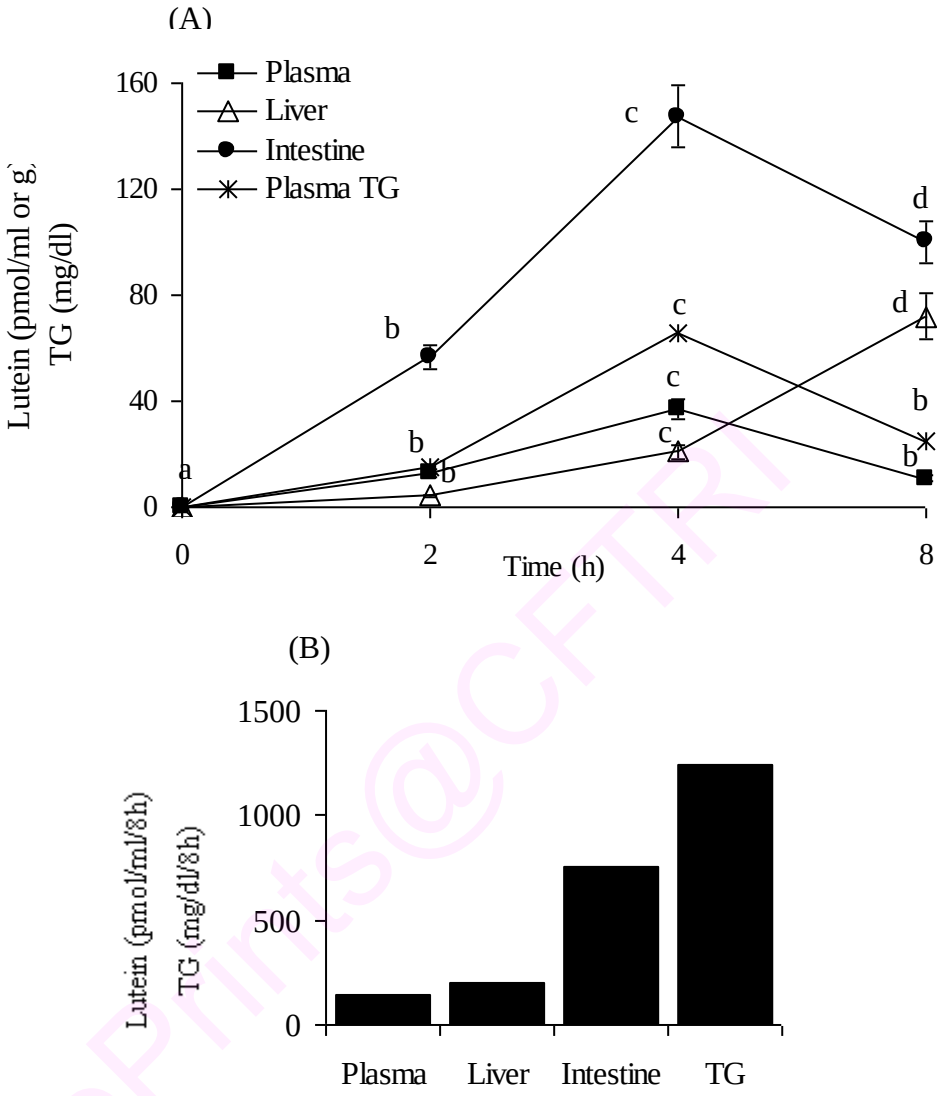


Figure 4.6. Lutein levels in plasma (pmol/ml), liver (pmol/g) and intestine (pmol/g) and triglycerides (TG) in plasma (mg/dl) (A) and area under the curve (AUC) values for lutein levels in plasma (pmol/ml/8h), liver, intestine (pmol/g/8h) and TG (mg/dl/8h) in plasma over 8h (B) of lutein fed rats. Data represent mean $\pm$ SD (n=5). Values at each time point within samples not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test after log transformation. RD group (baseline) value represents 0h.

Table 4.6. Total retinol levels in plasma (μmol/l), liver (nmol/g) and intestine (nmol/g) of RD rats gavaged with a single dose of lutein.

Parameter	0h (RD)	2h	4h	8h	AUC[#]
Plasma	0.38 ± 0.036^{a}	0.42 ± 0.03^{a}	0.45 ± 0.04^{a}	0.39 ± 0.03^{a}	3.35
Liver	3.05 ± 0.34^{a}	3.09 ± 0.23^{a}	3.10 ± 0.31^{a}	3.13 ± 0.25^{a}	24.79
Intestine	2.99 ± 0.31^{a}	2.98 ± 0.12^{a}	2.91 ± 0.22^{a}	2.67 ± 0.21^{a}	23.02

*Total retinol = retinol + ½ retinyl palmitate, RD = Retinol deficient.

[#]AUC=Area under the curve, Unit for AUC, plasma = pmol/ml/8h, liver = pmol/g/8h, intestine = nmol/g/8h, TG = mg/dl/8h.

Data represent mean ± SD (n=5). Values at each time point not sharing a common letter within a row are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

Fucoxanthin (FUCO)

Typical HPLC chromatogram of FUCO metabolites (fucoxanthinol, (FUOH) and amarouciaxanthin (AAx)) extracted from plasma and liver of rats fed FUCO is shown in Figure 4.7. Retinol levels in the plasma and liver of rats gavaged with FUCO is given in Table 4.7. The AUC values for the plasma and liver levels of FUCO metabolites are shown in Figure 4.8. The level of FUOH and AAx was found to peak at 6 h (1.1 pmol/ml) after gavage. Since no intact FUCO was detected in plasma and liver, the bioavailability of FUCO was calculated based on the levels of its metabolites FUOH and AAx. On gavage of FUCO, their levels were higher at 6 h (1.1 pmol/ml, 6 pmol/g) (Figure 4.8). Feeding FUCO to RD rats did not alter the plasma (0.58-0.62 pmol/ml) and liver (1.31-1.33 pmol/g) retinol levels ($p>0.05$) over 8 h as compared to RD group (0.53 pmol/ml, 0.96 pmol/g) (Table 4.6). Corresponding to the rise ($p<0.05$) in FUCO metabolites in plasma, there was a rise ($p<0.05$) in plasma TG levels, indicating the influx of FUCO from the intestine (Figure 4.8). Further, higher AUC value of FUCO in liver indicates that the intestinally absorbed FUCO may be stored in the liver (Figure 4.8).

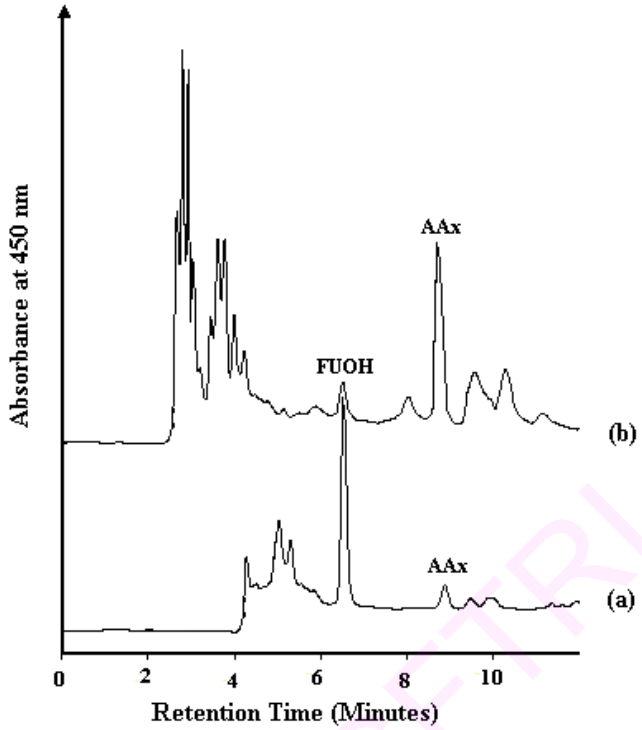


Figure 4.7. HPLC profile of fucoxanthin (FUCO) metabolites, fucoxanthinol (FUOH) and amarouciaxanthin (AAx) extracted from plasma (a) and liver (b) of rats at 6 h after gavage with FUCO.

Table 4.7. Total retinol levels in plasma (μmol/l) and liver (nmol/g) of RD rats gavaged with a single dose of fucoxanthin.

Parameter	0h (RD)	2h	4h	6h	8h	AUC[#]
Plasma	0.53±0.04[a]	0.58±0.04[a]	0.62±0.03[a]	0.62±0.05[a]	0.57±0.04[a]	4.74
Liver	0.96±0.05[a]	1.31±0.1[a]	1.32±0.05[a]	1.33±0.07[a]	1.32±0.05[a]	10.2

*Total retinol = retinol + ½ retinyl palmitate, RD = Retinol deficient.

[#]AUC = Area under the curve, plasma = pmol/ml/8h, liver = pmol/g/8h, TG = mg/dl/8h.

Data represent mean ± SD (n=5). Values at each time point within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA.

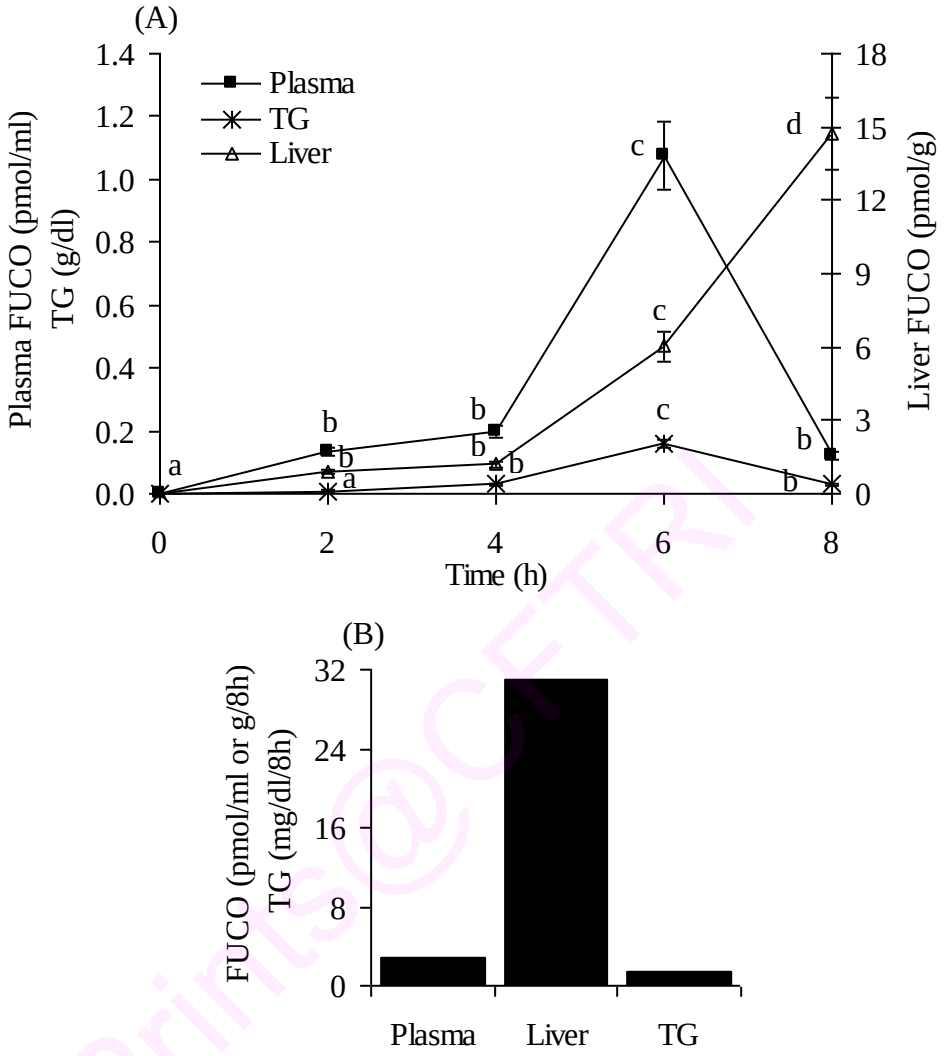


Figure 4.8. Fucoxanthin levels (fucoxanthinol + amarouciaxanthin) in plasma (pmol/ml) and liver (pmol/g), triglycerides (TG) in plasma (mg/dl) (A) and area under the curve (AUC) values for fucoxanthin levels in plasma (pmol/ml/8h), liver (pmol/g/8h) and plasma TG values (mg/dl/8h) over 8h (B) of fucoxanthin fed rats. Data represent mean ± SD (n=5). Values at each time point within a sample not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. Fucoxanthin values for RD group (baseline) values are represented at 0h. TG values are baseline corrected (120.8 mg/dl).

Comparative bioavailability and absorption kinetics of carotenoids

On gavage of a single dose of an equimolar concentration of β-carotene, lutein or astaxanthin, it was observed that the plasma, liver and intestine levels of astaxanthin were higher from 2 to 8 h after gavage followed by lutein and β-carotene. The comparative plasma AUC values of β-carotene, astaxanthin, lutein and fucoxanthin are shown in Figure 4.9. In accordance with the levels of intact carotenoids evaluated over a period of 8 h, the plasma AUC value for astaxanthin (356.5 pmol/ml/8h) was higher than β-carotene (not detected), lutein (140.8 pmol/ml/8h) and FUCO (4.74 pmol/ml/8h). β-Carotene on the other hand was not detected in plasma and its AUC level in liver was lower (21.4 pmol/g/8h) than other carotenoids.

The AUC values of plasma retinol (Figure 4.9) were significantly higher for β-carotene group while no difference was found among astaxanthin, lutein and fucoxanthin groups, indicating that bioconversion of β-carotene to retinol was significantly higher (ability to form retinol) than astaxanthin and lutein. The increased retinol levels in β-carotene group corresponded with increased monooxygenase activity (100.6-145.6 pmol retinal/h/mg protein) (Discussed later in this chapter). The results thus show that bioavailability of astaxanthin was higher than lutein and fucoxanthin while the bioefficacy was in the order of β-carotene > astaxanthin = lutein = fucoxanthin.

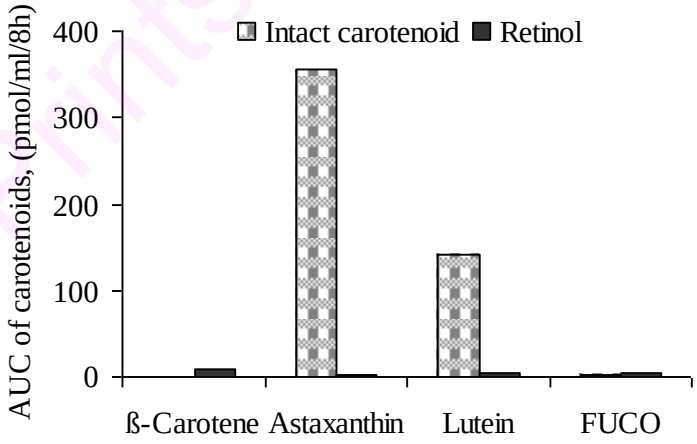


Figure 4.9. Area under the curve (AUC) values for intact carotenoid and retinol levels in plasma (pmol/ml/8h) of β-carotene, astaxanthin, lutein and fucoxanthin (FUCO) groups over a period of 8h.

The absorption kinetics of astaxanthin and lutein are shown in Figure 4.10 and Table 4.8. The absorption kinetic parameters of carotenoids revealed that the rate of

absorption and clearance (pmol/ml/h) from the absorption maxima are different for astaxanthin (20.4, 13.7), lutein (9.2, 6.5) and fucoxanthin (0.18, 0.96) (Table 4.8). Absorption kinetics of β-carotene is not presented, as it was not detected in plasma because of its complete conversion to retinol. Time taken for 50% absorbance ($t_{1/2A}$) and clearance ($t_{1/2C}$) was 2 hours for astaxanthin and lutein and time at which maximum concentration was recorded (t_{max}) was 4 h for both the groups whereas, $t_{1/2A}$, $t_{1/2C}$ and t_{max} was 3, 1 and 6 h for FUCO respectively. The AUC value (pmol/ml/h) was 44.6 for astaxanthin, 17.6 for lutein and 2.93 for FUCO. Therefore, bioavailability, rate of absorption and clearance of astaxanthin was greater than lutein, followed by FUCO.

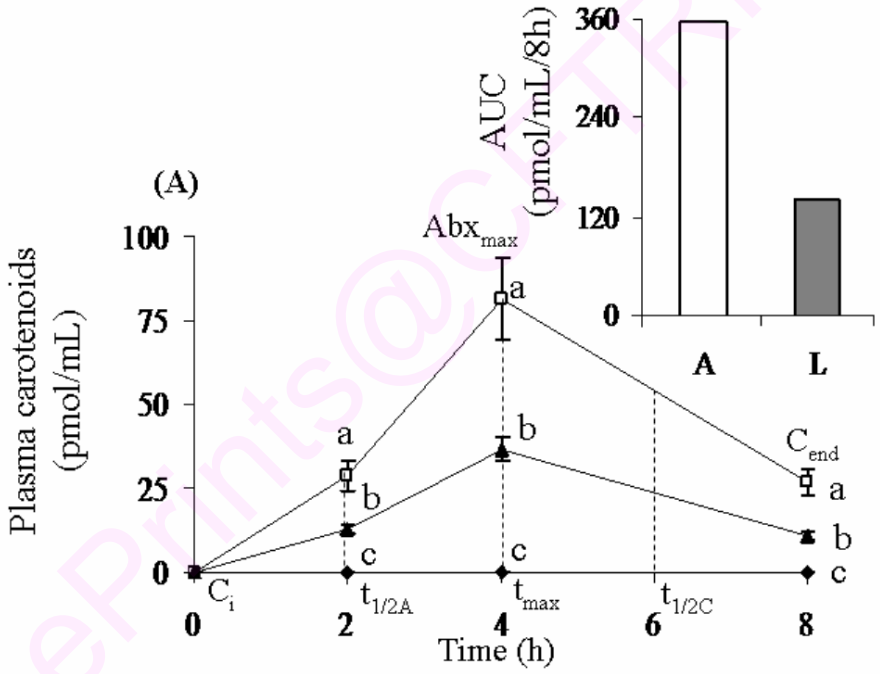


Figure 4.10. Postprandial plasma levels and kinetic characteristics of astaxanthin (□) and lutein (▲) after gavage with a single dose of carotenoids dissolved in peanut oil to respective groups. Data represent mean ± SD (n=5). Values at each time point within a group not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. Carotenoids were not detected at 0h. Inset: area under the curve (AUC) values for astaxanthin and lutein over a period of 8h. A=Astaxanthin, L=Lutein. Abx_{max} = maximum absorption, C_{end} = carotenoid level at the end of clearance phase, C_i = carotenoid level at base line, t = time, A = absorption phase, C = clearance phase.

Table 4.8. Absorption kinetic parameters of astaxanthin, lutein and FUCO over 8 h in RD rats.

Parameter	Astaxanthin	Lutein	FUCO
Abx_{max} (pmol/ml)	81.6 ± 10.2*	36.8 ± 7.5*	1.08 ± 0.01*
C_{end} (pmol/ml)	27.03 ± 2.35*	10.9 ± 1.22*	0.12 ± 0.009*
C_i (pmol/ml)	0.0	0.0	0.0
t_{max} (h)	4	4	6
t_{end} (h)	8	8	8
$t_{baseline}$ (h)	0	0	0
Rate of absorption (pmol/ml/h)	20.41	9.19	0.18
Rate of clearance (pmol/ml/h)	13.65	6.46	0.96
$T_{1/2A}$ (h)	2	2	3
$T_{1/2C}$ (h)	2	2	1
AUC (pmol/ml/8h)	356.6	140.8	2.93

* Data represent mean ± SD (n=5). Abx_{max} = maximum absorption of carotenoid, C_{end} = carotenoid level at end of clearance phase, C_i = carotenoid level at 0h (base line), t = time, A = absorption phase, C = clearance phase, AUC = area under the curve. Refer Figure 4.9 for graphical representation of parameters.

Repeated dose study: Bioavailability and bioefficacy of β-carotene, astaxanthin and lutein

RD rats were gavaged with an equal concentration of β-carotene, astaxanthin or lutein (0.1 mg/rat/day) for 7- and 15-days and sacrificed at the end of respective time intervals. The data was compared with the baseline values (RD group) while the data of RD and control groups were compared. The lower plasma retinol level of RD group (0.3 µmol/l) than the control groups (0.733 mmol/l) indicates the retinol deficient condition of the rats. Rats were weighed weekly and at the end of 7- and 15-days while food intake was measured daily. Results on the mean food

intake and gain-in-body weight of the control, experimental rats are shown in Figure 4.11. Retinol deficiency resulted in lowered body weight by 25% than the control while liver weight was unaffected. However, retinol level in the liver was depleted ($p<0.05$) to 2 nmol/g as a result of retinol deficiency when compared to control (7.7 µmol/g).

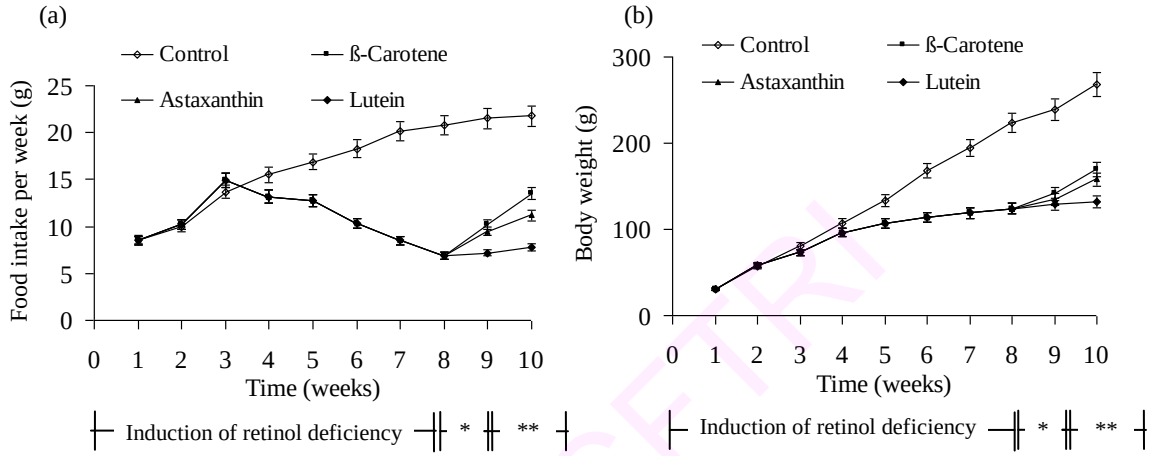


Figure 4.11. Effect of feeding vitamin A deficient diet on food intake (a) and growth (b) of rats and subsequent gavages with carotenoids, in comparison with control group that received retinol sufficient diet. Values are mean ± SD (n=5). * 7-days gavage. **15-days gavage.

β-Carotene

HPLC chromatogram of β-carotene and retinol extracted from the plasma, liver and intestine of RD rats gavaged with β-carotene for 7- and 15-days was found to be similar. Hence, the chromatograms obtained from the 15-days samples are shown in Figure 4.12. The β-carotene levels in plasma, liver and intestine after 7- and 15-days of gavage are shown in Figures 4.13 while retinol levels are given in Table 4.9. No β-carotene was detected in baseline group, while repeated intubations of β-carotene for 7- and 15-days resulted in an elevated β-carotene level in plasma (not detected, 0.37 µmol/l), liver (0.03, 1.32 µmol/g) and intestine (1.4, 3.1 µmol/g). Similarly, the retinol levels in plasma (5.7, 8.8 µmol/l), liver (12.6, 45.8 nmol/g) and intestine (10.7, 25.4 nmol/g) were increased from baseline values (0.3 µmol/l, 2 nmol/g and 3.1 nmol/g) after 7- and 15-days (Table 4.9). Results show that the retinol levels in plasma, liver and intestine

were higher ($p<0.05$) by >19, >6 and >3 fold respectively than the RD group. Further, the results show a time dependent increase in β-carotene and retinol levels in plasma, liver and intestine.

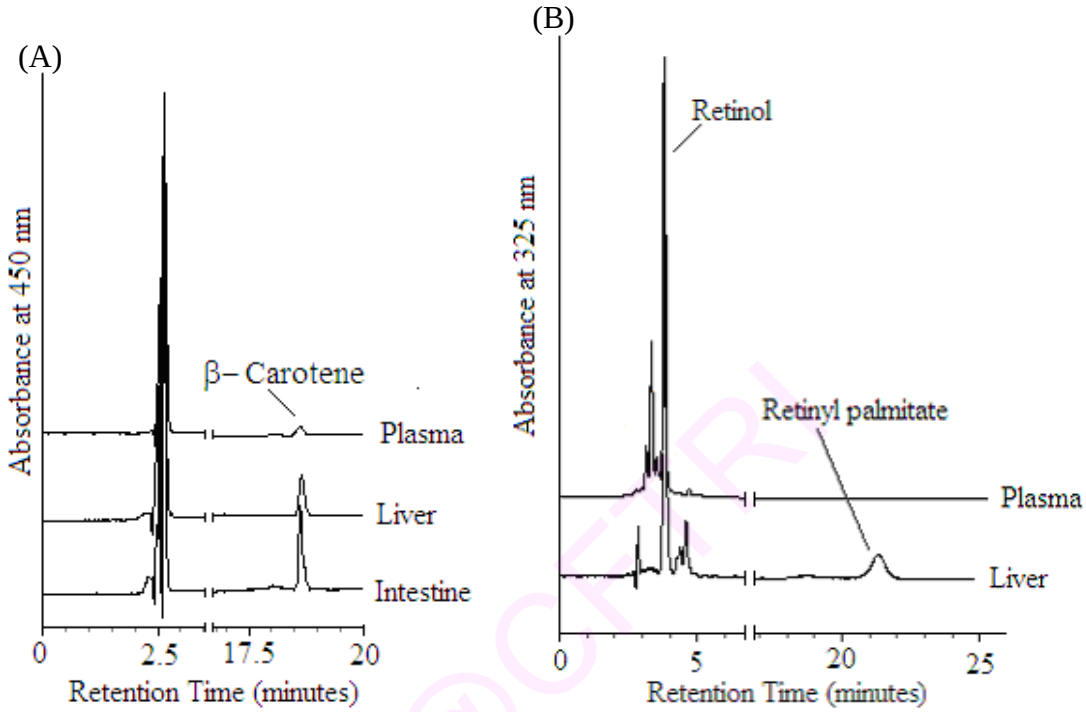


Figure 4.12. HPLC profile of β-carotene (A) and retinol (B) extracted from plasma, liver and intestine of RD rats gavaged with β-carotene for 15-days.

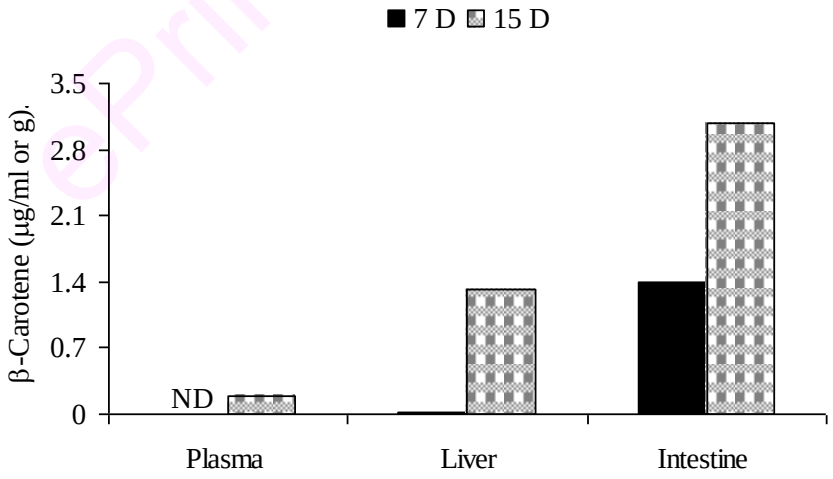


Figure 4.13. β-carotene levels in plasma (µg/ml), liver (µg/g) and intestine (µg/g) of RD rats gavaged with β-carotene for 7- and 15-days. Data represent mean ± SD (n=5), D = days.

Table 4.9. Total retinol* levels in plasma (μmol/l), liver (nmol/g) and intestine (nmol/g) of RD rats gavaged with β-carotene for 7- and 15-days.

Parameter	RD (baseline)	7-Days	15-Days
Plasma	0.3 ± 0.02^{a}	5.7 ± 0.3^{b}	8.8 ± 0.7^{c}
Liver	2.0 ± 0.02^{a}	12.6 ± 1.5^{b}	45.77 ± 5.82^{c}
Intestine	3.1 ± 0.03^{a}	10.7 ± 1.1^{b}	25.42 ± 2.95^{c}

*Total retinol = retinol + ½ retinyl palmitate, RD = Retinol deficient.

Data represent mean ± SD (n=5). Values between the groups not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

Astaxanthin

HPLC chromatogram of astaxanthin at 478 nm and β-carotene at 450 nm, extracted from the plasma, liver and intestine of RD rats gavaged with astaxanthin for 15-days are shown in Figure 4.14. The level of astaxanthin in plasma (0.93, 2.2 μg/ml), liver (0.22, 3.5 μg/g) and intestine (5.9, 8.9 μg/g) of the rats after 7- and 15-days feeding of astaxanthin is given in Table 4.10. Interestingly, β-carotene was also detected in the plasma (0.04, 0.94 μg/ml), liver (0.12, 1.2 μg/g) and intestines (0.4, 1.4 μg/g) of both groups (Table 4.10) indicating the conversion of astaxanthin to β-carotene under retinol deficiency. This corresponded with elevated retinol levels in plasma (4.9, 3.1 μmol/l), liver (5.3, 20.9 nmol/g) and intestine (7.3, 16 nmol/g) as compared to RD group (0.3 μmol/l, 2 nmol/g, 3.2 nmol/g). The percent increase in astaxanthin, β-carotene and retinol levels in the plasma, liver and intestine after 15-days gavages over 7-days was 63, 95 and 40% respectively (Figure 4.15). In addition, the β-carotene values in plasma, liver and intestine for 15-days samples was higher by 94, 86 and 50% over 7-days. Comparison of plasma retinol levels after 15-days revealed a 64% decrease while it was higher in the liver and intestine by 83 and 68% respectively than the 7-days repeated gavage with astaxanthin.

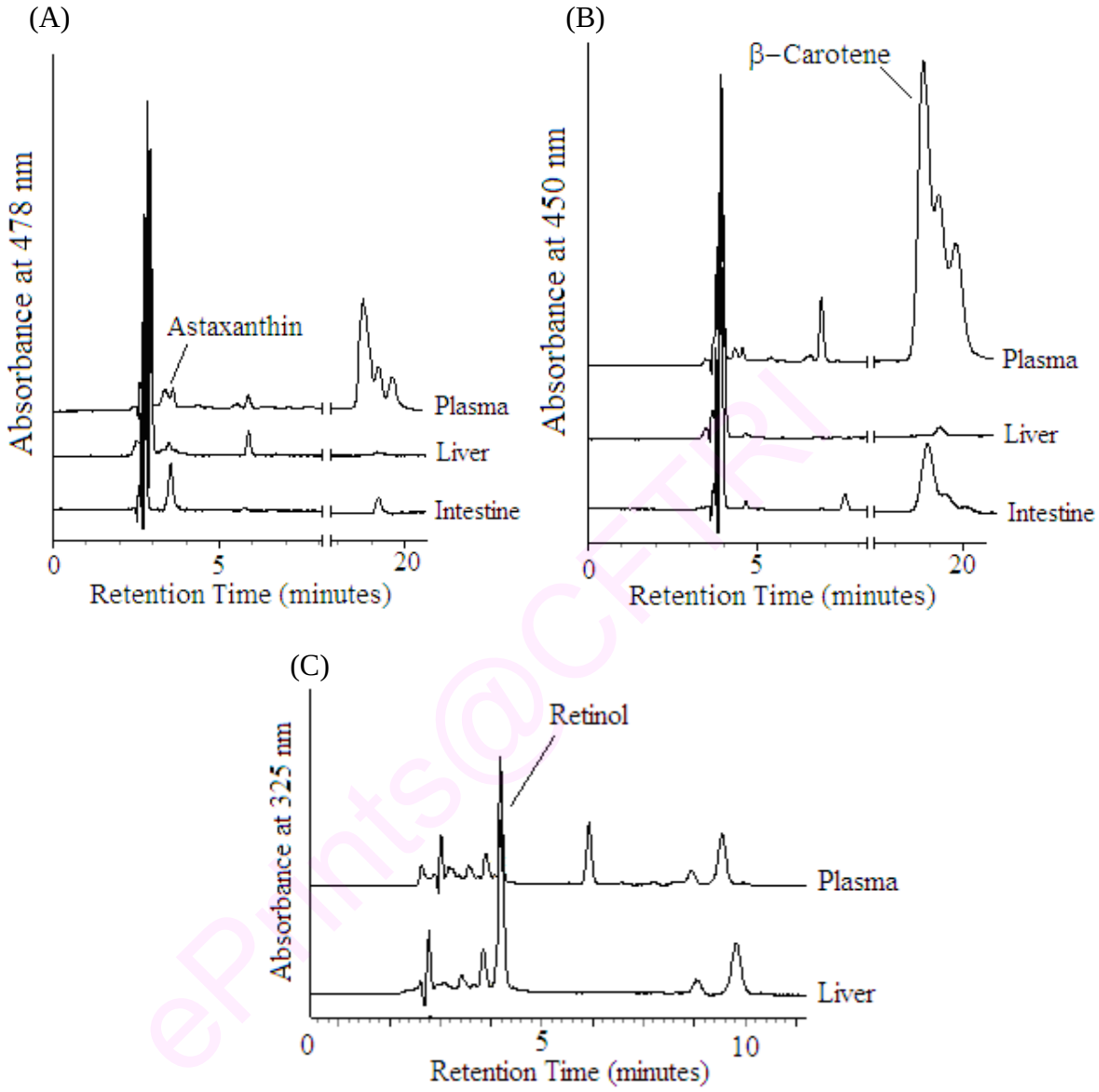


Figure 4.14. HPLC profile of astaxanthin (A), β-carotene (B) and retinol (C) extracted from plasma, liver and intestine of RD rats gavaged with astaxanthin for 15-days.

Table 4.10. Astaxanthin (µg/ml or g), β-carotene (µg/ml or g) and total retinol* (nmol/ml or g) levels in plasma, liver and intestine of RD rats gavaged with astaxanthin for 7- and 15-days.

Parameter	Astaxanthin		β-Carotene		Retinol**	
	7 days	15 days	7 days	15 days	7 days	15 days
Plasma	0.9 ± 0.05^{a}	2.4 ± 0.1^{b}	0.03 ± 0.001^{p}	0.5 ± 0.03^{q}	4.6 ± 0.3^{x}	2.8 ± 0.2^{y}
Liver	0.2 ± 0.01^{a}	3.9 ± 0.2^{b}	0.1 ± 0.001^{p}	0.7 ± 0.05^{q}	3.3 ± 0.2^{x}	18.9 ± 1.5^{y}
Intestine	5.9 ± 0.3^{a}	9.9 ± 0.8^{b}	0.4 ± 0.03^{p}	0.8 ± 0.04^{q}	4.2 ± 0.3^{x}	13.0 ± 1.1^{y}

*Total retinol = retinol + ½ retinyl palmitate. **Baseline corrected values (Plasma = 0.3 ± 0.02 nmol/ml, liver = 2 ± 0,02 nmol/g, intestine = 3.1 ± 0.03 nmol/g).

Data represent mean ± SD (n=5). Values between 7- and 15-days within a group not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA.

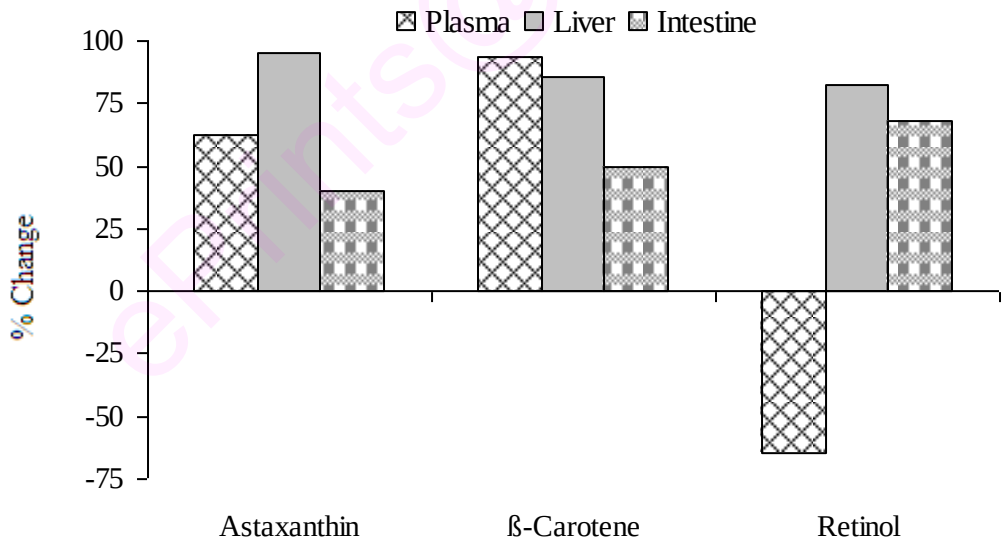


Figure 4.15. Percent change in astaxanthin, β-carotene and retinol levels after 15-days over 7-days gavages of astaxanthin.

Lutein

The HPLC elution profile and levels of lutein, zeaxanthin and retinol in plasma, liver and intestine of RD rats gavaged with lutein for 7- and 15-days are shown in Figure 4.16 and Table 4.11. The postprandial lutein (lutein + zeaxanthin) level in plasma, liver and intestine after 7-days of intubation was 3.3 µg/ml, 0.2 µg/g and 9.9 µg/g respectively, while it was 4.8 µg/ml, 8.9 µg/g and 11.7 µg/g after 15-days of intubation. It is seen from the results that the absorption and tissue accumulation of lutein is time dependent. No lutein was detected in the plasma of RD (baseline) group. Similarly, no change in the retinol level was evident in plasma, (0.6, 0.3 µmol/l), liver (3.1, 3.8 nmol/g) and intestine (3.9, 4.1 nmol/g) after 7- and 15-days of intubation of lutein, indicates that no retinol was formed from lutein. Further, the percent increase in the plasma, liver and intestinal lutein level after 15-days over 7-days was 32, 98 and 15% (Figure 4.17).

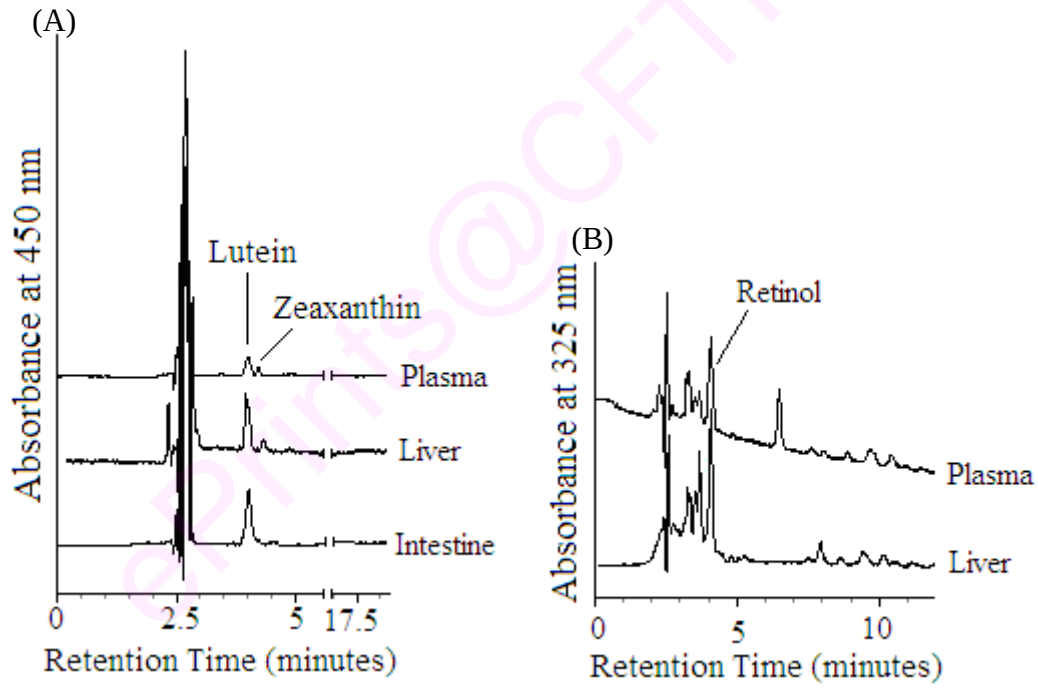


Figure 4.16. HPLC profile of lutein and zeaxanthin extracted from plasma, liver and intestine (A) and retinol in plasma and liver (B) of RD rats gavaged with lutein for 15-days.

Table 4.11. Lutein (μg/ml or g) and total retinol* (nmol/ml or g) levels in plasma, liver and intestine of RD rats gavaged with lutein for 7- and 15-days.

Parameter	Lutein		Retinol**	
	7 days	15 days	7 days	15 days
Plasma	3.26 ± 0.2^{a}	4.76 ± 0.3^{b}	0.6 ± 0.04^{p}	0.3 ± 0.01^{p}
Liver	0.21 ± 0.01^{a}	8.91 ± 0.6^{b}	1.1 ± 0.01^{p}	1.82 ± 0.1^{p}
Intestine	9.99 ± 0.7^{a}	11.73 ± 0.9^{a}	0.79 ± 0.06^{p}	0.97 ± 0.01^{p}

*Total retinol=retinol + ½ retinyl palmitate. **Baseline corrected values (Plasma = 0.3 ± 0.02 nmol/ml, liver = 2 ± 0,02 nmol/g, intestine = 3.1 ± 0.03 nmol/g).

Data represent mean ± SD (n=5). Values between 7- and 15-days not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA.

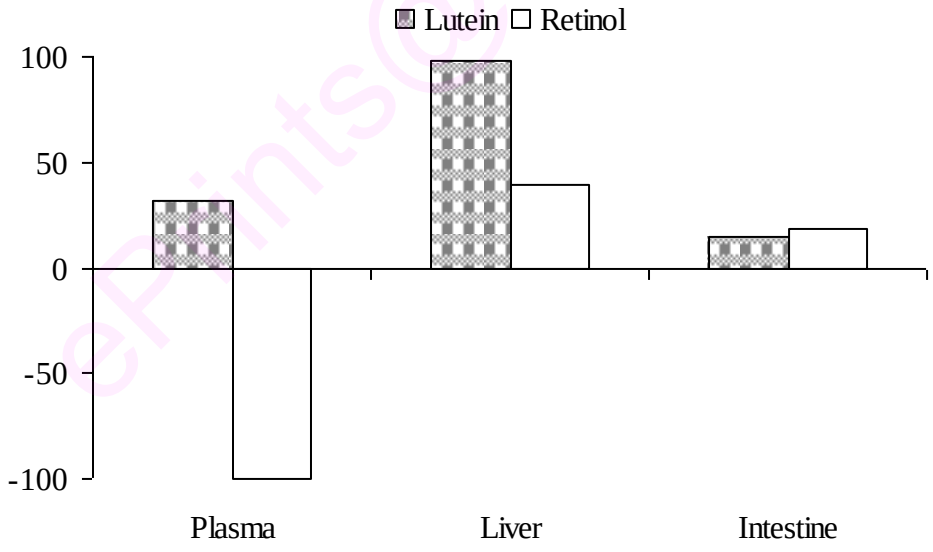


Figure 4.17. Percent change in lutein and retinol levels after 15-days over 7-days gavages of lutein.

Comparative bioavailability and bioefficacy of carotenoids

As a result of gavages of β-carotene for 7- and 15-days, the plasma retinol level of β-carotene group rose by 19 and 29 fold respectively and no β-carotene was detected in the 7-day samples. In addition, a 6 and 22 fold increase in retinol levels and a low concentration of β-carotene were detected in liver after 7- and 15-days respectively. Astaxanthin gavage to RD rats for 7- and 15-days resulted in elevation of retinol levels in plasma (16, 10 fold) and liver (2, 10 fold). Interestingly, apart from astaxanthin in plasma (0.9, 2.4 μg/mL) and liver (0.2, 3.9 μg/g), β-carotene was also detected in plasma (0.034, 0.51 μg/mL) and liver (0.12, 0.64 μg/g) of the group fed astaxanthin for 7- and 15-days. No change in the plasma (0.9, 0.5 μmol/l) and liver (2.1, 3.8 nmol/g) retinol levels were found in the case of lutein fed groups. The effect of retinol deficiency and subsequent feeding of carotenoids on the activity of intestinal monooxygenase is given later in the chapter. In brief, the activity of enzyme was higher in β-carotene (154.5, 169 pmol retinal/h/mg protein) and astaxanthin (146, 150.3 pmol retinal/h/mg protein) groups after 7- and 15-days as compared to RD group (47.2 pmol retinal/h/mg protein). Whereas, intubation of lutein did not show any change in the enzyme activity (44.4, 47.3 pmol retinal/h/mg protein) compared to the RD group. The results indicate conversion of β-carotene and astaxanthin to retinol as evidenced by the enhanced monooxygenase activity by 70 and 72% (β-carotene) and 68 and 69% (astaxanthin) as compared to RD group. Based on the results, the pathway for formation of retinol from astaxanthin is proposed and is described in detail in Chapter 5. Based on the retinol formed from carotenoids, the bioefficacy was in the order of β-carotene > astaxanthin > lutein.

Dietary Study: Bioavailability and bioefficacy of β-carotene, astaxanthin and lutein

Induction of retinol deficiency resulted in depleted retinol levels in plasma and liver of RD group (0.4 μmol/l, 2.96 nmol/g) as compared to control group (132.5 μmol/, 255.3 nmol/ml). RD rats were fed semi-synthetic diet supplemented with dried carrot (*Daucous carota)* powder (β-carotene source), purified astaxanthin and Indian dill leaves *(Peucedanum sowa)* powder (source of lutein) for 20 days. The food intake and gain-in-body weight of the experimental and control groups is shown in Figure 4.18. Retinol deficiency resulted in >25% lower ($p<0.05$) gain-in-body weight as compared to the control group. However, on feeding carotenoid or carotenoid sources, the gain in body weight was increased similarly in all experimental groups with no significant difference. The retinol levels in the plasma of RD rats were further lowered to 0.3

μmol/l during the 20 days, whereas, no change in retinol levels was observed in control (128.6 μmol/l) group.

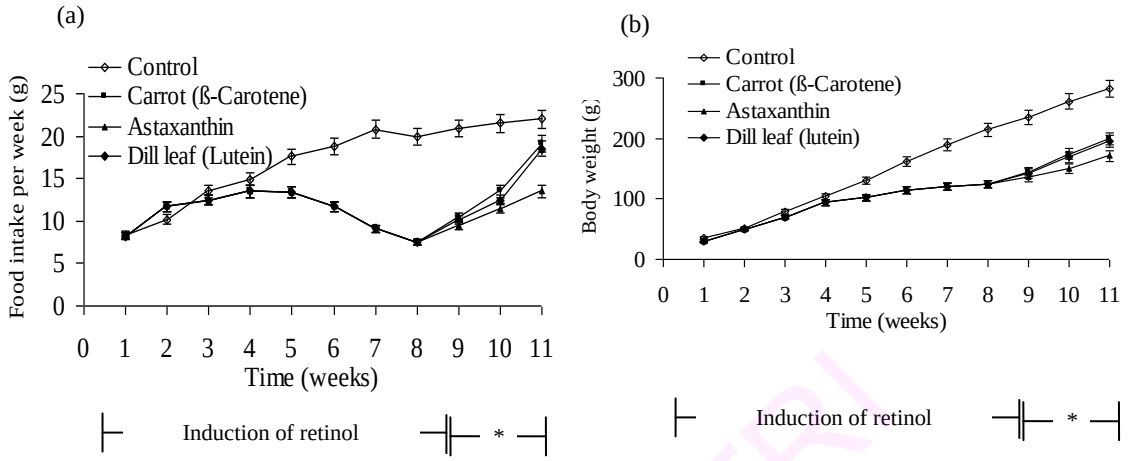


Figure 4.18. Effect of retinol deficiency and subsequent feeding with carotenoids supplemented diet, in comparison with control group on food intake (a) and growth (b) of rats. Values are mean ± SD (n=5). *Fed diet supplemented with carotenoids for 20 days.

β-Carotene

The RD rats were fed with β-carotene rich carrot powder supplemented diet for 20 days. HPLC chromatograms of the β-carotene extracted from the plasma, liver and intestine of the rats are shown in Figure 4.19. The β-carotene content of the carrot powder was estimated by HPLC prior to supplementation and it was 42 mg/ kg of the diet. In addition to the β-carotene, the carrot powder also contained α-carotene (29 mg/kg) and lutein (7.6 mg/ kg). The level of absorbed β-carotene in the plasma, liver and intestine of rats fed carrot powder supplemented diet is shown in Figure 4.20. The level of β-carotene, α-carotene and lutein in plasma (29.9, 13.7, 6.02 ng/ml), liver (89.1, 40.3, 16.8 ng/g) and intestine (121.4, 60.5, 60.6 ng/g) were detected whereas no carotenoids were detected in the RD group. Similarly, the retinol levels in plasma (10.9 μmol/l), liver (90.7 nmol/g) and intestine (65.7 nmol/g) of the rats were higher ($p<0.05$) after 20 days of feeding as compared to RD group (0.4. μmol/l, 3 nmol/g, 3.1 nmol/g) (Table 4.12). The results show that the plasma retinol level was higher ($p<0.05$) by 88% after 10 days feeding while, its level in plasma, liver and intestine was higher ($p<0.05$) by 96, 97 and 95% respectively after 20 days feeding than the RD group. Further, there were 68% higher ($p<0.05$) retinol levels in plasma of the 20-day samples over the 10-day samples.

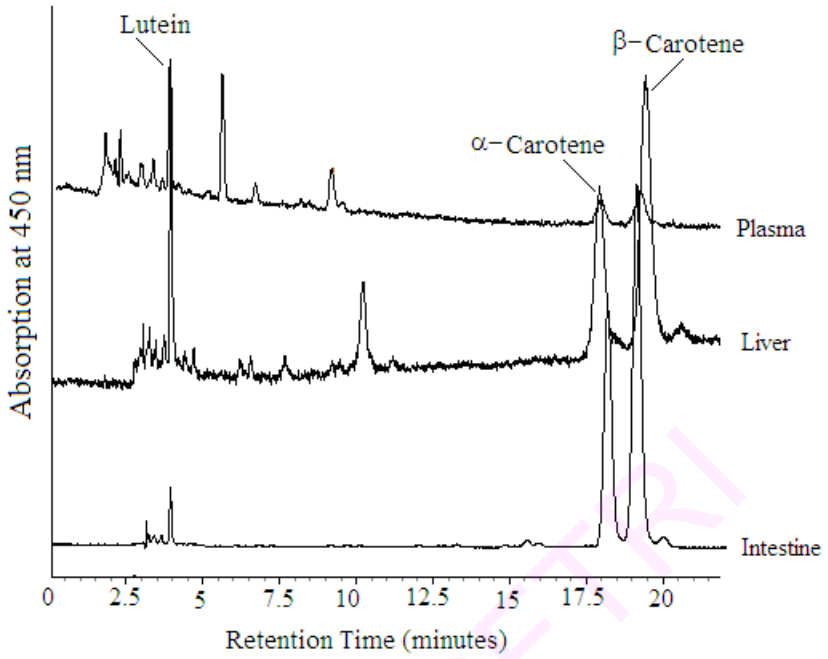


Figure 4.19. HPLC elution profile of carotenoids extracted from plasma, liver and intestine of RD rats fed diet supplemented with carrot powder for 20 days.

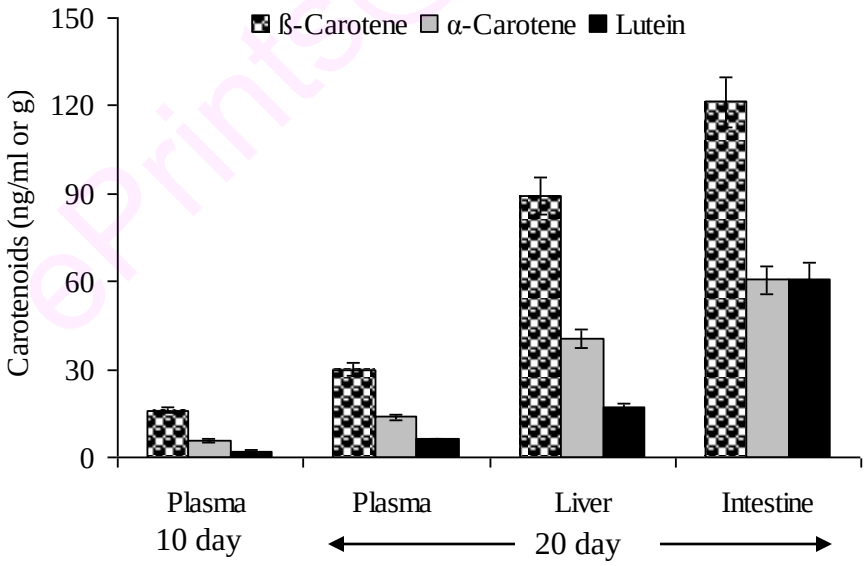


Figure 4.20. β-Carotene, α-carotene and lutein levels in plasma (ng/ml), liver (ng/g) and intestine (ng/g) of RD rats fed diet supplemented with carrot powder (β-carotene source) for 20 days. Data represent mean ± SD (n=5).

Table 4.12. Total retinol* levels in plasma (μmol/l), liver (nmol/g) and intestine (nmol/g) of RD rats fed diet supplemented with carrot powder (β-carotene source) for 20 days.

Samples	Duration of feeding	Groups	
		RD (baseline)	Carrot powder fed
Plasma	10 days	0.42 ± 0.04^{a}	3.37 ± 0.4^{b}
Plasma	20 days	0.40 ± 0.03^{a}	10.89 ± 1.2^{b}
Liver	20 days	2.96 ± 0.3^{a}	90.66 ± 10.4^{b}
Intestine	20 days	3.11 ± 0.3^{a}	65.70 ± 7.2^{b}

*Total retinol = retinol + ½ retinylpalmitate, data represent mean ± SD (n=5).

Values between the groups not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA. RD = retinol deficient.

Astaxanthin

Purified astaxanthin was supplemented with diet and fed to RD rats for a period of 20 days. After 10 days of feeding, blood was drawn from the orbital plexus of the eye for astaxanthin estimation while animals were sacrificed after 20 days of feeding for analysis of blood and tissue astaxanthin. The level of astaxanthin in the diet was 42 mg/kg and it was confirmed by HPLC prior to animal feeding. HPLC chromatograms of the astaxanthin and newly formed β-carotene from astaxanthin in plasma, liver and intestine of the rats are shown in Figure 4.21. The level of astaxanthin and β-carotene in plasma (72.1, 4.3 ng/ml), liver (142.9, 10.1 ng/g) and intestine (278.7, 20.0 ng/g) after 20 days feeding of astaxanthin was higher than the RD group (Figure 4.22). Interestingly, the results showed a detectable level of β-carotene in plasma and tissues of astaxanthin fed group indicating the formation of β-carotene from astaxanthin under retinol deficiency. This was not true in the case of control group. Astaxanthin feeding for 20 days also resulted in higher plasma (0.89 μmol/l), liver (4.6 nmol/g) and intestinal (6.5 nmol/g) retinol levels ($p<0.05$) than RD group (0.4 μmol/l, 3 nmol/g, 3.1 nmol/g) (Table 4.13). The increased retinol levels ($p<0.05$) indicate that retinol may be derived from astaxanthin and this may be due to the conversion of astaxanthin to β-carotene, which may have been cleaved to retinol to meet the retinol requirement, resulting in the higher retinol levels. On comparison, it was found that retinol levels increased by after 10 and 20 days of astaxanthin feeding, the plasma, liver and

intestine increased ($p<0.05$) by 29% after 10 days feeding and by 56, 35 and 52 % respectively after 20 days feeding over RD group. Further, the plasma retinol levels after 20 day feeding was higher by 33% over 10 days indicating a time dependent conversion of astaxanthin to retinol and β-carotene.

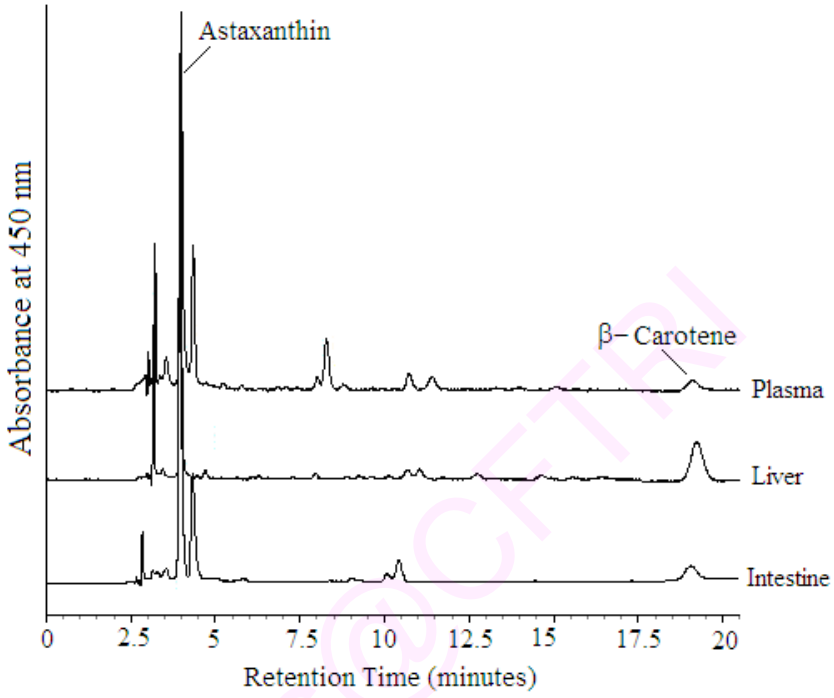


Figure 4.21. HPLC profile of astaxanthin and β-carotene extracted from plasma, liver and intestine of RD rats fed diet supplemented with astaxanthin for 20 days.

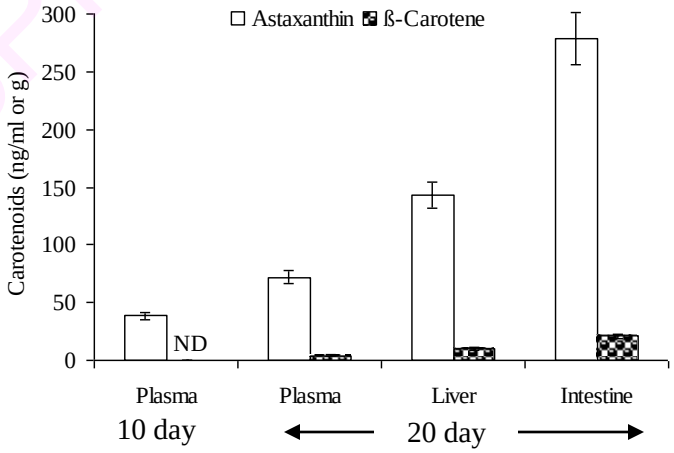


Figure 4.22. Astaxanthin and β-carotene levels in plasma (ng/ml), liver (ng/g) and intestine (ng/g) of RD rats fed diet supplemented with astaxanthin for 20 days. Data represent mean ± SD (n=5). ND = Not detected.

Table 4.13. Total retinol* levels in plasma (μmol/l), liver (nmol/g) and intestine (nmol/g) of RD rats fed diet supplemented with astaxanthin for 20 days.

Sample	Duration of feeding (days)	Groups	
		RD (baseline)	Astaxanthin fed
Plasma	10	0.42 ± 0.04^{a}	0.59 ± 0.06^{a}
Plasma	20	0.40 ± 0.03^{a}	0.89 ± 0.1^{b}
Liver	20	2.96 ± 0.3^{a}	4.59 ± 0.5^{b}
Intestine	20	3.11 ± 0.3^{a}	6.51 ± 0.5^{b}

*Total retinol=retinol + ½ retinyl palmitate, data represent mean ± SD (n=5).

Values within a row and between the groups not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA.

Lutein

Diet supplemented with powdered dill leaves *(P. sowa)* as lutein source was fed to RD rats for 20 days. The dill leaf powder was estimated for the lutein content of (42 mg/kg diet) by HPLC prior to use. HPLC chromatogram of the lutein and β-carotene extracted from the plasma, liver and intestine of the dill leaf powder fed rats are shown in Figure 4.23 while data on their levels are shown in Figure 4.24. There is a detectable level of β-carotene in dill leaf groups, which may be due to the presence of β-carotene in the leaves. On feeding dill leaf powder for 10 days, the plasma lutein and β-carotene levels were 19.6 and 8.4 ng/ml while, after 20 days feeding, their levels were further increased in plasma (43.9, 20.7 ng/ml), liver (159.8, 58.6 ng/g) and intestine (286.5, 101.3 ng/g) (Figure 4.24). Similarly, after 20 days of feeding, the retinol levels in plasma (4.4 μmol/l), liver (59.3 nmol/g) and intestine (50 nmol/g) of the rats was found to be higher as compared to RD group (0.4 μmol/l, 3 nmol/g, 3.1 nmol/g) (Table 4.14). The rise in retinol levels after 10 day in plasma was 67% while it was 91, 95 and 94 % after 20 days in plasma, liver and intestine respectively over RD group. The plasma retinol level after 20 days feeding of lutein source was found to be higher by 71% over 10 day plasma samples.

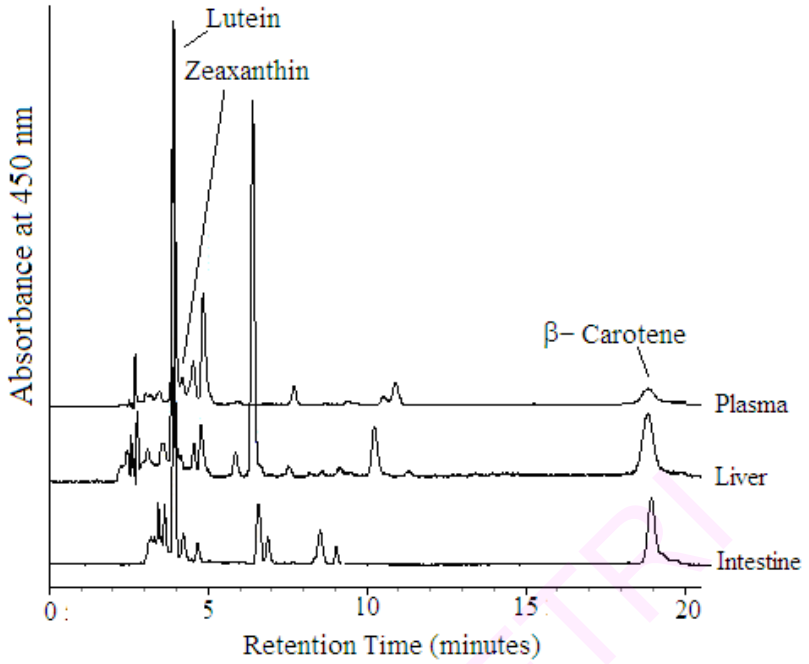


Figure 4.23. HPLC elution profile of lutein, zeaxanthin and β-carotene extracted from plasma, liver and intestine of RD rats fed diet supplemented with dill leaf powder for 20 days.

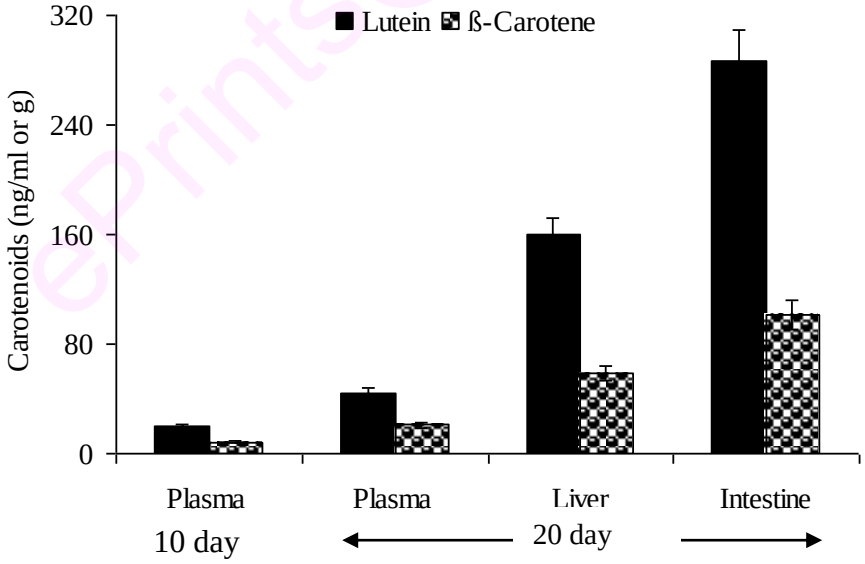


Figure 4.24. Lutein and β-carotene levels in plasma (ng/ml), liver (ng/g) and intestine (ng/g) of RD rats fed diet supplemented with dill leaf powder as lutein source for 20 days. Data represent mean ± SD (n=5).

Table 4.14. Total retinol* levels in plasma (μmol/l), liver (nmol/g) and intestine (nmol/g) of RD rats fed diet supplemented with dill leaf powder for 20 days.

Parameter	Feeding duration	Groups	
		RD (baseline)	Dill leaf powder fed
Plasma 10 D	10 days	0.42 ± 0.04^{a}	1.29 ± 0.1^{b}
Plasma 20 D	20 days	0.40 ± 0.03^{a}	4.44 ± 0.4^{b}
Liver	20 days	2.96 ± 0.3^{a}	59.29 ± 0.6^{b}
Intestine	20 days	3.11 ± 0.3^{a}	49.98 ± 0.6^{b}

*Total retinol=retinol + ½ retinyl palmitate, data represent mean ± SD (n=5).

Values at between the groups not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

Comparative bioavailability and efficacy of dietary carotenoids

Dietary feeding of carrot powder for 20 days to RD rats resulted in bioavailability of β-carotene and its conversion to retinol. The plasma levels of β-carotene and retinol was 29.9 ng/ml and 10.9 μmol/l, whereas, their level in liver was 89.1 ng/g and 90.7 nmol/g respectively. The level of intact β-carotene and retinol in the intestine of the rats was 121.4 ng/g and 65.7 nmol/g. The presence of β-carotene in the intestine indicates its absorption from the diet. The plasma, liver and intestinal levels of astaxanthin in rats after 20 days of feeding diet supplemented with astaxanthin was 72.1 ng/ml, 142.9 ng/g and 278.7 ng/g respectively. In addition, there was a detectable level of β-carotene in plasma (4.3 ng/ml), liver (10.1 ng/g) and intestine (200 ng/g). The presence of β-carotene in the astaxanthin fed group indicates its conversion to retinol to meet its requirement. Interestingly, the levels of retinol in plasma (0.89 μmol/l) and liver (4.6 nmol/g) were higher than the RD rats, which indicate the conversion of astaxanthin to retinol via β-carotene. On dietary feeding of dill leaves for 20 days, the plasma, liver and intestinal levels of lutein was 43.9 ng/ml, 159.8 ng/g and 286.5 ng/g respectively. The results show that the level of β-carotene in plasma and other tissues covered in this study was in the order of carrot powder fed group > dill leaf powder fed group > astaxanthin fed group. Whereas, the lutein level was in the order of dill leaf fed group > carrot powder fed group and no lutein was detected in astaxanthin group. In contrast, the levels of retinol in plasma and tissues was in the order of carrot powder fed

group > dill leaf fed powder fed group > astaxanthin fed group. Based on the results with respect to plasma levels of carotenoids and retinol from the dietary sources, the bioavailability and bioefficacy (retinol formation) of dietary carotenoids was in the order of carrot powder > dill leaves > astaxanthin.

The β-carotene, 15-15'-monooxygenase is involved in the cleavage of β-carotene to retinal, which is subsequently converted to retinol. The activity of the intestinal monooxygenase in control and experimental groups after single dose, repeated dose and dietary feeding is given in Table 4.15. In the absence of the substrate (provitamin A carotenoid) in control and RD groups, monooxygenase activity (pmol retinal/h/mg protein) was 45.7, 44.4 (single dose), 32.9, 47.2 (repeated dose) and 50.2, 48.8 (dietary feeding) respectively. In general, feeding of β-carotene and astaxanthin resulted in higher activity of monooxygenase than the control and RD groups indicating conversion of these carotenoids to retinol. The higher level of retinol in plasma, liver and intestine of these groups further supports the above results.

Discussion

Single dose study with β-carotene, astaxanthin, lutein and fucoxanthin

Rats fed retinol deficient diet for 8 weeks resulted in the depletion of retinol level in plasma and liver stores. Reports are available (Raju et al., 2006, Raju and Baskaran, 2009; Kaul and Krishnakantha, 1997) where a similar animal model was used to study the effect of retinol deficiency on biochemical changes at tissue level. They have also reported a drastic depletion of retinol in plasma and liver on induction of retinol deficiency over 8-10 weeks. The results of the present study corroborate well with the above-mentioned studies with respect to the decrease in food intake and growth rate as a result of retinol deficiency. The RD rats gavaged with a single dose of β-carotene showed a rise in retinol levels in plasma (40-78%) and liver (53-85 %) indicating the cleavage of β-carotene to retinol. This was supported by the enhanced (56-70%) monooxygenase activity in β-carotene fed group as compared to lutein, astaxanthin, fucoxanthin, baseline (RD) and control groups. Raju and Baskaran (2009), Raju et al. (2006) have reported a similar increase with higher intestinal monooxygenase activity on feeding β-carotene to RD rats. Interestingly, no intact β-carotene was found in the plasma after any of the time interval (2h, 4h and 8h) and was detected in the liver after 8 h of gavage. In contrast, Raju et al. (2009) reported presence of intact β-carotene in plasma after a dose of micellar β-carotene. The possible reason for the absence of intact β-carotene in the plasma may have been due to its complete conversion to retinol, which may have been efficient in RD rats, resulting in the

Table 4.15. Intestinal β-carotene, 15-15'-monooxygenase activity (pmol retinal/h/mg protein) in the control, RD and carotenoid fed rats.

Study	Time point	Groups				
		Control	RD	β-Carotene	Astaxanthin	Lutein
Single dose[#]	2h	45.7 ± 4.2[a]	44.4 ± 4.6[a]	100.6 ± 10.1[b]	47.8 ± 4.6[a]	45.1 ± 4.2[a]
	4h	45.7 ± 4.2[a]	44.4 ± 4.6[a]	122.7 ± 10.9[a]	42.9 ± 5.9[a]	46.6 ± 4.4[a]
	8h	45.7 ± 4.2[a]	44.4 ± 4.6[a]	145.64± 11[b]	45.6 ± 4.9[a]	44.8 ± 5[a]
Repeated dose	7 D	32.9±15[a]	47.2±11[a]	154.5±47[b]	146.0±67[b]	44.4±16[a]
	15 D	42.7 ± 4.2[a]	45.8 ± 3.7[a]	169 ± 10.4[b]	150.3 ± 12.5[b]	47.3 ± 4.5[a]
Dietary Study	20 D	50.2 ± 3.2[a]	48.8 ± 5.2[a]	*182.5 ± 10.1[b]	155.3 ± 9.1[c]	**177.9 ± 14.6[b]

[#]Monooxygenase activity (pmol retinal/h/mg protein) in fucoxanthin group at 2, 4, 6 and 8 h after gavage was 43.5 ± 4.2[a], 45.8 ± 5.1[a], ± 47.1 ± 4.1[a] and 44.8 ± 3.8[a] respectively.

*Fed carrot powder as β-carotene source.

**Fed dill leaf powder as lutein source.

Data in retinol deficient (RD) group) represents baseline values for each group. Data represent mean ± SD (n=5). Values in a row between the groups not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

elevated retinol levels in plasma. Barua and Olson (2000) have reported 3 times higher serum retinoid levels in vitamin A deficient rats as compared to vitamin A sufficient rats when fed a single dose of β-carotene. Grolier et al. (1995) have also reported better conversion of β-carotene to retinol in vitamin A deficient rats while Ribaya-Mercado et al. (2000) reported an inverse relationship between vitamin A status and bioconversion of plant carotenoids to retinol in children. They have speculated that this may be due to a feed back mechanism. Additionally, the increased retinol levels and monooxygenase activity found in this study corroborate well with the report of Parvin and Sivakumar (2000), who have reported enhanced intestinal monooxygenase activity and thus carotene conversion to retinol in RD rats. Whereas, in the present study, a single gavage of astaxanthin and lutein did not result in significant rise in retinol levels in plasma at any of the time intervals, indicating that a single dose may not be sufficient for the enzyme to recognize them as substrate for cleavage to retinol. This is further supported by comparatively lower monooxygenase activity as in the case of RD and control groups. In fact, the retinol levels in plasma, liver, intestine and microsomes of astaxanthin and lutein groups are lower than the RD group indicating that animals may have used the stored retinol.

In contrast, repeated gavages of astaxanthin resulted in partial conversion of it to retinol via β-carotene. Repeated gavages of astaxanthin over a period of 7-days may have led the RD rats to make use of nonprovitamin-A astaxanthin as a substrate for conversion to retinol. The higher level of plasma and tissue retinol and monooxygenase activity of this group further supports the above result. Further, it is speculated that this may have been the reason for the provitamin-A activity of astaxanthin as reported in fish (Moren et al., 2002). Thus, in the current study, over a period of 8 hours, β-carotene alone exhibited provitamin A activity resulting in raised retinol levels in plasma and liver over a period of 8 h along with increased monooxygenase activity. Raju and Baskaran (2009) have also observed increased retinol levels and elevated monooxygenase activity on feeding β-carotene to RD rats.

The bioavailability of carotenoids was measured in terms of the area under the curve (AUC) over a period of 8 h. β-Carotene was not detected in the plasma and was detected only in liver after 8h. Hence, AUC of β-carotene is not comparable with the AUC of astaxanthin and lutein. This is in contrast with the report of Raju and Baskaran, (2009) who detected intact β-carotene in plasma and liver of the rats fed on semi-synthetic diet with sufficient retinol from 1h to 6 h. Results of the present study demonstrate that RD rats convert the entire single dose of β-carotene to retinol and hence no β-carotene was detected in the blood. The higher AUC values for plasma, liver and intestine of astaxanthin group indicate that astaxanthin was more bioavailable

than lutein. The AUC values of plasma triglycerides further support the higher bioavailability of astaxanthin as compared to lutein. Additionally, the plasma triglyceride levels in the β-carotene group was higher than astaxanthin and lutein groups, indicating that β-carotene is probably more bioavailable and the lack of intact β-carotene can be attributed to its conversion to retinol. Similar association of triglycerides with absorbed β-carotene (Raju and Baskaran, 2009) and lutein (Lakshminarayana et al., 2009) was observed with higher triglyceride levels where plasma carotenoid levels were higher. The absorption kinetics of astaxanthin and lutein reveal that time taken to reach maximum concentration (t_{max}= 4h), 50% absorption ($t_{1/2A}$= 2h) and 50% clearance ($t_{1/2C}$= 2h) was similar, while the rate (pmol/ml/h) of absorption (20.4, 9.2) is greater than that of clearance (13.7, 6.5) for these carotenoids. In contrast, Lakshminarayana et al. (2006) and Baskaran et al. (2003) have reported maximum absorption of lutein at 2h in retinol sufficient rats and mice respectively. The results of the present study clearly demonstrate that the rate of absorption and clearance of astaxanthin was greater than lutein. The AUC values (pmol/ml/h) also show a similar trend as in the case of astaxanthin group (44.6) displaying higher levels than lutein group (17.6). These data further support the result that astaxanthin is more bioavailable than lutein in retinol deficient rats.

Gavages of FUCO to RD rats resulted in no change ($p>0.05$) in plasma retinol level demonstrating FUCO is not converted to retinol or retinoid like compounds as reported for astaxanthin (Moren et al., 2002) and lutein (Matsuno, 1991; Goswami and Barua, 1985). FUCO and its metabolites (FUOH and AAx) were not detected in the plasma of 0h group (base line) thereby confirming that metabolites found were derived from FUCO. FUOH and AAx were detected within 2 h of intubation with FUCO and reached maximum at 6 h and remained at detectable levels up to 8 h. In both plasma and liver, the main peaks ascribed to metabolites of FUCO were detected at 6.9 and 8.4 min in the HPLC analysis. UV-VIS spectrum of peak detected at 6.9th min was consistent with FUOH, the hydrolytic deacetylation product of FUCO as reported by Sugawara et al. (2002). LC-MS analysis revealed positive ions (m/z 617) corresponding to $(M+1)^+$ for FUOH. Thus, FUOH was identified as a prime metabolite of FUCO in rats in this study and mice (Asai et al. 2004, Sugawara et al. 2002). The UV-VIS and mass spectra of peak that eluted at 8.4 min was different from that of FUOH with molecular ion $(M+1)^+$, at *m/z* 615, and corroborates with earlier report (Asai et al. 2004). Interestingly, it was found that AAx was a major metabolite of FUCO in liver whereas in plasma it was FUOH demonstrating that liver enzymes may play a role in hydrolyzing the FUOH to AAx (Asai et al. 2004). FUCO bioavailability was calculated by estimating the sum of FUOH and AAx in plasma and liver. The absorption kinetics of FUCO in plasma as compared to astaxanthin and lutein

reveals that it was less bioavailable than other carotenoids. The time taken by FUCO to reach maximum concentration was 6 h unlike 4 h for other carotenoids. Also, FUCO was rapidly cleared after 6 h and reached low concentrations by 8 h. The rate (pmol/ml/h) of clearance (0.96) for FUCO was greater than that for absorption (0.18), whereas, for astaxanthin and lutein, the rate of absorption is greater than the rate of clearance over a period of 8h. The AUC values confirm that bioavailability of FUCO was less than astaxanthin and lutein.

Repeated gavage of carotenoids for 7-days showed that the postprandial plasma and liver levels of astaxanthin were lower than lutein and β-carotene which may be due to a part of astaxanthin being converted to β-carotene and thereafter to retinol. The elevated retinol level in plasma and liver of astaxanthin fed group supports the above hypothesis. As in the case of β-carotene group (70%), the activity of monooxygenase in the intestinal mucosa of astaxanthin group was higher (68%) than RD group. This further supports the conversion of astaxanthin to β-carotene and then to retinol. This may be the reason for the presence of β-carotene in astaxanthin group. Whereas, Matsuno et al. (1991) proposed a metabolic pathway for the formation of retinol from astaxanthin through zeaxanthin and 3-dehydroretinol in rats. They have suggested that 3-dehydroretinol formed from astaxanthin may give rise to retinol. Raju and Baskaran (2009) also reported an increase in intestinal monooxygenase activity and elevated retinol levels in plasma of rats fed β-carotene. Absence of intact β-carotene, elevated plasma retinol level and higher activity of β-carotene, 15-15'monooxygenase in the intestinal mucosa of β-carotene group (70%) compared to RD group observed in this study may be attributed to its bioconversion to retinol.

On repeated gavage of β-carotene to RD rats for 15-days, as anticipated, the plasma and liver retinol levels increased to 8.8 nmol/ml and 45.7 nmol/g which corresponded with a lower plasma level of β-carotene (0.37 nmol/ml,) indicating its conversion to retinol (8.39 nmol/ml) (Table 4.9). Interestingly, RD rats fed on astaxanthin also showed an increase in plasma (3.1 nmol/ml) and liver (20.9 nmol/g) retinol levels. A detectable level of β-carotene (0.94 nmol/ml, 1.2 nmol/g) was also found in the plasma and liver of astaxanthin group, which further supports the speculation that retinol, may have been formed by the bioconversion of astaxanthin to β-carotene. The biosynthesis process of carotenoids in micro algae indicates that astaxanthin is derived from β-carotene (Rodriguez-Amaya, 2001). In contrast, in RD rats, astaxanthin seems to be converted to β-carotene and subsequently to retinol. Gross and Budowski (1966) have reported a similar conversion of astaxanthin in fish. The newly formed retinol in β-carotene and astaxanthin groups is further supported by the increased activity of the intestinal monooxygenase (73, 70%) compared to RD rats (Table 4.15). This corroborates well with Raju and Baskaran

(2009) who have also reported increased monooxygenase activity on feeding β-carotene to RD rats. In contrast, lutein group did not exhibit any change in retinol levels and the activity of monooxygenase activity, hence higher levels of lutein were detected in plasma (8.36 nmol/ml) and liver (15.7 nmol/g). Matsuno (1991) and Moren et al. (2002) have reported formation of retinol from xanthophylls via reductive pathways with the formation of 3-dehydroretinol in fish. However, no such intermediate compounds as seen in their proposed pathways were detected in this study. In fact, β-carotene detected in astaxanthin group indicates that the astaxanthin may be converted to β-carotene, which in turn cleaved into retinol. Based on the results of this study, the probable pathway of astaxanthin bioconversion to retinol (via β-carotene) is proposed and shown in Chapter 5.

Diet supplemented with either carrot powder (source of β-carotene) or astaxanthin or dill leaf powder (source of lutein) was fed to RD rats. Carrot powder contained α-carotene and lutein in addition to β-carotene, which was predominant while dill leaf powder contained β-carotene in addition to lutein. Due to the presence of provitamin A carotenoids in both the sources, both groups had elevated retinol levels as compared to RD group. However, the rise in retinol levels was greater in carrot-supplemented than dill leaf diet. This difference is explained by the composition of the diets where, carrot powder diet contained higher level of β-carotene and α-carotene, while the dill leaf diet contained higher lutein. Hence, the elevation of retinol levels in both these groups is explained by the presence of provitamin A carotenoids in the diet and carrot fed rats had significantly higher retinol levels than other groups. Moreover, it has been reported that carotenoids from vegetable and fruit sources are more bioavailable and a better source of retinol than leafy greens (de Pee et al., 1998), indicating that differences exist in the bioavailability and bioefficacy of carotenoids. Astaxanthin fed group also showed increased retinol levels as compared to RD group, however, it was significantly lower than the carrot and dill leaf fed groups. This corresponded well with the repeated dose studies for astaxanthin, which resulted in formation of retinol along with the formation of β-carotene.

The HPLC analysis of the carotenoids extracted from 10 day and 20 day plasma and liver tissue homogenate of RD rats fed with carrot supplemented diet showed that β-carotene concentration (16, 29.9 ng/ml, 89.1 ng/g) was highest, followed by α-carotene (5.7, 13.7 ng/ml, 40.3 ng/g) and lutein (2.1, 6 ng/ml, 16.8 ng/g), while in dill leaf group, lutein levels (11.2, 25 ng/ml, 90.0 ng/g) were higher followed by β-carotene. In case of astaxanthin group, astaxanthin was the major carotenoid detected (23.1, 43 ng/ml, 85.3 ng/g) along with β-carotene (0, 2.3 ng/ml, 5.4 ng/g). Monooxygenase activity in all the 3 groups was elevated as compared to RD

group. However, amongst the three, it was highest in carrot-supplemented diet followed by dill leaf diet and astaxanthin diet. This corresponds well with the increased retinol levels in the respective groups, indicating that β-carotene and astaxanthin absorbed from diet were cleaved to retinol. Raju and Baskaran (2009) and Barua and Olson (2000) have also reported increased retinol levels and monooxygenase activity on feeding β-carotene to RD rats, indicating its conversion to retinol. The increased β- and α-carotene, lutein and retinol levels in the carrot and dill leaf supplemented diets indicate that carotenoids from these sources are bioavailable. With respect to alleviation of retinol deficiency, carrot was seen to have better effect, probably as it contained higher levels of provitamin A carotenoids (β- and α-carotene). Also, it has been reported earlier that bioavailability and bioefficacy of carotenoids from fruits and other vegetables is greater than those from leafy greens (de Pee et al., 1998; 1995). Carrillo-Lopez et al. (2010) have suggested that specific conversion factors for groups of horticultural crops be considered, as their study on bioconversion of carotenoids in 5 fruits and vegetables (including spinach, carrot, mango, parsley and papaya) indicated that there was no relation between carotenoids' content in fruit and vegetable based diet and retinol status in plasma. This indicates that bioavailability and bioefficacy of carotenoids from different vegetable sources can vary, which is in agreement with the present results. Similar to feeding of carrot powder and dill leaf powder, dietary astaxanthin also relieved retinol deficiency as it yielded retinol but it was significantly lower than carrot and dill leaf groups. Moreover, the plasma retinol levels were significantly increased (>1.29 μmol/l) after 10 days of feeding carrot and dill leaf diet while for astaxanthin group it was below the 0.7 μmol/l threshold value for retinol deficiency (Olson, 1984) and rose above the threshold value to 0.89 μmol/l only after 20 days of feeding.

In conclusion, the single dose study revealed that over a period of 8h, only β-carotene showed provitamin A activity by its cleavage to retinol, while astaxanthin, lutein and FUCO did not yield retinol. Among the xanthophylls studied, astaxanthin was more bioavailable than lutein, followed by FUCO. However, repeated gavages of β-carotene and astaxanthin to RD rats showed provitamin A activity (along with formation of β-carotene in astaxanthin group), indicating that the body may recognize astaxanthin as a precursor for vitamin A in vitamin A deficiency. Repeated gavages of lutein however did not result in retinol formation and hence no provitamin A activity was observed for it. The increased retinol levels in β-carotene and astaxanthin groups were associated with their lowered levels in plasma and liver while lutein levels were higher with no raised retinol levels. Results of dietary feeding of astaxanthin was similar to repeated dose studies with respect to provitamin A activity, where astaxanthin, β-carotene and retinol were

detected, indicating the probable conversion of astaxanthin to β-carotene and thereafter to retinol. Feeding carrot and dill leaves to RD rats resulted in raised retinol levels in both groups and alleviated the retinol deficiency and this was more effective for carrots than for dill leaf. Therefore, amongst the two vegetables, carrot was more efficient in formation of retinol and alleviation of vitamin A deficiency. Moreover, astaxanthin was found to yield retinol and thus can be an alternate source of vitamin A in the diet and may be used for prevention and alleviation of vitamin A deficiency.

CHAPTER 5: Elucidation and characterization of metabolites of β-carotene, astaxanthin, lutein and fucoxanthin in vitamin A deficient rats

Introduction

Retinol plays many important functions and its deficiency leads to a variety of changes in the functions of the body. Retinol is an essential micronutrient that is supplied to the body either in the preformed state or as carotenoids (precursors). Of the many carotenoids known, only few have provitamin A activity, they cleave *in vivo* to yield retinol. Xanthophylls have many health benefits, but except for β-cryptoxanthin, do not exhibit provitamin A activity. Some of the health benefits of carotenoids include enhancement of immune responses, gap junction communication, carcinogen-metabolizing enzyme activity (Wang, 1994; Stahl et al., 1997), cardiovascular disease (Gaziano et al. 1995) and cancer (van Poppel 1996). β-, α- and γ-carotenes are used to treat VAD as they cleave to retinol *in vivo* (Olson 1994) while lutein and zeaxanthin are used against the progression of age related macular degeneration (Klein et al., 1995). Carotenoids are thought to have a similar absorption pathway as lipids as they are lipophilic (Furr and Clark, 1999), however, their metabolic fate in the body is poorly understood. Hence, it is hypothesized that metabolites of carotenoids may exert health benefits superior to parent molecules. Therefore, it is important to elucidate and characterize the structures of carotenoid metabolites.

β-Carotene is one of the most commonly occurring carotenoids present in foods and is cleaved by the action of β-carotene 15,15'-monooxygenase (previously known as β-carotene 15,15'-dioxygenase). It is present predominantly in intestine although its activity has been observed in liver as well. When β-carotene is cleaved centrally, it gives two molecules of retinal. In eccentric cleavage, apocarotenals such as β-apo-8'-, 10'-, 12'and 14'-carotenals are formed and these can further be converted to retinol or retinoic acid. Both centric and eccentric cleavage of carotenoids is known to exist in biological systems (Lemke et al., 2003). Barua and Olson (2000) detected retinoids as major metabolites and β-apocarotenoids to a lesser extent (<5%) in the intestine of RD rats. They have reported the centric and eccentric cleavage of β-carotene and concluded that central cleavage is the main pathway for retinol formation in healthy mammals.

Astaxanthin is a potent antioxidant. Its provitamin A activity in lower vertebrates has been explored by many researchers (Moren et al., 2002; Yamashita et al., 1996; Matsuno, 1991).

Moren et al. (2002) postulated that retinol is the primary product of carotenoid conversion in the Atlantic halibut, whereas, Yamashita et al. (1996), Matsuno (1991) have reported that astaxanthin was converted to 3-dehydroretinol in various fish species. Bjerkeng et al. (2000) has reported presence of astaxanthin, iodaxanthin, crustaxanthin and their isomers, as well as very low levels of tunaxanthin, zeaxanthin and lutein-like metabolites in Arctic charr. Wolz et al. (1999) have concluded from their study in rat hepatocyte cultures that astaxanthin was metabolized into (rac)-3-hydroxy-4-oxo-β-ionone, and its reduced form (rac)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone independent of the xenobiotic-metabolizing enzymes induced by astaxanthin. Østerlie et al. (2000) have studied the pharmacokinetics of astaxanthin in men fed with a single dose of astaxanthin and have reported E/Z isomers of astaxanthin in plasma and lipoproteins. Coral-Hinostroza et al. (2004) have reported the plasma appearance of unesterified astaxanthin geometrical E/Z and optical R/S isomers in men given single doses of a mixture of optical isomers of astaxanthin fatty acyl diesters. Matsuno (1991) has reported conversion of astaxanthin to zeaxanthin and 3-dehydroretinol in rat.

Lutein is commonly found in fruits and vegetables. Lutein and zeaxanthin are often referred to as macular pigments due to their specific role in the macula of the eye (Schalch and Dayhaw-Barker, 1999). Lutein, zeaxanthin and meso-zeaxanthin have been isolated and characterized in macula by HPLC (Bone et al., 2001). Apart from dietary lutein, 3′-epilutein, 3′-dehydrolutein, (3R,3′S)-meso-zeaxanthin, 3′-oxolutein, and 3-methoxy-zeaxanthin have also been reported in human ocular tissues and serum (Bhosale et al., 2007; Bhosale and Bernstein, 2005; Khachik et al., 2002; Bernstein et al., 2001; Khachik et al., 1997; Bone et al., 1993). 3′-Oxolutein was reported as a major oxidative derivative formed by direct oxidation of lutein and zeaxanthin (Bhosale and Bernstein, 2005; Khachik et al., 2002; Bernstein et al., 2001; Khachik et al., 1997; Bone et al., 1993). Lakshminarayana et al. (2008) have reported anhydrolutein, lutein diepoxide and other metabolites of lutein in rats fed dietary lutein. Khachik et al. (1996) suggested that a complete characterization of carotenoids and their metabolites in retina might help in understanding their functional properties. However, only few studies are available on the oxidative breakdown/metabolism of non-provitamin A carotenoids with an objective to elucidate their vitamin A activity (Astorg et al., 1994; Gradelet et al., 1996). There are studies on the metabolism and bioconversion of lutein in lower vertebrates such as fish. Yamashita et al. (1996), Matsuno (1991) and Goswami and Barua (1986) have reported the conversion of lutein to 3-dehydroretinol in freshwater and marine fish such as yellowtail, black bass, ayu and *H. fossilis.* A similar reductive pathway was reported in rat fed with lutein (Matsuno, 1991). Metabolism of

lutein may be varied based on the adaptability of the biological system and elucidation of the metabolites, thus, may improve our understanding of lutein metabolism under VAD condition.

Fucoxanthin (FUCO) is a predominant carotenoid found in brown algae. Previously, Sugawara et al. (2002) and Asai et al. (2004) have reported that FUCO hydrolyzed to fucoxanthinol (FUOH) and amarouciaxanthin (AAx) in mice and PC-3 human prostrate cancer cells and the metabolites exhibit higher antioxidant property than FUCO *in vitro* (Sachindra et al., 2007). Studies on metabolism of β-carotene to retinol/retinoic acid (Bachmann et al. 2002; Barua and Olson, 2000), lutein to zeaxanthin, oxalolutein and epilutein (Bhosale and Bernstein, 2005; Khachik et al., 2002; Bone et al., 2001), lycopene to apo-8-, and apo-12-lycopenal (Gajic et al., 2006) and astaxanthin to retinol (Moren et al., 2002; Matsuno, 1991) in lower and higher vertebrates are available, while metabolites of FUCO are scarcely known. FUCO and its metabolites have been found to have potent antioxidant activity *in vitro* and *in vivo* (Shiratori et al., 2005; Sachindra et al., 2007). Hence, elucidation and characterization of FUCO metabolites may help to understand its functional properties in physiological systems. Moreover, no reports are available where an attempt has been made to study the bioconversion of FUCO to retinod-like compounds. Therefore, FUCO fed to retinol deficient mammals may yield interesting and novel information, which may improve our understanding of the mechanisms underlying the action of FUCO *in vivo.* It is evident from the literature that studies on possible provitamin A activity and metabolism of lutein, astaxanthin and fucoxanthin in VAD condition are lacking. Hence, studies were undertaken to elucidate and characterize the metabolites formed as a result of feeding β-carotene, astaxanthin, lutein and fucoxanthin to retinol deficient rats. The schematic representation showing the methodology adapted from the above objective is given in Figures 2.3-2.5 in Chapter 2.

Results

Metabolism of β-Carotene

RD rats were gavaged with a pharmacological dose of β-carotene, sacrificed and blood and liver was collected after 2,4, 6 and 8 h. No β-carotene or its metabolites were detected in control and RD group. Subsequent to gavage with β-carotene, it was not detected in the plasma and liver of the rats except in the liver of the 8 h rats. Typical HPLC chromatograms of the retinol and β-carotene extracted from the plasma and liver of rats gavaged with β-carotene are shown in Figure 5.1. The presence of retinol and β-carotene was further confirmed with their respective spectra and λ_{max} (Figure 5.2). Results show elevated retinol levels in plasma and liver of the rats

compared to RD group, which may be formed by the cleavage of the β-carotene. This was evidenced with enhanced monooxygenase activity in β-carotene fed group than control and RD groups (Chapter 4). The retinol levels in plasma peaked at 4 h after intubation and were used for LC-MS analysis for its confirmation (m/z 287.53 $(M+H)^+$) (Figure 5.3A). In liver, retinol levels were higher 8 h after intubation along with the presence of β-carotene and hence the 8 h liver samples were subjected to LC-MS analysis (Figure 5.3B). The HPLC and MS data confirmed that β-carotene (m/z 537 $(M+H)^+$) was cleaved into retinol (m/z 287 $(M+H)^+$) and resulted in its elevated levels. Quantitative data on the plasma and tissue retinol and β-carotene levels are given in Tables 4.2 and 4.3 (Chapter 4). Under the analytical conditions adopted, the major metabolite of β-carotene detected was retinol. Few other peaks were also detected in the mass spectra of plasma and liver but were not considered as their magnitude was designated as too low to be confirmatory. β-Carotene was not detected in plasma and liver of 0 h group (base line).

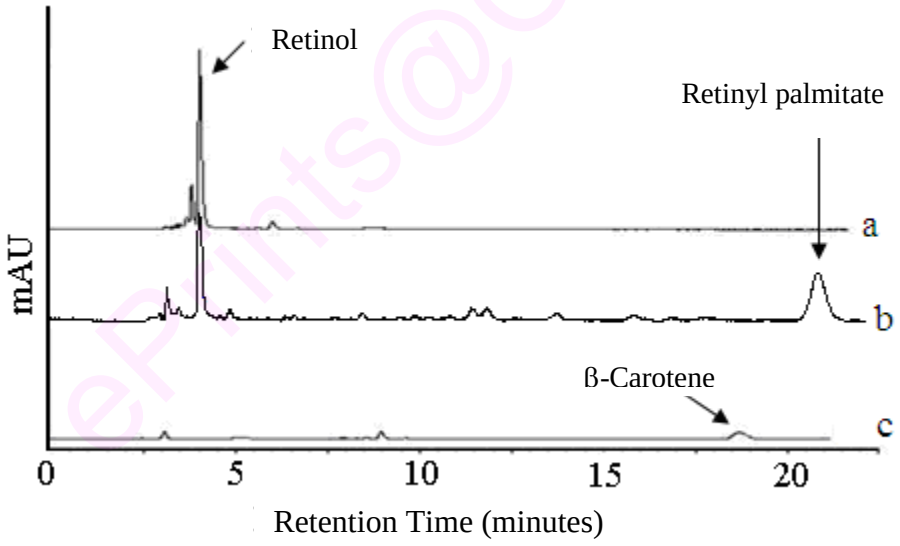


Figure 5.1. HPLC chromatogram of retinol in plasma (a) and liver (b) at 325 nm and β-carotene at 450 nm (c) extracted from liver of RD rats gavaged with β-carotene.

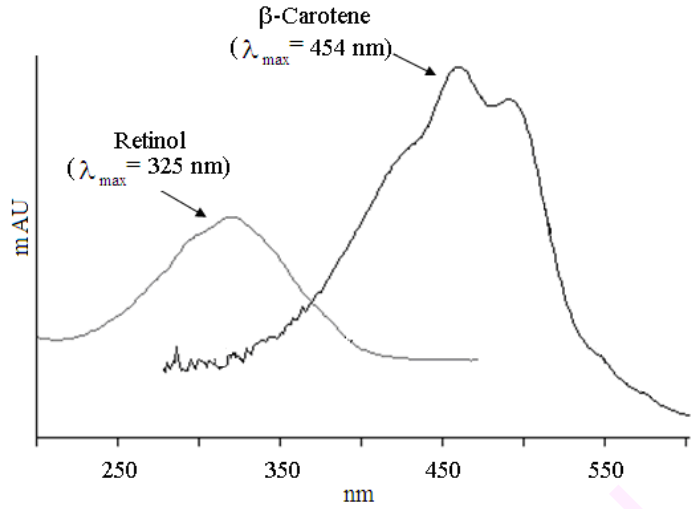


Figure 5.2. Characteristic spectra of retinol and β-carotene extracted from liver of RD rats gavaged with β-carotene. Absorption maxima (λ_{max}) are given in parentheses.

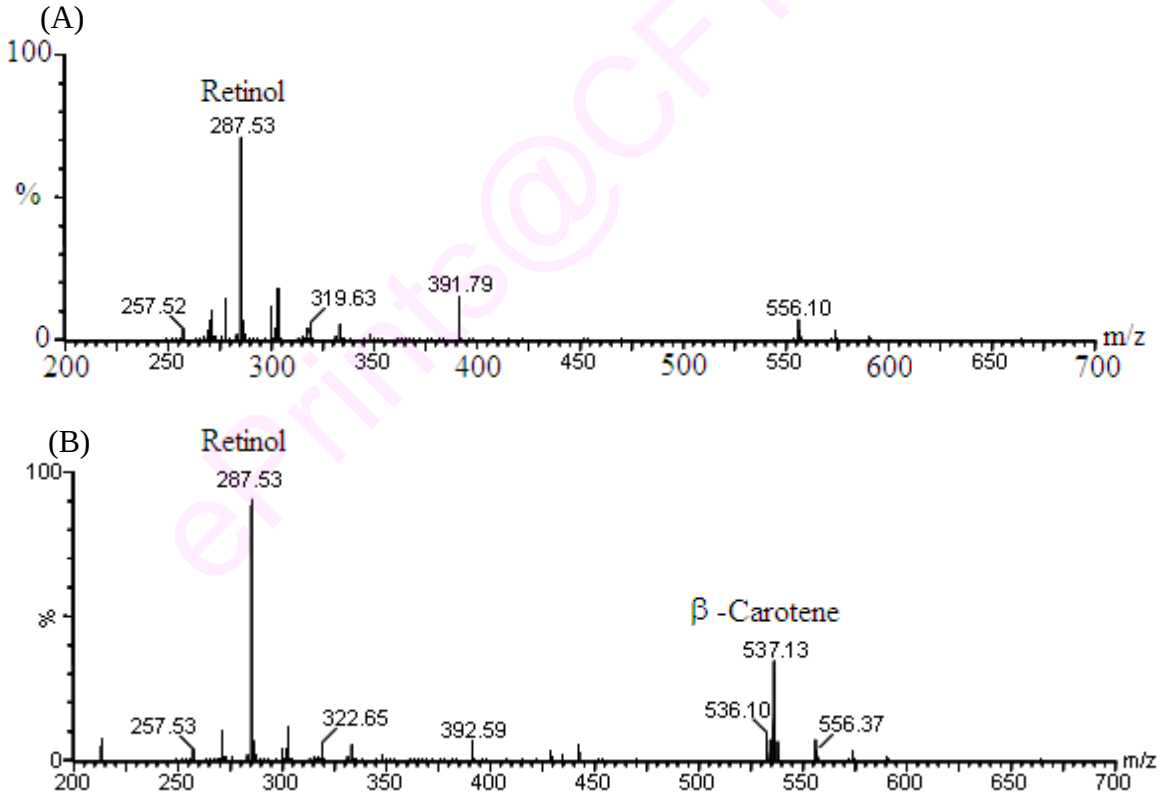


Figure 5.3. LC-MS (APCI) profile of retinol in the plasma (A) and retinol and β-carotene in liver (B) of rats fed with β-carotene. LC-MS conditions: APCI source temperature 130°C, probe temperature 500°C, corona voltage 5KV, HV lens voltage 0.5KV, cone voltage 30V, nitrogen as sheath (100 l/h) and drying (300 l/h) gas.

Similarly, retinol was the predominant metabolite on repeated gavage with β-carotene for 7-days and intact β-carotene was not detectable. Whereas, intact β-carotene and retinol was detected in the plasma of the rats after 15-days of repeated gavage and 20 days dietary feeding of β-carotene. No carotenoids were detected in the control or baseline group, indicating that β-carotene feeding resulted in its presence and elevated retinol levels over RD group. The schematic pathway for β-carotene metabolism in RD rats is given in Figure 5.4.

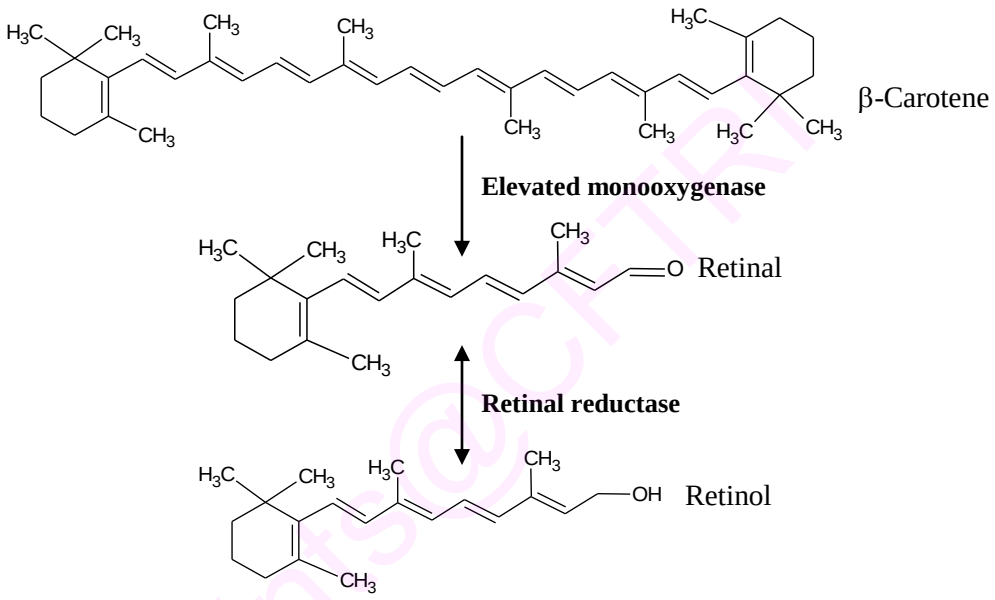


Figure 5.4. Pathway for the metabolism of β-carotene to retinol (Scheme 1).

Metabolism of Astaxanthin

No metabolites of astaxanthin were detected with a single dose, whereas, repeated gavage of astaxanthin for 15-days to RD rats resulted in detectable levels of its metabolites in blood (plasma) and liver by HPLC and LC-MS. No astaxanthin or its metabolites were detected in control and RD group, indicating that metabolites identified were formed from astaxanthin. As a result of astaxanthin intubations to RD rats, β-carotene and elevated retinol levels were detected in plasma and liver. HPLC chromatograms of retinol, astaxanthin and β-carotene extracted from plasma are shown in Figure 5.5 and they were confirmed with their UV-visible spectra (Figure 5.6). Astaxanthin and β-carotene were not detected in plasma and liver of RD group (base line). The presence of astaxanthin (m/z 597 $(M+H)^+$), β-carotene (m/z 537 $(M+H)^+$) and retinol (m/z 287 $(M+H)^+$) was confirmed by LC-MS analysis (Figure 5.7 and 5.8). LC-MS analysis revealed

an intermediate compound in liver, which was characterized as echinenone (m/z 551 $(M+H)^+$). The proposed pathway for the formation of β-carotene and retinol via echinenone from astaxanthin is shown in Figure 5.9 (Scheme 2). Monooxygenase activity was not elevated as compared to RD group on gavage of a single dose of astaxanthin, whereas, repeated gavages resulted in its enhanced activity which further confirms the formation of retinol by cleavage of β-carotene. The results obtained for the monooxygenase activity are described and discussed elsewhere (Chapters 4 and 6), while plasma and liver retinol levels on astaxanthin feeding are given in Chapter 4 (Table 4.4).

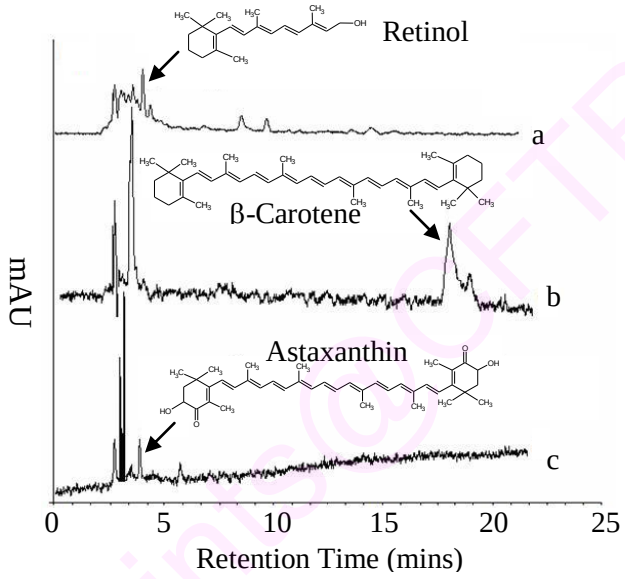


Figure 5.5 HPLC chromatogram showing the presence of retinol and β-carotene in plasma extract of astaxanthin group at 325 nm (a), 450 nm (b) and 478 nm (c). HPLC conditions are described in Methods section of Chapter 2.

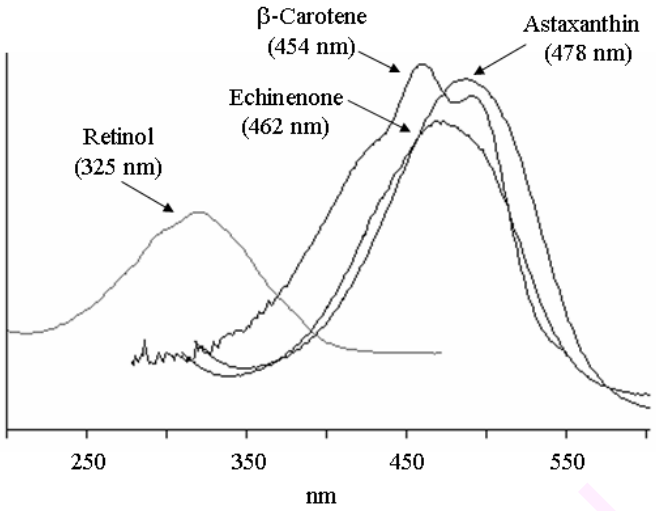


Figure 5.6 Overlaid characteristic spectra of astaxanthin and its metabolites, echinenone, β-carotene and retinol in RD rats. Absorption maxima (λ_{max}) are given in parenthesis.

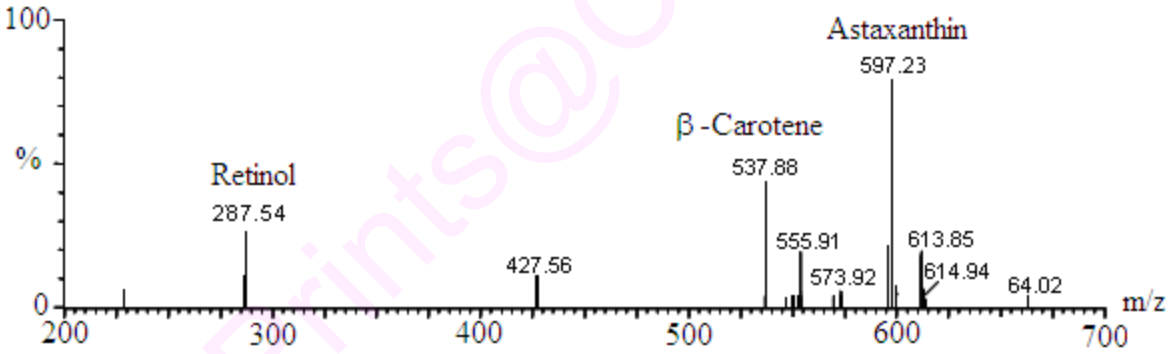


Figure 5.7. LC-MS (APCI) profiles of retinol and β-carotene detected in plasma of rats fed with astaxanthin. LC-MS conditions: APCI source temperature 130°C, probe temperature 500°C, corona voltage 5KV, HV lens voltage 0.5KV, cone voltage 30V, nitrogen as sheath (100 l/.h) and drying (300 l/h) gas.

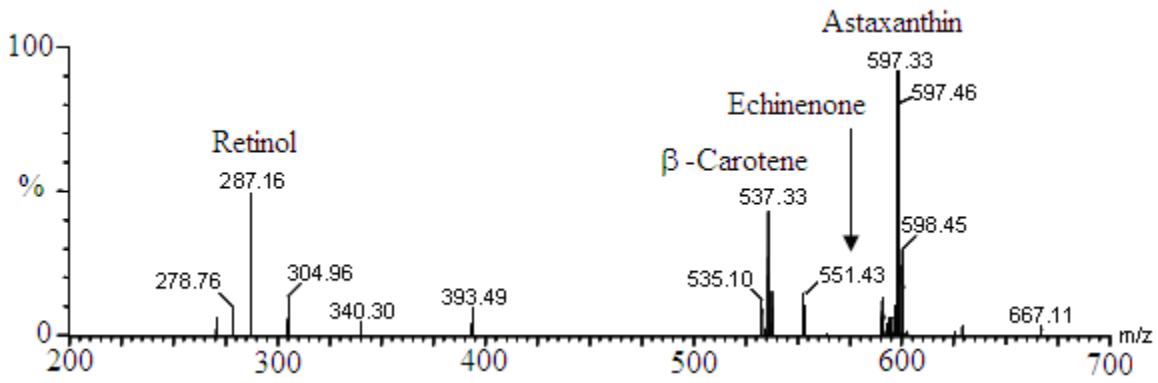


Figure 5.8. LC-MS (APCI) profiles of β-carotene, retinol, echinenone and astaxanthin detected in liver of rats fed with astaxanthin. Refer Figure 5.5 for LC-MS conditions.

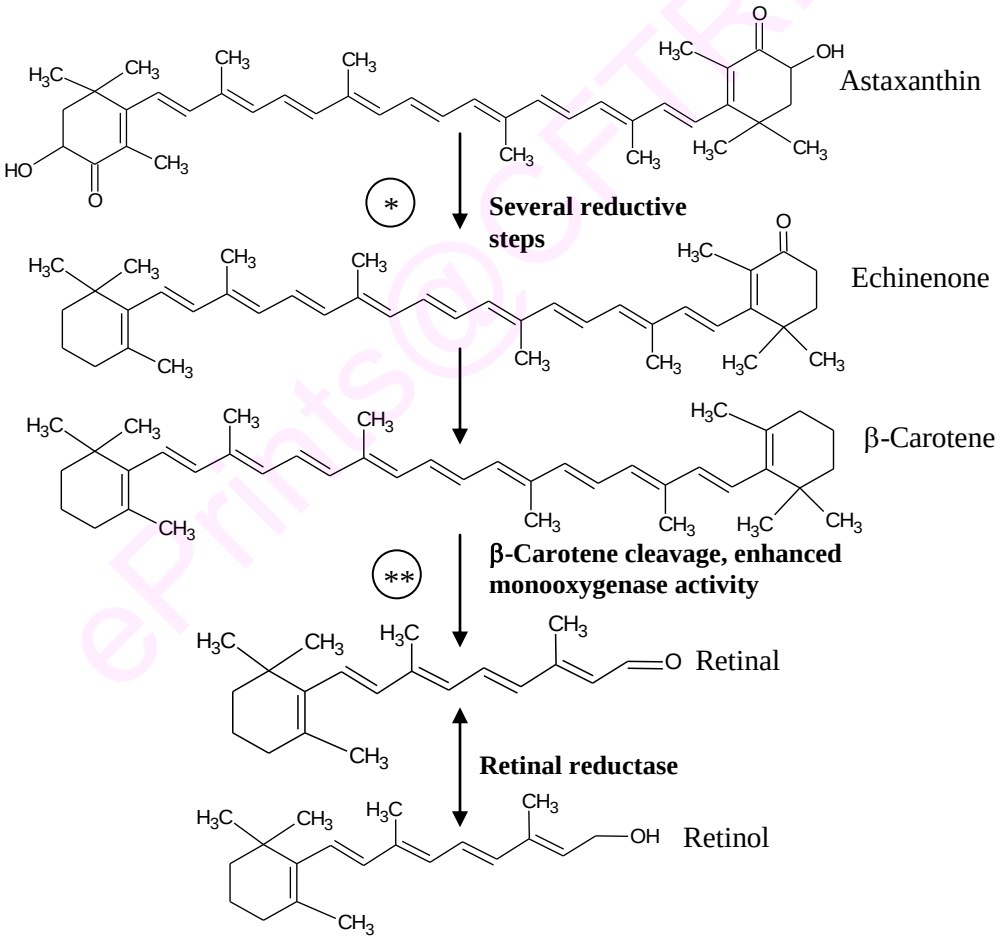


Figure 5.9. Proposed pathway for metabolism of astaxanthin to β-carotene and retinol in RD rats. (Scheme 2). ***** Short lived or unstable metabolites that may not have been detected as they may have been below detectable levels. **Higher cleavage activity of intestinal monooxygenase activity discussed elsewhere and given in Table 4.14 in Chapter 4.

Metabolism of Lutein

Lutein was gavaged to RD rats for 15-days, blood and liver were collected and analyzed by HPLC and LC-MS for its metabolites. Lutein gavages did not increase the plasma and liver retinol levels (0.5mmol/l and 3.8 nmol/g), while lutein, zeaxanthin and other metabolites were detected. No lutein or its metabolites were detected in control and RD group, indicating that administered lutein was metabolized. Under the conditions adopted (Chapter 2), a prominent peak at m/z 551.5 was detected for lutein despite its molecular weight of 568.9 indicating the elimination of water molecule from the protonated 569 $(M+1)^+$ ion, which is characteristic of hydroxy- carotenoids like lutein (Figure 5.10 and 5.11). The unidentified metabolites were characterized based on their mass spectra and have been designated as A, B, C, D and E (Figures 5.10 and 5.11). The identified base peak B corresponds to lutein diepoxide (5,6, 4', 5'-diepoxy-5, 6; 4', 5'-tetrahydro-β,β-carotene 3,3'-diol), and C (B-X+O) to 5,6 epoxy-3 hydroxy-12'-β,ε-carotene-12'al. These metabolites may be formed due to lutein oxidation in the liver itself. Other characteristic peaks D (A-Y) corresponding to (2E, 4E)-3-methyl-5- (2,6,6-trimethylcyclohexa-2, 4-dien-1-yl) penta-2, 4-dien-1-ylium and E (A-Z) to 2,6,6-trimethylcyclohex-2-ene-1, 4-bis (ylium) could have been formed from fragmentation of anhydrolutein (A). Zeaxanthin, an isomer of lutein was also identified in liver. The molecular mass, chemical structure and formula of the metabolites are given in Table 5.1. The probable pathway for the formation of these metabolites is shown in Figure 5.12 (Scheme 3).

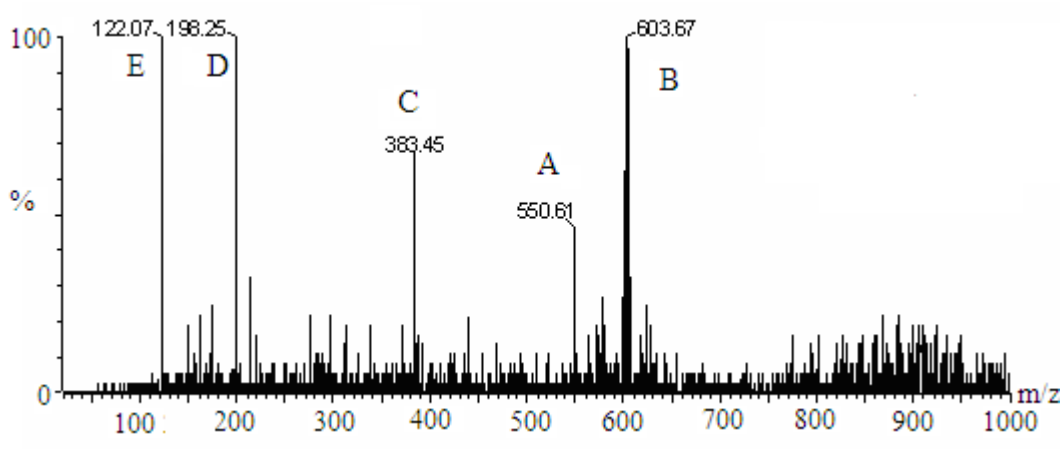


Figure 5.10. LC-MS (APCI) profiles of lutein and its metabolites detected in plasma of rats fed with lutein. LC-MS conditions: APCI source temperature 130°C, probe temperature 500°C, corona voltage 5KV, HV lens voltage 0.5KV, cone voltage 30V, nitrogen as sheath (100 l/.h) and drying (300 l/h) gas.

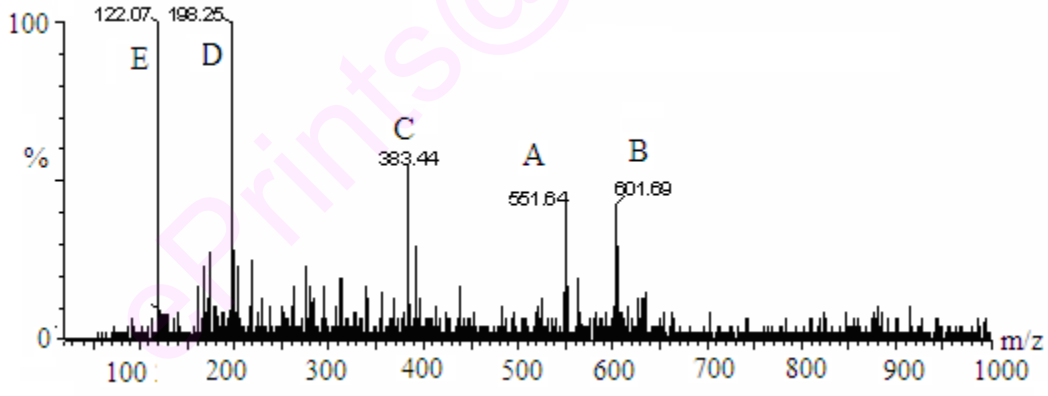
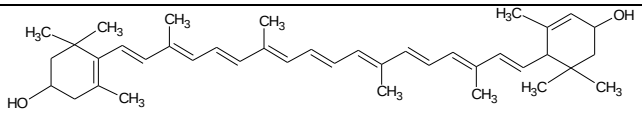
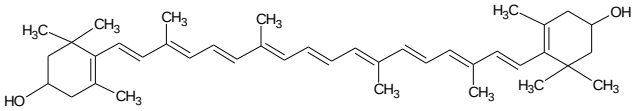
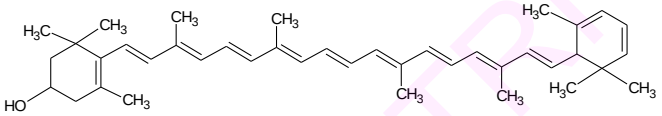
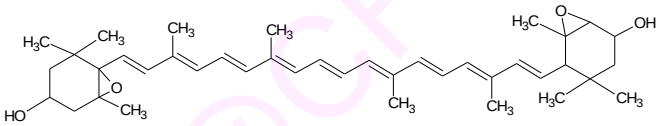
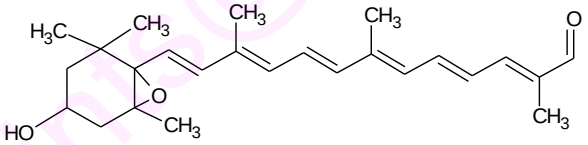
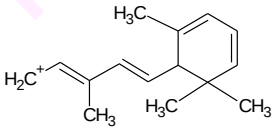
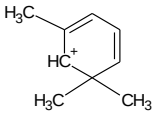
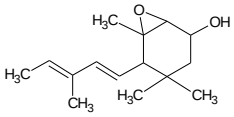
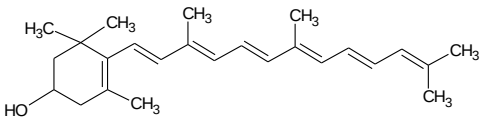
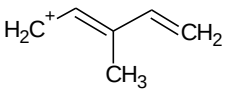


Figure 5.11. LC-MS (APCI) profiles of lutein and its metabolites detected in liver of rats fed with lutein. Refer Figure 5.7 for LC-MS conditions.

Table 5.1. Mass spectral fragments of lutein and its metabolites detected from plasma and liver of RD rats intubated with lutein.

Molecular Mass	Derivation	Chemical structure and name	Chemical Formula
568.9	-	 Lutein	$C_{40}H_{56}O_2$
568.9	-	 Zeaxanthin	$C_{40}H_{56}O_2$
551.5	A; Lutein-H_2O	 Anhydrolutein	$C_{40}H_{56}O$
601.8	B; Lutein + O_2	 Lutein diepoxide	$C_{40}H_{56}O_4$
382.5	C; B-X* + O	 5,6-Epoxy-3-hydroxy-12'-β,ε-carotene-12'-al	$C_{25}H_{34}O_3$
201.3	D; A- Y*	 (2*E*,4*E*)-3-methyl-5-(2,6,6-trimethylcyclohexa-2,4-dien-1-yl)penta-2,4-dien-1-ylium	$C_{15}H_{21}$
121.2	E; C-Z*	 2,6,6-trimethylcyclohexa-2,4-dienylium	C_9H_{13}

*X=  Y=  Z= 

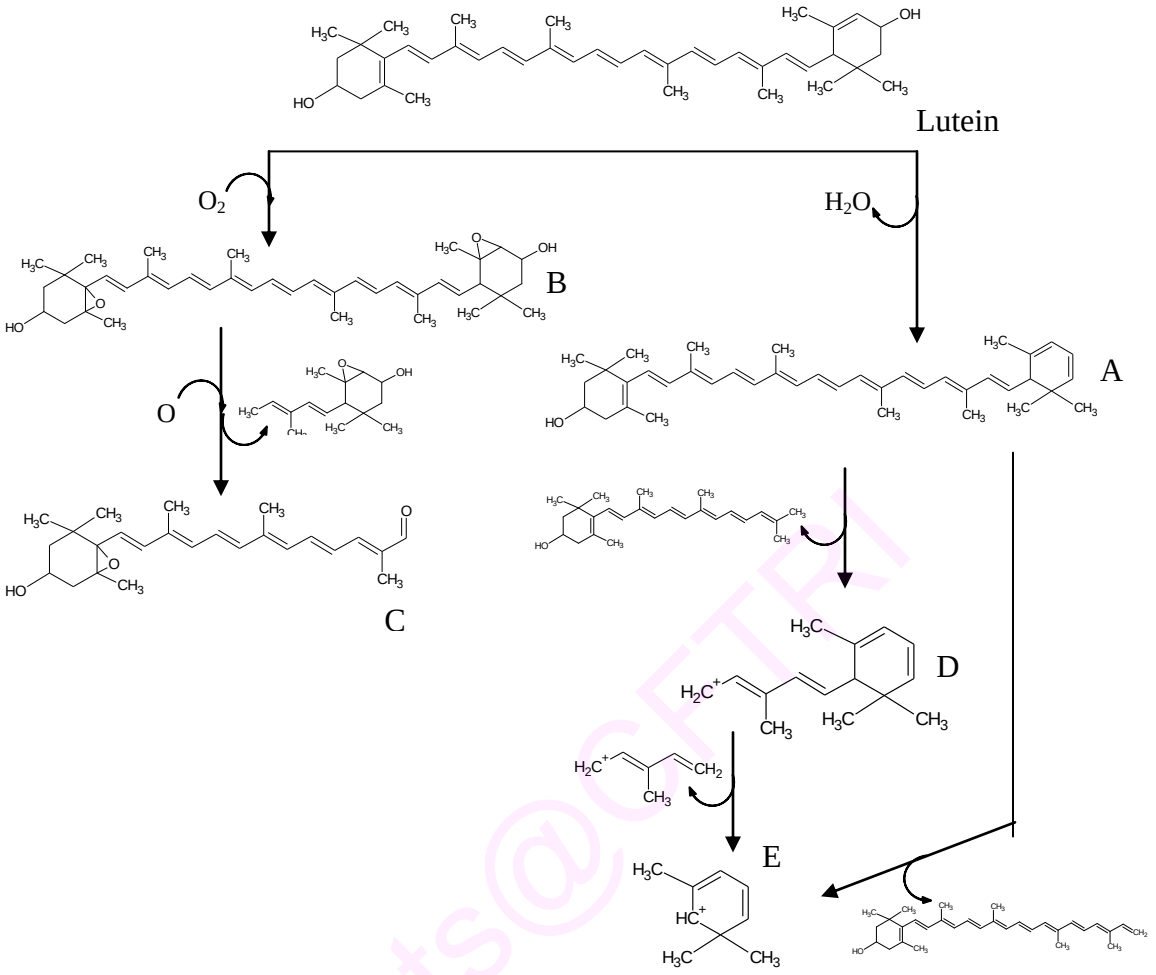


Figure 5.12. Proposed pathway for lutein metabolism in plasma and liver of RD rats (Scheme 3). Refer Table 5.1 for chemical names, molecular mass and molecular formula of metabolites corresponding to the alphabets.

Metabolism of Fucoxanthin

Retinol deficient (RD) rats were gavaged with a single dose of fucoxanthin (FUCO). Metabolites were extracted from the plasma and liver and subjected to HPLC and LC-MS analysis. Although FUCO metabolites were detected in plasma and liver at 2 h after its intubation, their levels were maximum at 6 h. Hence, 6h samples were used for the LC-MS analysis. The peak that appeared after 2 h in the FUCO fed group was identified as fucoxanthinol (FUOH), (m/z 617 $(M+H)^+$). Using APCI in positive ion mode, FUOH, amarouciaxanthin (AAx) and several unidentified metabolites were detected between 0 to 1000 *m/z* (Figures 5.13 and 5.14). The possible chemical structures, molecular mass and formula of metabolites detected based on their mass spectrum are presented in Tables 5.2 and 5.3, respectively. The characteristic

fragmented ions (unidentified metabolites) obtained from plasma were designated as A, B, C, D and E (Table 5.2, Figure 5.13) and those in liver coded as A, E, F, G, H, I, J, K, L, M and N (Table 5.3, Figure 5.14).

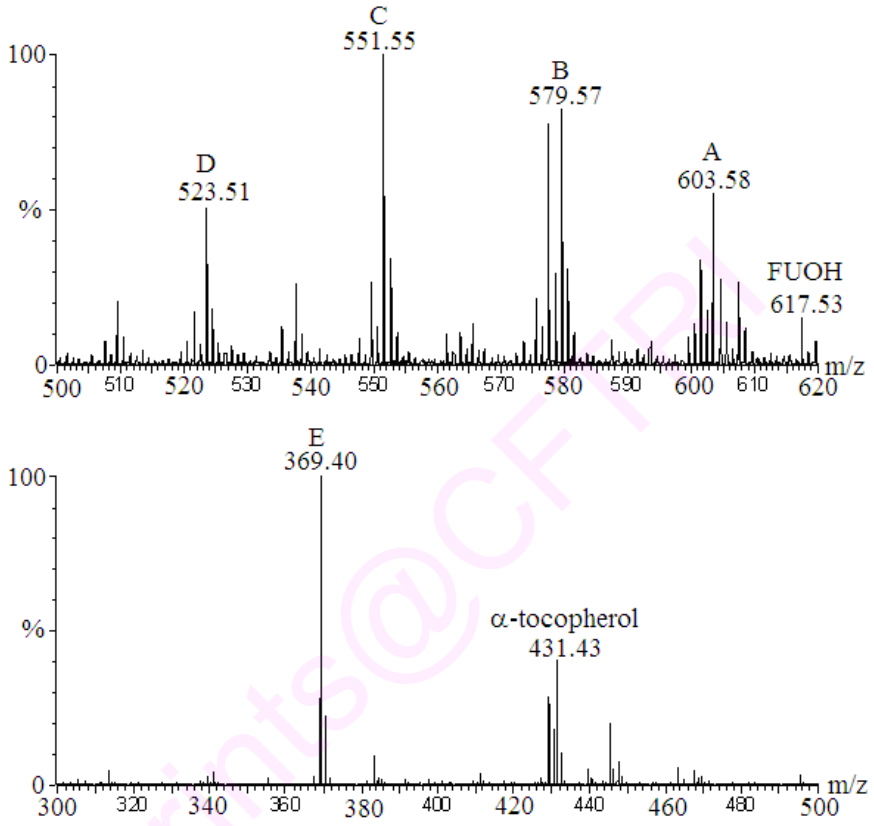


Figure 5.13. LC-MS (APCI) profiles of FUCO metabolites detected in plasma of rats fed with FUCO. LC-MS conditions: APCI source temperature 130°C, probe temperature 500°C, corona voltage 5KV, HV lens voltage 0.5KV, cone voltage 30V, nitrogen as sheath (100 l/.h) and drying (300 l/h) gas. Refer Table 5.2 for chemical names and molecular formula of metabolites corresponding to the alphabets. (*m/z* 431 represents α-tocopherol, which was used during extraction). FUOH = Fucoxanthinol

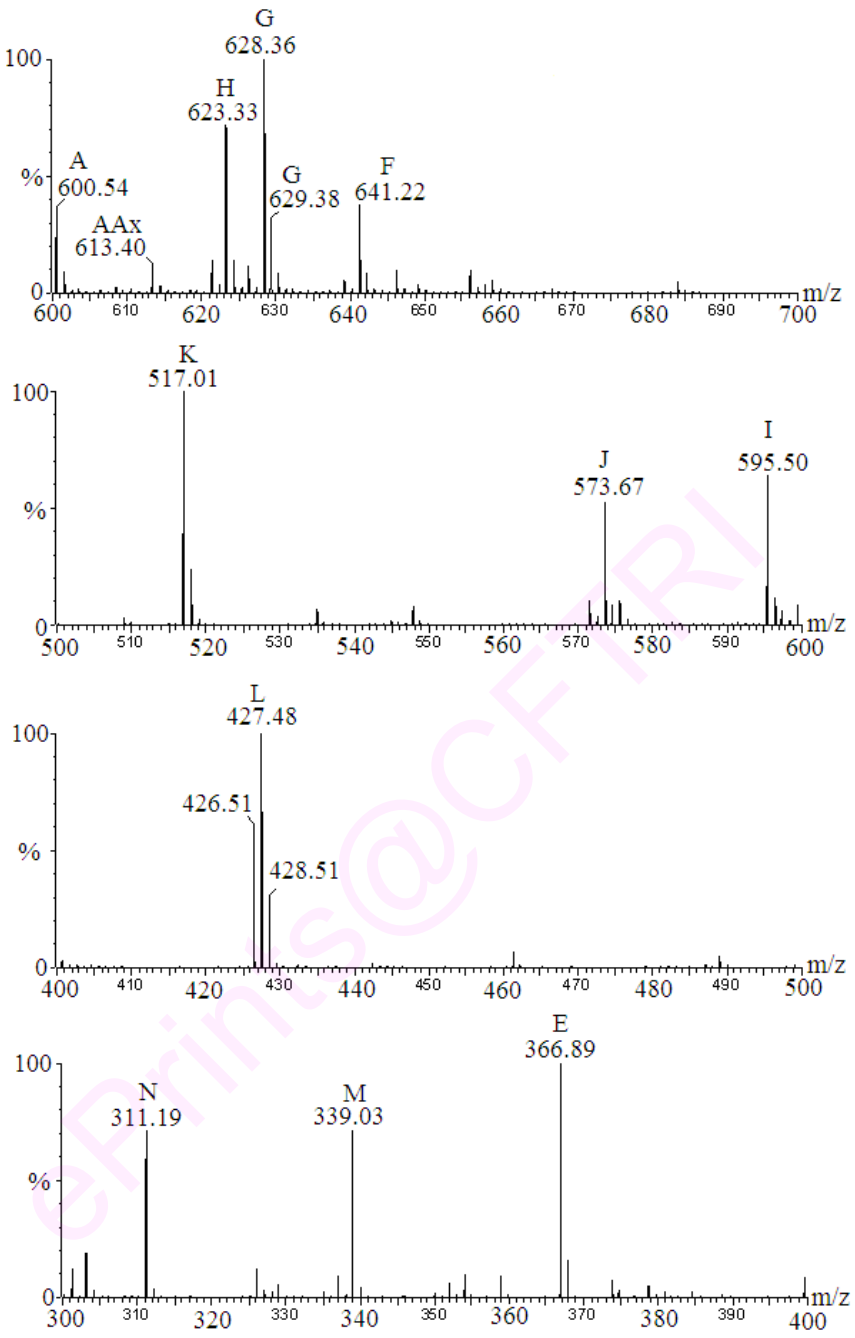
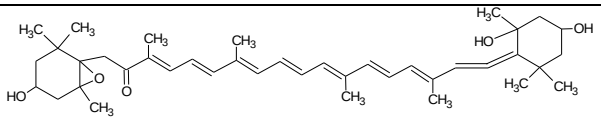
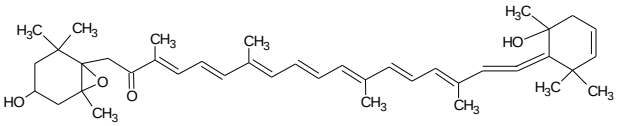
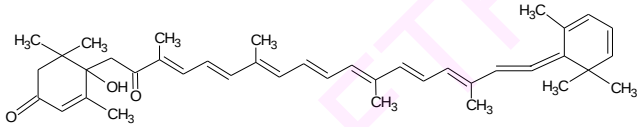
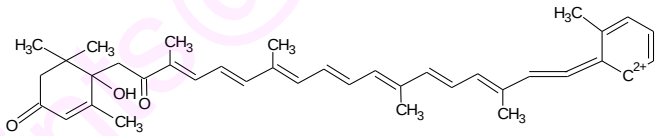
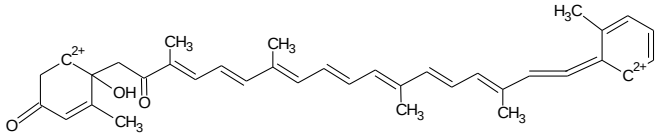
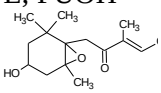
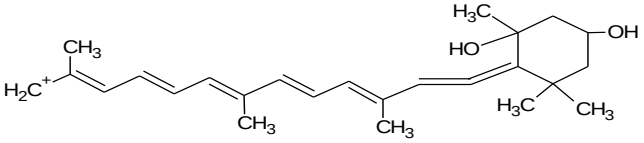


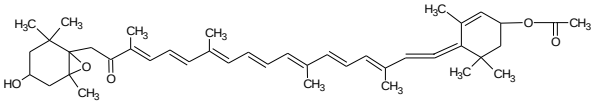
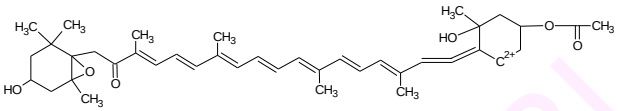
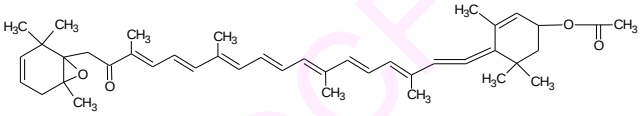
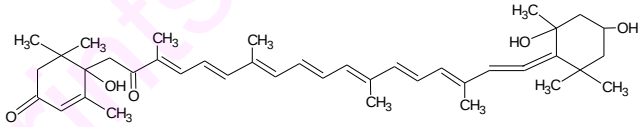
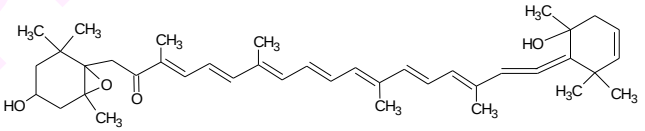
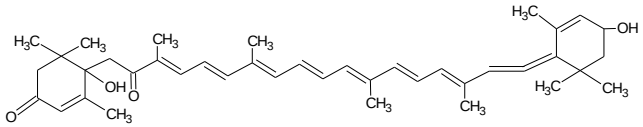
Figure 5.14. LC-MS (APCI) profiles of FUCO metabolites detected in liver (m/z 300-700) of rats fed with FUCO. Refer Figure 5.9 for LC-MS conditions. Refer Table 5.3 for chemical structures, names and molecular formula of metabolites corresponding to the alphabets. AAx = Amarouciaxanthin.

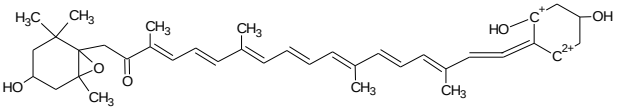
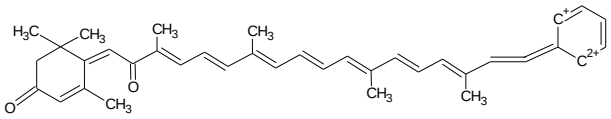
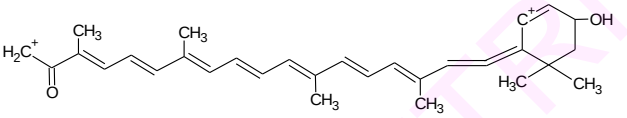
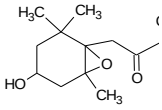
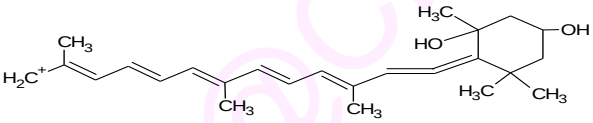
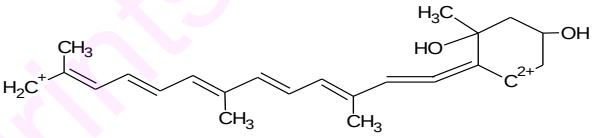
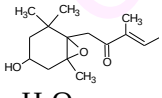
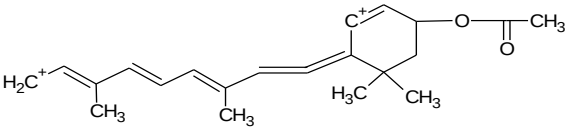
Table 5.2. Mass spectral fragments, molecular formula and chemical structures of FUCO metabolites detected from plasma of rats gavaged with FUCO.

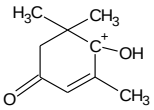
Mass	Derivation	Structure and Name	Molecular formula
617	FUOH[a]	 Fucoxanthinol	$C_{40}H_{56}O_5$
603	A; FUOH – H_20	 (3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*)-1-(4-hydroxy-2,2,5a-trimethylperhydro-1-benzoxiren-1-yl)-18-(6-hydroxy-2,2,6-trimethyl-3-cyclohexenyliden)-3,7,12,16-tetramethyl-3,5,7,9,11,13,15,17-octadecaoctaen-2-one	$C_{40}H_{54}O_4$
579	B; AAx[b] – $2H_2O$	 (3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*)-1-(1-hydroxy-2,6,6-trimethyl-4-oxo-2-cyclohexenyl)-3,7,12,16-tetramethyl-18-(2,6,6-trimethyl-2,4-cyclohexadienyliden)-3,5,7,9,11,13,15,17-octadecaoctaen-2-one	$C_{40}H_{50}O_3$
551	C; B – $2CH_3$	 (3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*)-1-(1-hydroxy-2,6,6-trimethyl-4-oxo-2-cyclohexenyl)-3,7,12,16-tetramethyl-18-(2-methyl-2,4-cyclohexadienyliden)-3,5,7,9,11,13,15,17-octadecaocta- en-2-one	$C_{38}H_{44}O_3$
523	D; B – $4CH_3$	 (3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*)-1-(1-hydroxy-2-methyl-4-oxo-2-cyclohexenyl)-3,7,12,16-tetramethyl-18-(2-methyl-2,4-cyclohexadienyliden)-3,5,7,9,11,13,15,17-octadecaocta- en-2-one	$C_{36}H_{38}O_3$
369	E; FUOH – 	 1,5,5-trimethyl-6-[(3*E*,5*E*,7*E*,9*E*)-3,7,12-trimethyl-1,3,5,7,9,11 -tridecahexaenylidene]-3-cyclohexen-1-ol	$C_{25}H_{35}O_2$

[a] - Fucoxanthinol; [b] - Amarouciaxanthin

Table 5.3. Mass spectral fragments, molecular formula and chemical structures of FUCO metabolites detected from liver of rats intubated with FUCO.

Mass	Derivation	Structure and Name	Molecular formula
641	F; FUCO[p] – H_2O	 4-[(3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*)-18-(4-hydroxy-2,2,5a-trimethylperhydro-1-benzoxiren-1-yl)-3,7,12,16-tetramethyl-17-oxo-1,3,5,7,9,11,13,15-octadecaoctaenylidene]-3,5,5-trimethyl-2-cyclohexenyl acetate	$C_{42}H_{56}O_5$
629 628	G; FUCO – $2CH_3$	 4-[(3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*)-18-(4-hydroxy-2,2,5a-trimethylperhydro-1-benzoxiren-1-yl)-3,7,12,16-tetramethyl-17-oxo-1,3,5,7,9,11,13,15-octadecaoctaenylidene]-3-methyl-2-cyclohexenyl acetate	$C_{40}H_{52}O_6$
623	H; F – H_2O	 4-[(3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*)-18-(2,2,5a-trimethyl-1a,2,5,5a-tetrahydro-1-benzoxiren-1-yl)-3,7,12,16-tetramethyl-17-oxo-1,3,5,7,9,11,13,15-octadecaoctaenylidene]-3,5,5-trimethyl-2-cyclohexenyl acetate	$C_{42}H_{54}O_4$
615	AAx[q]	 Amarouciaxanthin	$C_{40}H_{54}O_5$
601	A; FUOH[r] – H_2O	 (3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*)-1-(4-hydroxy-2,2,5a-trimethyl perhydro-1-benzoxiren-1-yl)-18-(6-hydroxy-2,2,6-trimethyl-3-cyclohexenyliden)-3,7,12,16-tetramethyl-3,5,7,9,11,13,15,17-octadecaoctaen-2-one	$C_{40}H_{54}O_4$
596	I; AAx – H_2O	 (3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*)-18-(4-hydroxy-2,6,6-trimethyl-2-cyclohexenyliden)-1-(1-hydroxy-2,6,6-trimethyl-4-oxo-2-cyclohexenyl)-3,7,12,16-tetramethyl-3,5,7,9,11,13,15,17-octadecaoctaen-2-one	$C_{40}H_{52}O_4$

574	J; FUOH – $3CH_3$	 (3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*)-18-(2,4-dihydroxycyclohexyliden)-1-(4-hydroxy-2,2,5a-trimethylperhydro-1-benzoxiren-1-yl)-3,7,12,16-tetramethyl-3,5,7,9,11,13,15,17-octadecaoctaen-2-one	$C_{37}H_{47}O_5$
517	K; AAx – $3H_2O$ – $3CH_3$	 (3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*)-18-(2,4-cyclohexadienyliden)-3,7,12,16-tetramethyl-1-(2,6,6-trimethyl-4-oxo-2-cyclohexenyliden)-3,5,7,9,11,13,15,17-octadecaoctaen-2-one	$C_{37}H_{39}O_2$
427 429 430	L; AAx – Z^s – H_2O – CH_3	 (3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*)-18-(4-hydroxy-6,6-dimethyl-2-cyclohexenyliden)-3,7,12,16-tetramethyl-3,5,7,9,11,13,15,17-octadecaoctaen-2-one	$C_{30}H_{36}O_2$
367	E; FUOH – 	 1,5,5-trimethyl-6-[(3*E*,5*E*,7*E*,9*E*)-3,7,12-trimethyl-1,3,5,7,9,11-tridecahexaenylidene]-3-cyclohexen-1-ol	$C_{25}H_{35}O_2$
339	M; G – 2 CH_3	 1-methyl-6-[(3*E*,5*E*,7*E*,9*E*)-3,7,12-trimethyl-1,3,5,7,9,11-tridecahexaenylidene]-1,3-cyclohexanediol	$C_{23}H_{29}O_2$
311	N; FUCO -  - H_2O – CH_3	 4-[(3*E*,5*E*,7*E*)-3,7-dimethyl-1,3,5,7-nonatetraenylidene]-3,3-dimethylcyclohexyl acetate	$C_{21}H_{26}O_2$

[p]- Fucoxanthin; [q] – Amarouciaxanthin; [r] – Fucoxanthinol; [s] - 

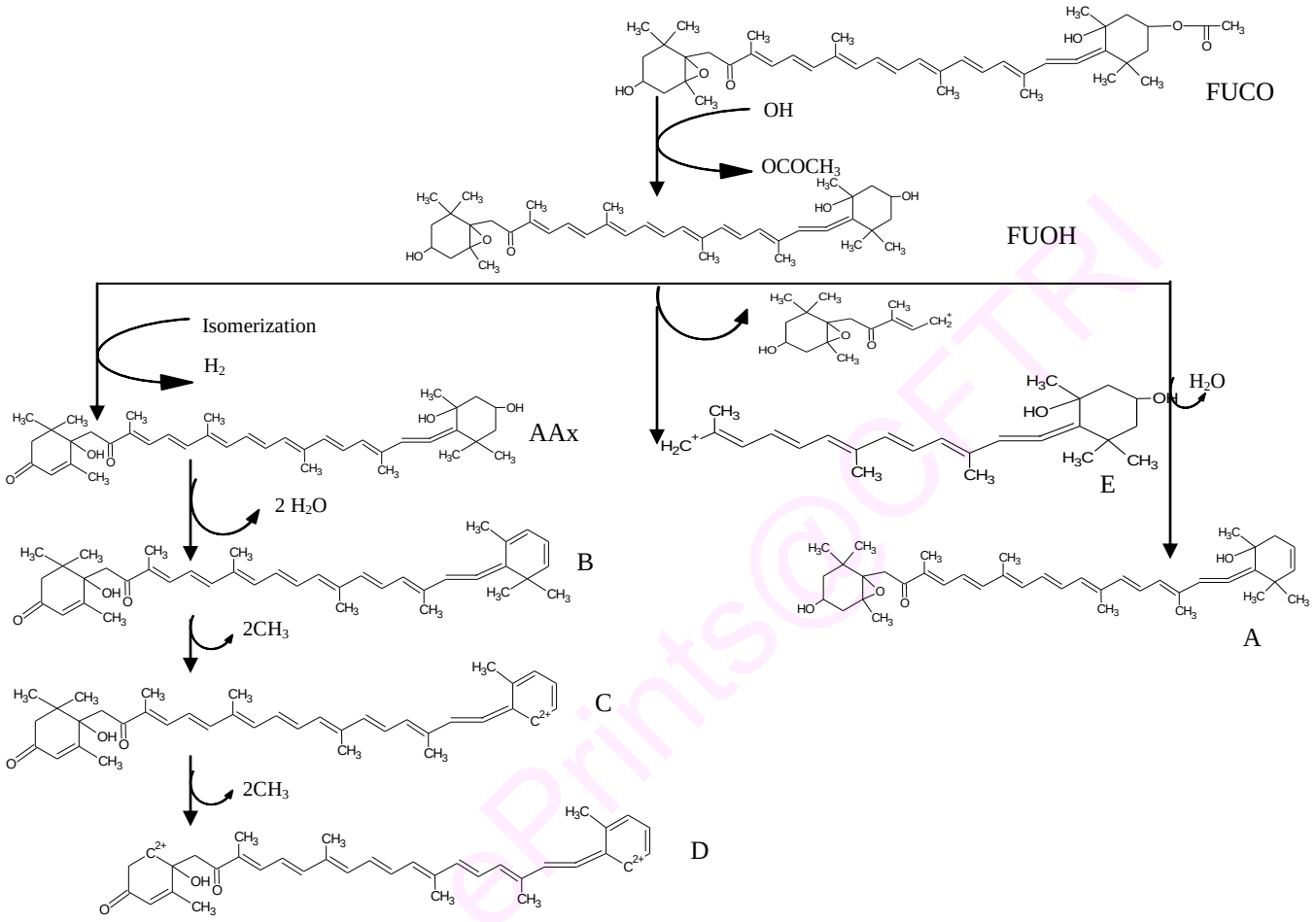


Figure 5.15. Proposed pathway for fucoxanthin metabolism in rat plasma. Refer Table 5.2 for chemical names, molecular mass and molecular formula of metabolites corresponding to the alphabets (Scheme 4).

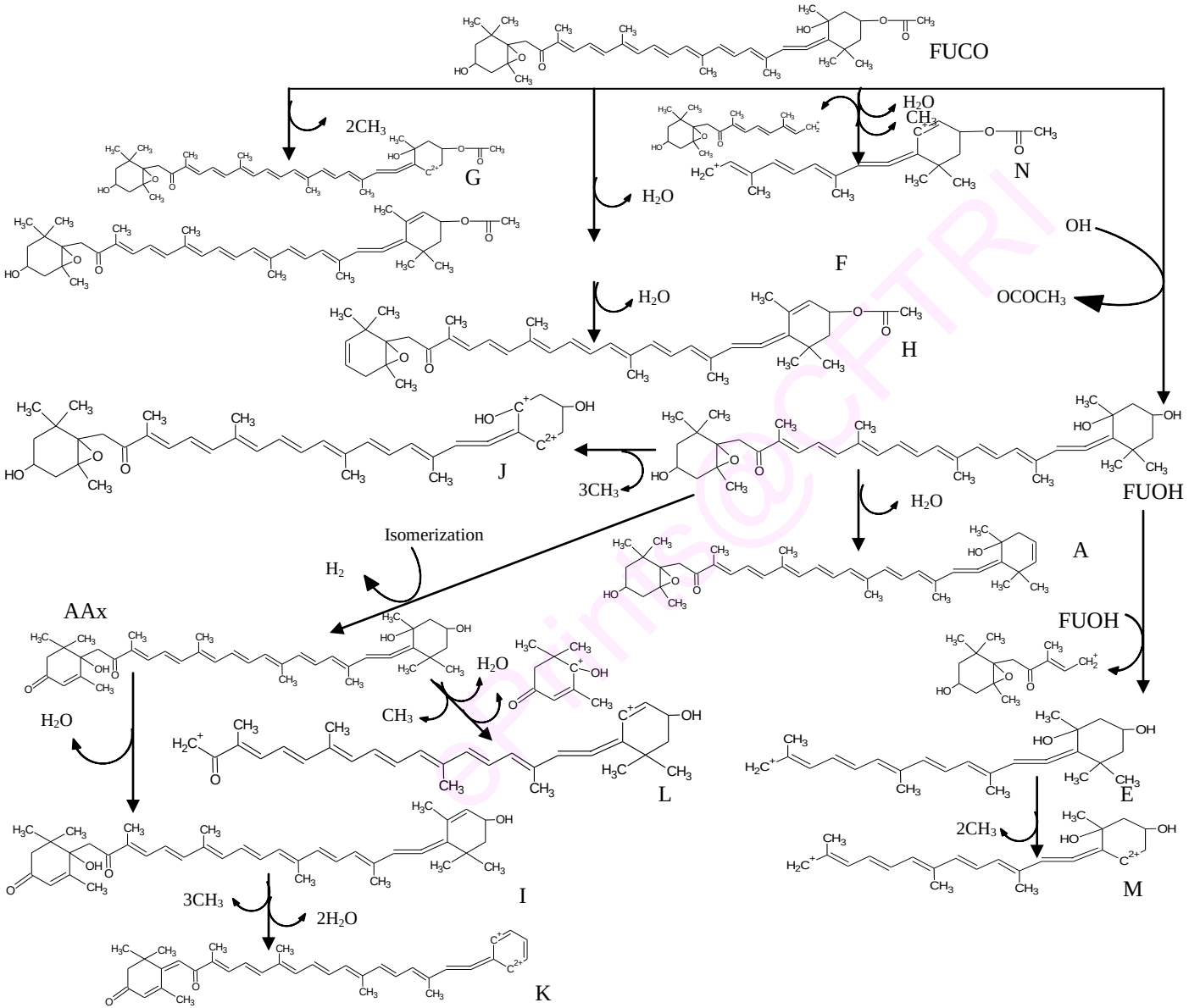


Figure 5.16. Proposed pathway for fucoxanthin metabolism in rat liver. Refer Table 5.3 for chemical names, molecular mass and molecular formula of metabolites corresponding to the alphabets (Scheme 5).

Discussion

RD rats were administered β-carotene, astaxanthin, lutein and fucoxanthin to ascertain provitamin A activity if any. Hence, the metabolites formed as a result of carotenoid administrations were characterized by HPLC and LC-MS. No carotenoid or their metabolites were detected in the baseline and control groups. This indicated that on feeding carotenoids to RD rats, they were either cleaved or oxidized or fragmented (metabolites). The unknown metabolites were also identified and characterized, however not quantified due to the lack of standards.

Gavage with a single dose of β-carotene to RD rats resulted in elevated retinol levels in plasma and liver over a period of 8 h. β-Carotene in its intact form was not detected in plasma while it was detected in liver only 8 h after intubation. This indicated that β-carotene was cleaved to its major metabolite, retinol. This is in agreement with several other studies that also reported the formation of retinol from β-carotene (Lemke et al., 2003; Bachmann et al., 2002, Barua and Olson, 2000). However, no apocarotenals were detected in this study. Either the β-carotene or apocarotenals may rapidly degrade to retinol or else the centric cleavage of β-carotene may have been predominant resulting in retinol. Similarly, Barua and Olson (2000) have reported very low apo-carotenal formation (<5%) in vitamin A deficient and sufficient rats on feeding β-carotene. Thus, the major metabolite of β-carotene found in this study was retinol in retinol deficient rats as evidenced by the elevated plasma and liver retinol levels.

Similar to single dose study, repeated gavage of β-carotene to RD rats resulted in a rise in plasma and liver retinol levels which corresponded with a lower level of β-carotene in plasma indicating its conversion to retinol. Interestingly, astaxanthin gavages to RD rats also showed increased plasma and liver retinol levels. The presence of β-carotene in the plasma of astaxanthin group further supports the speculation that retinol may have been formed by the bioconversion of astaxanthin to β-carotene via reductive pathways as reported in lower vertebrates. In fact, the biosynthesis process of carotenoids in micro algae indicates that astaxanthin is derived from β-carotene (Rodriguez-Amaya, 2001). In contrast, in RD rats, astaxanthin seems to be converted to β-carotene and subsequently to retinol in the present study. The newly formed retinol in β-carotene and astaxanthin groups in the present study is further supported by the increased activity of monooxygenase (73, 70%) compared to RD rats. This corroborates well with Raju and Baskaran (2009) who have also reported increased monooxygenase activity on feeding β-carotene to RD rats. In contrast, when compared to RD group, lutein group exhibited no change in retinol levels and activity of monooxygenase. Matsuno (1991) and Moren et al. (2002) have reported

formation of retinol from xanthophylls such as astaxanthin, canthaxanthin, lutein, zeaxanthin and tunaxanthin via reductive pathways with the formation of zeaxanthin and 3-dehydroretinol in fish. Whereas, the present results did not indicate the presence of these intermediate compounds in rats. On the contrary, LC-MS analysis of liver samples revealed a compound whose mass corresponds with echinenone (m/z 551 $(M+H)^+$). The identity of the compound was further confirmed by its characteristic UV-Vis spectrum, recorded by the PDA detector. It is predicted that astaxanthin and its hydroxyl derivatives may undergo reduction reactions leading to dehydroxylation with the removal of two hydroxyl-groups and one keto-group to form echinenone by the action of reducing enzymes such as reductase, dehydrogenase and dehydroxylase. It is probable that the intermediate compounds of these reactions are unstable and short-lived and therefore not detected. Echinenone in turn may have been reduced to β-carotene, which may be cleaved by the monooxygenase to retinal. The newly formed retinal may be further very rapidly converted to retinol in the body. Reactions suggesting the probable mechanism of astaxanthin metabolism in RD rats have been depicted in Scheme 2 (Figure 5.9). Matsuno (1991) have observed the formation of echinenone from canthaxanthin, while astaxanthin resulted in formation of zeaxanthin and 3-dehydroretinol in their study. It is suggested that due to the clinical retinol deficient condition of the rats in the present study, in contrast to previous reports, astaxanthin may have been reduced to echinenone and β-carotene to meet the requirement of retinol under RD condition. Such reaction was not evidenced in retinol sufficient rats fed with astaxanthin. Thus, this is the first study to report the formation of echinenone and β-carotene from astaxanthin by reductive pathways, demonstrating the provitamin A activity of astaxanthin in RD rats.

Unlike β-carotene and astaxanthin, gavage of lutein to RD rats did not result in change in liver or plasma retinol levels, indicating that it was not cleaved to retinol. However, other metabolites of lutein were identified in the samples including zeaxanthin, anhydrolutein and diepoxylutein. The metabolites formed in liver and plasma were almost similar. Only few reports are available on epoxy-carotenoids in plasma of rodents fed on lutein (Lakshminarayana et al. 2006; Asai et al. 2004; Barua and Olson, 1998). The present results are in agreement with the observations of Barua and Olson (2001) who also reported epoxy carotenoids and zeaxanthin *in vitro* and *in vivo* (rats). Lutein oxidation in liver may have resulted in the formation of the metabolites designated as B (lutein diepoxide) (5,6, 4', 5'-diepoxy-5, 6; 4', 5'-tetrahydro-β,β-carotene 3,3'-diol), and C (5,6 epoxy-3 hydroxy-12'-β,ε-carotene-12'al). Fragmentation of A (anhydrolutein) may have resulted in the formation of the metabolites designated as D (2*E*, 4E)-3-methyl-5- (2,6,6-trimethylcyclohexa-2, 4-dien-1-yl) penta-2, 4-dien-1-ylium and E (B-Z) to 2,6,6-trimethylcyclohex-2-ene-1, 4-bis (ylium). Thus, lutein and its diepoxides were identified in liver

and plasma. The diepoxides might be formed in liver itself or mobilized from intestine via plasma. Degradation of lutein in the body indicates that lutein is involved in various biochemical reactions and acts as an antioxidant in various oxidative reactions.

Khachik et al. (2002), have suggested that the transport and the metabolic inter-conversion between lutein and zeaxanthin in the eyes are most probably induced by sunlight as evidenced *in vitro*. Hartmann et al. (2004) reported that dosing zeaxanthin to human volunteers resulted in considerable accumulation of *all-E*-3-dehyro-lutein in plasma and postulated that since lutein concentration remains unaffected by zeaxanthin dosing, the increase in *all-E*-3-dehydro-lutein might have derived from zeaxanthin. Another study demonstrated that long-term intake of lutein resulted in an accumulation of 3`dehydro-lutein in human plasma (Thurman et al., 2005). Lutein may be converted into several metabolites *in vivo*. In liver and plasma, oxidized molecules like B and C may be formed due to oxidative reactions, which indicate that lutein is involved in the chain breaking peroxyl- radical or quenching of the singlet oxygen (Stratton and Liebler, 1997; Yamauchi et al., 1998). The formation of oxidized product B indicates the formation of lutein epoxide, which is very similar to neoxanthin, and may have more antioxidative capacity. This may be the reason for the higher antioxidant property of lutein evidenced in rats as compared to β-carotene (Chapter 6). It has been reported earlier that increase in the number of hydroxyl-groups in the carotenoid molecule amplifies the antioxidant capacity. Apart from these two compounds, A, D and E were also formed in the plasma, and liver. D and E may have been formed due to fragmentation of anhydrolutein. Based on these results, a probable scheme of formation of the above-described molecules is proposed (Figure 5.12, Scheme 3). The present study shows that lutein is degraded in the system by oxidative reactions and the metabolites/oxidized products formed could be excreted from the biological samples (Bausch et al., 1999). Degradation of lutein in the body indicates that lutein is involved in various photochemical reactions (in eyes) and as an antioxidant in various oxidative reactions (other tissues). Based on the results we have proposed metabolic pathways for conversion of dietary lutein to its oxidation products. This study emphasizes the essentiality of maintaining dietary lutein status to function as an antioxidant since it is easily degraded. Further research is needed to identify lutein metabolites in detail in biological samples in order to evaluate their possible biological significance with reference to age related macular degeneration.

No FUCO metabolites were detected in the plasma of rats at 0h group (base line) thereby confirming that metabolites found were derived from FUCO. In plasma and liver, the UV-visible spectrum of the peak detected at 6.9 min was ascribed to be FUOH, the hydrolytic product of FUCO as reported by Sugawara et al. (2002) *in vitro* (Caco-2 cell line) and *in vivo* (mice). The

metabolite eluted at 6.9th min was the de-acetylation product of FUCO and its mass spectra produced the same UV-Visible spectrum and positive m/z ions at 617 which corresponds to $(M+1)^+$ for FUOH. Thus, FUOH was identified as a prime metabolite of FUCO in rats in this study and mice (Sugawara et al., 2002; Asai et al., 2004). The UV-visible and mass spectrum of peak that eluted at 8.4 min was different from that of FUOH with molecular ion $(M+1)^+$, at *m/z* 615 which corresponds to AAx as reported by Asai et al. (2004) *in vitro* (PC-3 human prostrate cancer cell line) and *in vivo* (mice). Interestingly, AAx was a major metabolite of FUCO in liver whereas, in plasma it was FUOH, demonstrating that liver enzymes may play a role in hydrolyzing the FUOH to AAx (Asai et al., 2004). However, detailed studies in this regard are necessary. From the LC-MS spectra, metabolites other than FUOH and AAx were also characterized in plasma and liver samples. Interestingly, metabolites bearing molecular mass at *m/z* 600 (A) and *m/z* 367 (E) were found in both plasma and liver samples. Similar to earlier reports in mice model (Asai et al., 2004), the conversion of FUCO to FUOH and/or AAx was higher in rat liver, which may be due to dehydrogenation and/or isomerization of 5,6-epoxy-3-hydroxy-2,4-dihydro-β end group of FUOH to 6-hydroxy-3-oxo-ε end group of AAx by liver dehydorgenase (Sugawara et al., 2002; Asai et al., 2004). These metabolites may act as functional molecules *in vivo*, similar to lutein metabolites (Bhosale et al., 2007). FUCO seems to be rapidly hydrolyzed in the intestine to FUOH, AAx, FUOH-H_20 and AAx-H_20. Metabolites at m/z 641 (FUCO-H2O), m/z 629 (FUCO-2CH_3) found in the liver were different from those in plasma. This is probably due to hydrolysis of FUCO to FUOH and other metabolites by gastro-intestinal enzymes lipase, cholesterol esterase and carboxyl esterase and subsequently transported to liver (Asai et al., 2004; Strand et al., 1998). Metabolites found in plasma (A & B) and liver (A, F, H, I & K) could have resulted from either FUOH or AAx by removal of water molecules due to enzymatic reactions involving oxygenases (Olson, 1994). Metabolites (E, L, M & N) could partly be due to enzymatic retroaldol cleavage (Bhosale et al., 2007). Enzymatic demethylation of FUOH or AAx could be the reason for the metabolites C, D, G, J, K, L, M & N. Strand et al. (1998) reported that FUOH but not FUCO was transported to egg yolk of laying hens fed with a diet containing 15% brown algal meal. Based on present findings, the possible FUCO metabolic pathway in plasma and liver of RD rats are proposed.

Therefore, it is seen from the results that rats could convert β-carotene to retinol under RD but not lutein and fucoxanthin. Metabolites formed in plasma and liver of RD rats as a result of feeding carotenoids is summarized in Table 5.4.

Table 5.4 Metabolites formed as a result of administration of carotenoids to RD rats.

Carotenoid fed	Experiment duration	Metabolites					
		Retinol	β-Carotene	Intermediate	Zeaxanthin	Lutein	Astaxanthin
β-Carotene	Single dose	+	+	-	-	-	-
Astaxanthin	15 days	+	+	+ [a]	-	-	+
Lutein	15 days	-	-	+ [b]	+	+	-
Fucoxanthin	Single dose	-	-	+ [c]	-	-	-

[a] Refer Figure 5.9

[b] Refer Table 5.1

[c] Refer Tables 5.2 and 5.3

CHAPTER 6: Influence of dietary carotenoids (β-carotene, astaxanthin, lutein and fucoxanthin) on vitamin A deficiency induced biochemical changes

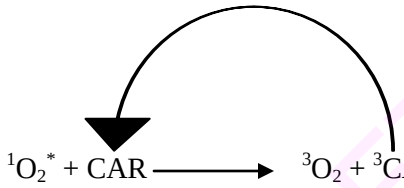
Introduction

Consumption of carotenoid rich foods is associated with a reduced risk of cancers, cardiovascular disease and age related macular degeneration (Seddon et al., 1994; Van Poppel, 1996). These effects could be related to their role as precursors of vitamin A or as antioxidants (Olson, 1999). A possible explanation for the protective effects associated with dietary fruits and vegetables is that carotenoids inhibit the onset of disease due to their antioxidant properties (Rice-Evans et al., 1997). Carotenoids are generally recognized as safe for human health. Thus, the potential use of carotenoids as supplements to ameliorate biochemical changes caused by chronic diseases in addition to their use in preventing vitamin A deficiency has stimulated interest in the field of carotenoids research. Raju and Baskaran (2009) and Raju et al. (2006) studied the effect of VAD on the monooxygenase in retinol deficient and sufficient rats. Parvin and Sivakumar (2000) investigated the role of monooxygenase in conversion of β-carotene to retinol in retinol deficient rats and retinol sufficient rats. Thus, measuring the activity of the intestinal monooxygenase serves as a marker molecule for determining the provitamin A activity of carotenoids. Further, retinol levels can be confirmed by estimating the circulating serum retinol binding protein (RBP) that helps in its transport to target organs. It has been demonstrated that RBP secretion from hepatocytes is regulated by retinol (Bellovino et al., 2003) and hence serum RBP levels are a good indicator for retinol status. Retinol-RBP circulates in the bloodstream in a 1:1 molar complex with transthyretin, a 55 kDa protein that is synthesized and secreted from the liver (Monaco et al., 1995). This ternary complex prevents retinol-RBP excretion by the kidney (van Bennekum et al., 2001). Although the major site of RBP synthesis in the body is the hepatocyte, other organs and tissues are reported to express RBP.

The potent antioxidant potential of carotenoids is well known (Britton, 1995) and can be attributed to their unique chemical structure with alternating double bonds on the polyene chains and functional groups on the β-ionone rings. There are studies available on the antioxidant property of carotenoids. Bhosale and Bernstein (2005) have studied the protective effect of zeaxanthin against lipid peroxidation *in vivo* (human) while Cantrell et al. (2003) have investigated the effect of lycopene, β-carotene, astaxanthin and canthaxanthin in quenching

singlet oxygen radicals *in vitro* (liposomes). Chitchumroonchokchai et al. (2004) have studied UV-induced lipid peroxidation and stress signaling in human lens epithelial cells by lutein, zeaxanthin and astaxanthin. Kennedy and Liebler (1992) have studied the role of β-carotene *in vitro* in protection of lipid layers against lipid peroxidation.

Equation 1 given below explains the antioxidant activity of carotenoids by quenching singlet molecular oxygen ($^1O_2^*$) as described by Edge et al. (1997). Foote and Denny (1968) first demonstrated that β-carotene could inhibit photosensitized oxidation and was, therefore, an efficient quencher of $^1O_2^*$. The carotenoid triplet state ($^3CAR^*$) is produced via electronic energy transfer. Once $^3CAR^*$ is produced, it can easily return to the ground state dissipating the energy as heat. Thus, the carotenoid acts as a catalyst deactivating $^1O_2^*$.



$$^1O_2^* + CAR \longrightarrow {}^3O_2 + {}^3CAR^* \qquad (1)$$

Peroxyl radicals are generated during the course of auto-oxidation of lipids. However, the chain-reaction of lipid auto-oxidation can be interrupted by carotenoids. Carotenoids scavenge lipid peroxyl radicals forming a resonance-stabilized antioxidant radical. Carotenoids may scavenge radicals in the initial step involving one or more of the following three possibilities namely, electron transfer, allylic hydrogen abstraction, and addition as shown in equations (2), (3) and (4) (El Agamey et al., 2004).

$$CAR + ROO^* \longrightarrow CAR^{*+} + ROO^- \qquad (2)$$

$$CAR + ROO^* \longrightarrow CAR^{*+} + ROOH \qquad (3)$$

$$CAR + ROO^* \longrightarrow ROOCAR \qquad (4)$$

The above mentioned antioxidant property of carotenoids indicates that they can reverse the biochemical changes caused by the nutritional deficiency at cellular level. Retinol has been known as an essential micronutrient (Olson, 1999). Yet, its deficiency is still a major public health problem in developing countries (Underwood, 1994). A large section of the human population depends on dietary carotenoids as the primary source for vitamin A. The role of carotenoids as precursors of vitamin A is still of public health importance although the issue of bioavailability of dietary provitamin A carotenoids has been raised (de Pee and West, 1996). Vitamin A deficiency (VAD) is known to increase oxidative stress and cause alterations in the

biochemical parameters *in vivo* (Anzulovich et al., 2000; Oliveros et al., 2000; Kaul and Krishnakantha, 1997). Due to its provitamin A activity, β-carotene would obviously help to relieve the alterations caused by VAD. However, by virtue of their antioxidant property, it was of interest to study the effect of astaxanthin, lutein and fucoxanthin on oxidative stress and other biochemical parameters in retinol deficiency.

The objective of this study was to examine the effect of VAD on lipid peroxidation (Lpx), activity of antioxidant molecules, lipid profile (total cholesterol, HDL-cholesterol, LDL+VLDL-cholesterol, phospholipids, triglycerides and fatty acid profile) and retinol binding protein (RBP) in rats. In single dose study, retinol deficient (RD) rats were gavaged with a dose of β-carotene, astaxanthin, lutein and fucoxanthin to study their time course effect over period of 8 hours. In the repeated dose study, RD rats were gavaged with repeated doses of β-carotene, astaxanthin or lutein for 7- and 15-days. In dietary study, groups of RD rats were fed diet supplemented with carrot powder (β-carotene source), purified astaxanthin and dill leaf powder (lutein source) for 20 days. A control and baseline (RD) group was maintained for each experiment. Blood was collected in heparinized (for plasma separation) and non-heparinized (for serum separation) tubes. Lipid peroxides, glutathione and triglyceride levels, activity of superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) was estimated in plasma while total cholesterol, HDL-cholesterol, LDL+VLDL-cholesterol and phospholipids were estimated in serum. Native PAGE electrophoresis was run for serum RBP to compare the RBP levels between RD, control and experimental groups. Further, it was quantified using ELISA to confirm the retinol levels. The carotenoid metabolizing enzyme, monooxygenase activity was estimated in the intestines of the rats. Lipid peroxides, glutathione, cholesterol, phospholipid and triglyceride levels as well as activity of SOD, CAT and GST were estimated in the liver of the rats to determine the effect of carotenoid feeding on biochemical alterations induced by retinol deficiency.

Results

Single dose study

Rats were made retinol deficient (RD), which was confirmed by their plasma retinol levels (0.38 ± 0.036 μmol/l). RD rats were gavaged with β-carotene, astaxanthin, lutein or fucoxanthin (FUCO). A separate group of RD (baseline) and control (fed diet containing retinol) rats were maintained throughout the experimental runs. Thus, the effect of vitamin A deficiency and subsequent carotenoid feeding on biochemical parameters as compared to control was studied.

Activity of 15-15' β-carotene monooxygenase

Results on the effect of retinol deficiency and gavage of carotenoids on the activity of intestinal monooxygenase are given in Table 6.1. No difference was found in the activity of 15-15' β-carotene monooxygenase (pmol/h/mg protein) between RD (45.7) and control (44.4) groups. On β-carotene gavage, the activity of monooxygenase was significantly higher (56-70%) over a period 8 h as compared to RD group (Figure 6.1). Whereas, no significant difference was observed in its activity (pmol/h/mg protein) in astaxanthin (43.9-47.8), lutein (44.8-46.6) and FUCO (43.5-45.3) groups compared to RD group (45.7) indicating that β-carotene may be converted to retinol but not the other carotenoids.

Lipid peroxidation and activity of antioxidant enzymes and molecules

Retinol deficiency resulted in increased lipid peroxides (Lpx) level in plasma (81.5nmol/ml) and liver (2.6μmol/g) as compared to control (8.7 nmol/ml, 0.4μmol/g) group (Table 6.2). Gavage of β-carotene, astaxanthin, lutein and FUCO resulted in decreased Lpx in plasma over a period of 2 to 8 h by 49-69, 56-77, 51-72, 3-74% and in liver by 33-65, 42-76, 39-69, 25-72% as compared to RD group (Table 6.2, Figure 6.2).

Retinol deficiency resulted in decreased activity of superoxide dismutase (SOD, 68%), catalase (CAT, 84%) and reduced glutathione (GSH, 63%) levels in plasma as compared to control (Figure 6.3). As in the case of plasma, the activity of SOD (69%), CAT (72%), GST (54%) and GSH (72%) levels in liver was also decreased as compared to control (Figure 6.3). In contrast, a dose of β-carotene, astaxanthin, lutein and FUCO (8 h) resulted in increased activity of plasma SOD (4-37, 17-55, 14-44, 14-56%), CAT (35-58, 47-71, 44-66, 91-95%) and GSH (2-19, 9-37, 5-29, 7-42%) as compared to RD group (Table 6.1 and 6.3). Similarly, the activity of SOD (0-34, 4-46, 7-39, 11-48%), CAT (0-47, 17-62, 9-57, 17-66%), GST (2-31, 3-44, 1-37, 26-19 %) and GSH (2-26, 9-43, 5-34, 23-51 %) in liver was enhanced in carotenoid fed groups as compared to RD group (Table 6.1 and 6.3). The above results indicate the potential antioxidant property of carotenoids in ameliorating the Lpx and antioxidant molecules altered by retinol deficiency.

Table 6.1. Effect of retinol deficiency and gavage with a dose of carotenoids on the activity of intestinal 15-15' β-carotene monooxygenase and antioxidant molecules in rats.

Time (h)	Groups					
	Control	RD[#]	β-Carotene	Astaxanthin	Lutein	FUCO
Intestinal 15-15' β-carotene monooxygenase (pmol retinal/h/mg protein)						
2	45.7 ± 4.2 [a]	44.4 ± 4.6 [a]	100.6 ± 10.1 [b]	47.8 ± 4.6 [a]	45.1 ± 4.2 [a]	43.5 ± 4.1 [a]
4	45.7 ± 4.2 [a]	44.4 ± 4.6 [a]	122.7 ± 10.9 [b]	43.9 ± 5.9 [a]	46.6 ± 4.4 [a]	44.6 ± 5.1 [a]
8	45.7 ± 4.2 [a]	44.4 ± 4.6 [a]	145.6 ± 11.0 [b]	45.6 ± 4.9 [a]	44.8 ± 5.0 [a]	45.3 ± 4.2 [a]
Plasma Superoxide Dismutase (U/mg protein)						
2	7.4 ± 0.6 [a]	2.4 ± 0.2 [b]	2.5 ± 0.1 [b]	2.9 ± 0.1 [b]	2.8 ± 0.2 [b]	2.8 ± 0.1 [b]
4	7.4 ± 0.6 [a]	2.4 ± 0.2 [b]	3.1 ± 0.2 [c]	4.1 ± 0.2 [d]	3.6 ± 0.1 [b]	3.9 ± 0.1 [d]
8	7.4 ± 0.6 [a]	2.4 ± 0.2 [b]	3.8 ± 0.3 [c]	5.3 ± 0.3 [d]	4.3 ± 0.3 [c]	5.5 ± 0.2 [d]
Liver Superoxide Dismutase (U/mg protein)						
2	8.0 ± 0.5 [a]	2.5 ± 0.2 [b]	2.5 ± 0.2 [b]	2.6 ± 0.2 [b]	2.7 ± 0.1 [b]	2.8 ± 0.2 [b]
4	8.0 ± 0.5 [a]	2.5 ± 0.2 [b]	3.2 ± 0.2 [c]	3.9 ± 0.3 [d]	3.5 ± 0.2 [c]	4.0 ± 0.2 [d]
8	8.0 ± 0.5 [a]	2.5 ± 0.2 [b]	3.8 ± 0.3 [c]	4.6 ± 0.2 [d]	4.1 ± 0.3 [c]	4.8 ± 0.3 [d]
Plasma Catalase (μmol/min/mg protein)						
2	7.46 ± 0.3[a]	0.79 ± 0.2[b]	1.2 ± 0.1 [c]	1.5 ± 0.1 [d]	1.4 ± 0.1 [d]	8.41±0.2 [e]
4	7.46 ± 0.3[a]	0.79 ± 0.2[b]	1.5 ± 0.1 [c]	1.9 ± 0.1 [d]	1.8 ± 0.2 [d]	14.53±1.0 [e]
8	7.46 ± 0.3[a]	0.79 ± 0.2[b]	1.9 ± 0.2 [c]	2.7 ± 0.1 [d]	2.3 ± 0.2 [c]	8.33±0.5 [e]
Liver Catalase (μmol/min/mg protein)						
2	3.6 ± 0.3 [a]	1.0 ± 0.1 [b]	1.0 ± 0.1 [b]	1.2 ± 0.1 [b]	1.1 ± 0.1 [b]	1.2 ± 0.1 [b]
4	3.6 ± 0.3 [a]	1.0 ± 0.1 [b]	1.5 ± 0.1 [c]	1.9 ± 0.2 [d]	1.8 ± 0.2 [d]	2.3 ± 0.2 [e]
8	3.6 ± 0.3 [a]	1.0 ± 0.1 [b]	1.9 ± 0.1 [c]	2.6 ± 0.2 [d]	2.3 ± 0.1 [c]	2.9 ± 0.1 [d]
Liver Glutathione Transferase (μmol/min/mg protein)						
2	22.9 ± 2.1 [a]	10.5 ± 1.0 [b]	10.7 ± 0.9	10.8 ± 1.0	10.6 ± 0.9	14.2±0.3[c]
4	22.9 ± 2.1 [a]	10.5 ± 1.0 [b]	11.4 ± 0.8	14.2 ± 1.2	13.1 ± 0.9	14.2±0.8[c]
8	22.9 ± 2.1 [a]	10.5 ± 1.0 [b]	15.1 ± 1.1	18.6 ± 0.9	16.5 ± 0.9	13.0±0.2[d]
Plasma Glutathione (mg/ml)						
2	11.2 ± 0.9 [a]	4.2 ± 0.3 [b]	4.3 ± 0.4 [b]	4.6 ± 0.3 [b]	4.4 ± 0.4 [b]	4.5 ± 0.4 [b]
4	11.2 ± 0.9 [a]	4.2 ± 0.3 [b]	4.7 ± 0.4 [c]	5.5 ± 0.4 [d]	5.0 ± 0.2 [c]	5.9 ± 0.5 [d]
8	11.2 ± 0.9 [a]	4.2 ± 0.3 [b]	5.2 ± 0.3 [c]	6.7 ± 0.3 [d]	5.9 ± 0.5 [e]	7.2 ± 0.6 [d]
Liver Glutathione (mg/g)						
2	14.2 ± 1.1 [a]	4.0 ± 0.3 [b]	4.1 ± 0.4 [b]	4.4 ± 0.3 [b]	4.2 ± 0.3 [b]	5.2 ± 0.5 [c]
4	14.2 ± 1.1 [a]	4.0 ± 0.3 [b]	4.6 ± 0.4 [b]	5.5 ± 0.4 [c]	5.0 ± 0.4 [c]	6.4 ± 0.5 [d]
8	14.2 ± 1.1 [a]	4.0 ± 0.3 [b]	5.4 ± 0.4 [c]	7.0 ± 0.3 [d]	6.1 ± 0.5 [c]	8.1 ± 0.6 [d]

[#]RD represents value at 0 h; Data represent mean ± SD (n=5/time point). Values in a row between the groups with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test. RD = retinol deficient, FUCO = fucoxanthin.

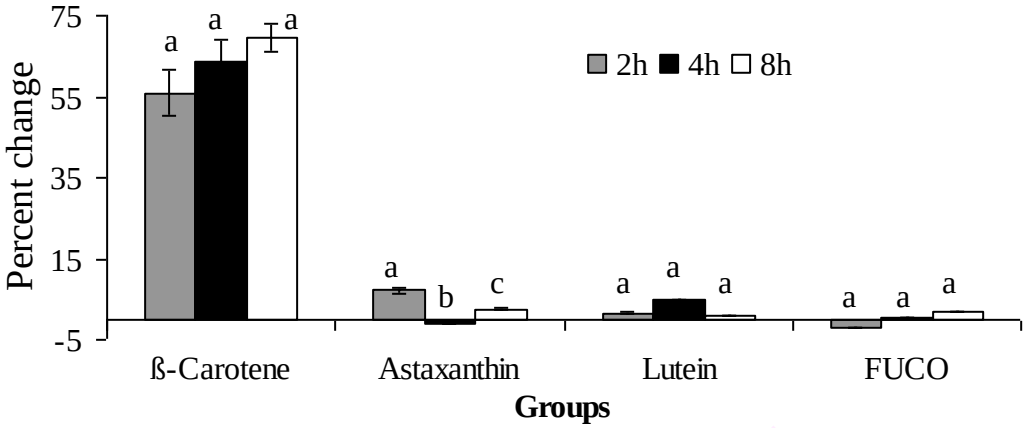


Figure 6.1. Percent change in intestinal 15-15' β-carotene monooxygenase activity in carotenoid fed groups as compared to RD group. The value for RD group was 45.7 4.2 pmol retinal/h/mg protein. Values between time points in a group with similar superscript are not significantly different (p>0.05) as determined by one-way ANOVA followed by Tukey's test. FUCO = fucoxanthin.

Table 6.2. Effect of retinol deficiency and gavage with a dose of carotenoids on plasma and liver levels of lipid peroxides.

Time (h)	Groups					
	Control	RD[#]	β-Carotene	Astaxanthin	Lutein	Fucoxanthin
Plasma lipid peroxides (nmol TMP/ml)						
2	8.7 ± 4.2^{a}	81.5 ± 8.2^{b}	41.6 ± 4.1^{c}	35.8 ± 3.6^{c}	40.2 ± 4.0^{c}	79.5 ± 4.1^{b}
4	8.7 ± 4.2^{a}	81.5 ± 8.2^{b}	33.7 ± 3.6^{c}	22.7 ± 2.5^{d}	25.6 ± 2.4^{d}	32.6 ± 3.1^{c}
8	8.7 ± 4.2^{a}	81.5 ± 8.2^{b}	25.6 ± 3.0^{c}	18.8 ± 1.9^{c}	22.9 ± 3.0^{c}	21.3 ± 2.2^{c}
Liver lipid peroxides (μmol TMP/g)						
2	0.4 ± 0.03^{a}	2.6 ± 1.9^{b}	1.74 ± 0.2^{c}	1.5 ± 0.2^{c}	1.6 ± 0.2^{c}	1.96 ± 0.2^{d}
4	0.4 ± 0.03^{a}	2.6 ± 1.9^{b}	1.49 ± 0.1^{c}	1.1 ± 0.1^{d}	1.33 ± 0.1^{c}	1.18 ± 0.1^{c}
8	0.4 ± 0.03^{a}	2.6 ± 1.9^{b}	0.9 ± 0.08^{c}	0.62 ± 0.05^{c}	0.81 ± 0.07^{c}	0.73 ± 0.06^{c}

[#]RD represents value at 0 h; Data represent mean ± SD (n=5/time point). Values in a row between the groups with similar superscript are not significantly different (p>0.05) as determined by one-way ANOVA followed by Tukey's test. RD = retinol deficient, FUCO = fucoxanthin.

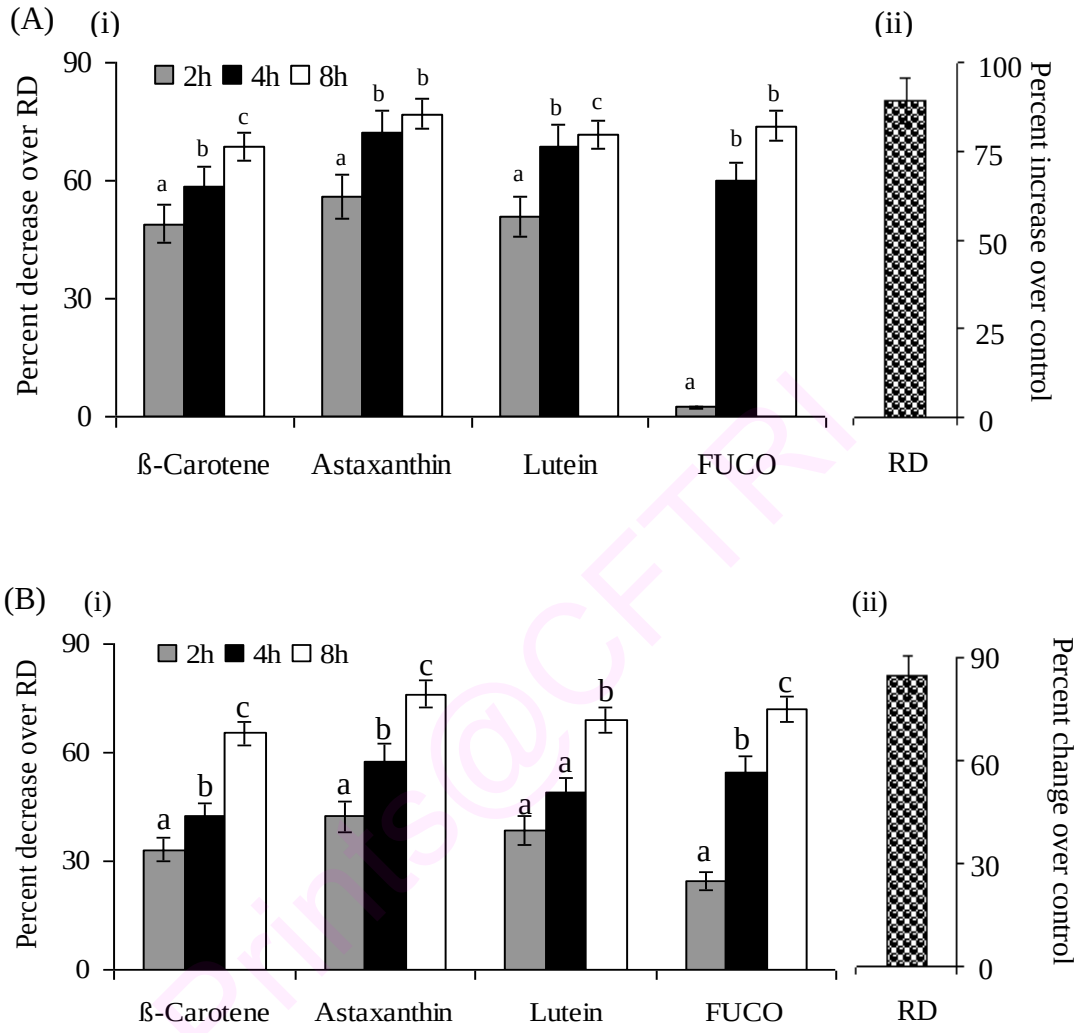


Figure 6.2. Percent decrease in lipid peroxidation over RD group in plasma (A) and liver (B) of β-carotene, astaxanthin, lutein and fucoxanthin gavaged groups (i). Percent increase in plasma lipid peroxidation as a result of retinol deficiency over control group (ii). Values represent mean (n=5/time point). Values within a group at different time points with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test. RD = retinol deficient, FUCO = fucoxanthin.

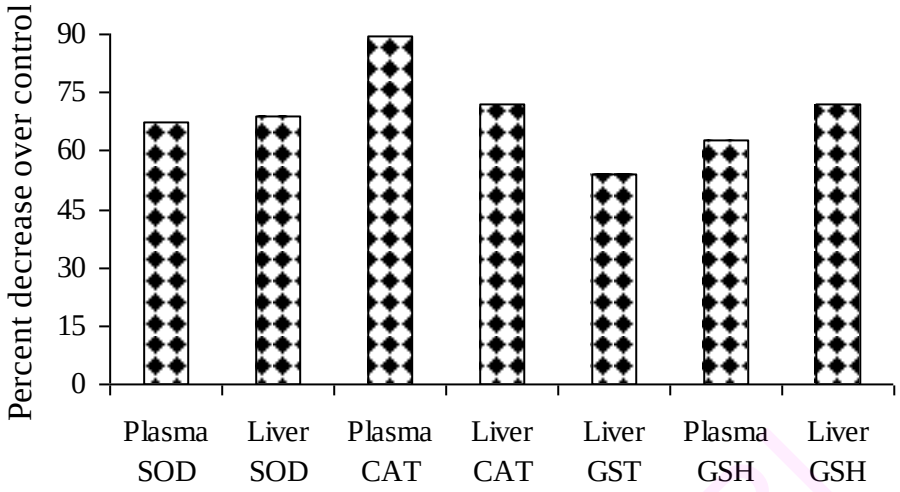


Figure 6.3 Percent decrease in activities of antioxidant molecules in RD rats over control.

Lipid Profile

Induction of retinol deficiency resulted in altered fatty acid profile in plasma. Results revealed an increase ($p<0.05$) in saturated fatty acids (SFA; 38%) and decrease ($p<0.05$) in mono- and poly-unsaturated fatty acids (MUFA; 40% and PUFA; 55%) as compared to control (Table 6.4, Figure 6.4). On gavages with β-carotene, astaxanthin, lutein or FUCO, the SFA levels were decreased by 18-34, 9-27, 6-26, 20-33% while MUFA and PUFA were increased by 23-37, 20-34, 19-33, 14-31% and 28-48, 27-41, 21-40, 51-57% respectively over 8h than RD group (Figure 6.5). The blood cholesterol, phospholipids and triglyceride values of experimental and control groups are presented in Table 6.5. Although the change in total cholesterol and LDL+VLDL-cholesterol is not significantly ($p>0.05$) differed for carotenoid fed groups, the distribution of the cholesterol in HDL fraction (mg/dl) showed alteration ($p<0.05$) in astaxanthin (45-49) and lutein (44-47) groups while in FUCO group, the difference was seen only after 8 h of gavage (45).

Table 6.3. Percent increase in activity of SOD, CAT, GST and GST over RD group in plasma and liver of RD rats intubated with carotenoids.

Time (h)	Groups			
	β-Carotene	Astaxanthin	Lutein	Fucoxanthin
Plasma Superoxide Dismutase (%)				
2h	4.00[a]	17.24[a]	14.29[a]	14.29[a]
4h	22.58[b]	41.46[b]	33.33[b]	38.46[b]
8h	36.84[c]	54.72[b]	44.19[c]	56.36[c]
Liver Superoxide Dismutase (%)				
2h	0.00[a]	3.85[a]	7.41[a]	10.71[a]
4h	21.88[b]	35.90[b]	28.57[b]	37.50[b]
8h	34.21[c]	45.65[c]	39.02[b]	47.92[c]
Plasma Catalase (%)				
2h	34.17[a]	47.33[a]	43.57[a]	90.61[a]
4h	47.33[b]	58.42[a]	56.11[b]	94.56[a]
8h	58.42[c]	70.74[b]	65.65[c]	90.52[a]
Liver Catalase (%)				
2h	0.00[a]	16.67[a]	9.09[a]	16.67[a]
4h	33.33[b]	47.37[b]	44.44[b]	56.52[b]
8h	47.37[c]	61.54[c]	56.52[b]	65.52[b]
Liver Glutathione-S-Transferase (%)				
2h	1.87[a]	2.78[a]	0.94[a]	26.06[a]
4h	7.89[b]	26.06[b]	19.85[b]	26.06[a]
8h	30.46[c]	43.55[c]	36.36[c]	19.23[a]
Plasma Glutathione (%)				
2h	2.33[a]	8.70[a]	4.55[a]	6.67[a]
4h	10.64[b]	23.64[b]	16.00[b]	28.81[b]
8h	19.23[c]	37.31[c]	28.81[c]	41.67[c]
Liver Glutathione (%)				
2h	2.44[a]	9.09[a]	4.76[a]	23.08[a]
4h	13.04[b]	27.27[b]	20.00[b]	37.50[b]
8h	25.93[c]	42.86[c]	34.43[c]	50.62[c]

Values are represented as mean (n=5/time point). Values in a row between groups with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test.

The phospholipid levels (mg/dl) were elevated ($p<0.05$) in β-carotene (108-110), astaxanthin (106-107), lutein (103-106) and FUCO (102-106) groups as compared to RD group (88.4) over a period of 8 h, but were comparable ($p>0.05$) with control (115.4). In case of plasma triglyceride levels (mg/dl), no significant change was noticed up to 2h in the carotenoid fed groups compared with RD group (100.2), whereas, the level was significantly elevated after 4h in β-carotene (160-205), astaxanthin (162-200), lutein (146-186) and FUCO (143-151) groups.

Cholesterol and triglycerides (mg/g) levels in liver of carotenoid groups were not significantly different as compared to RD group (13.8, 17.6) except for triglycerides in 8h of astaxanthin (25.9) and lutein (23.5) groups, which were significantly higher (Table 6.6). No difference was found in the phospholipids level (mg/g) after 2 h compared to RD group (15.6) while it was significantly higher after 4h and 8h in β-carotene (19.8, 21.03), astaxanthin (19.1, 21.2) and FUCO (18.9, 21.1) groups except for lutein group (higher at 8 h, 19.7) as compared to RD group. The results demonstrate the hypolipidemic effect of carotenoids and a favourable change in the essential fatty acid profile.

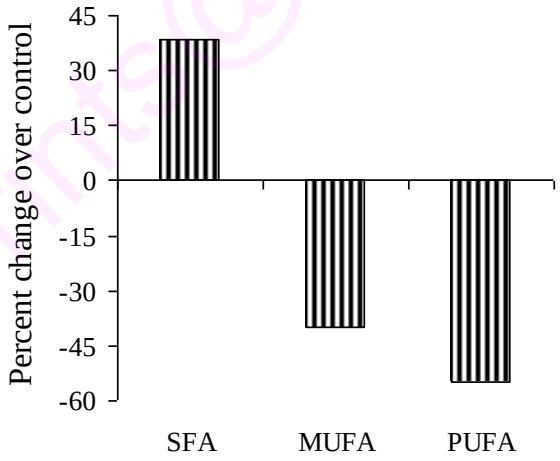


Figure 6.4. Percent change in saturated fatty acid (SFA), mono-unsaturated fatty acid (MUFA) and poly-unsaturated fatty acid (PUFA) in RD rats as compared to control.

Table 6.4. Effect of retinol deficiency and carotenoid gavages on plasma fatty acid profile.

Fatty acids	Time (h)	Groups					
		Control	RD[#]	β-Carotene	Astaxanthin	Lutein	FUCO
12:0	2	0.42	0.60	1.1	1.15	1.2	1.25
	4	0.42	0.60	0.9	0.98	1.0	0.8
	8	0.42	0.60	0.7	0.8	0.9	0.9
14:0	2	0.27	0.45	0.6	0.7	0.7	0.75
	4	0.27	0.45	0.45	0.52	0.57	0.5
	8	0.27	0.45	0.39	0.42	0.45	0.35
16:0	2	22.30	34.25	30.5	31.5	32.4	28.1
	4	22.30	34.25	25.2	28.3	27.6	23.1
	8	22.30	34.25	23.9	25.8	25.6	24.1
18:0	2	9.50	17.45	11.1	14.7	15.1	12.05
	4	9.50	17.45	10.7	12.2	14.03	11.05
	8	9.50	17.45	9.6	11.3	12.2	10.95
16:1	2	1.95	2.20	2.3	2.5	2.61	2.6
	4	1.95	2.20	2.11	2.4	2.5	2.1
	8	1.95	2.20	2.0	2.1	2.4	2.4
18:1	2	31.00	17.55	23.5	22.3	21.9	20.35
	4	31.00	17.55	25.8	24.04	23.8	23.65
	8	31.00	17.55	29.2	27.9	27.2	26.3
18:2	2	19.27	6.20	10.1	9.5	9.1	9
	4	19.27	6.20	13.7	10.6	10.3	10.8
	8	19.27	6.20	14.8	12.5	11.9	11.3
20:4	2	10.95	7.40	8.9	9.1	8.2	22.35
	4	10.95	7.40	9.5	9.9	9.3	17.9
	8	10.95	7.40	11.1	10.5	10.9	16.35
SFA	2	32.49^{a}	52.75^{b}	43.3^{c}	48.05^{b}	49.4^{b}	42.15^{c}
	4	32.49^{a}	52.75^{b}	37.25^{c}	42^{d}	43.2^{d}	35.45^{a}
	8	32.49^{a}	52.75^{b}	34.59^{a}	38.32^{b}	39.15^{b}	36.3^{b}
MUFA	2	32.95^{a}	19.75^{b}	25.8^{c}	24.8^{c}	24.51^{c}	22.95^{c}
	4	32.95^{a}	19.75^{b}	27.91^{c}	26.44^{c}	26.3^{c}	25.75^{c}
	8	32.95^{a}	19.75^{b}	31.2^{a}	30^{a}	29.6^{a}	28.7^{a}
PUFA	2	30.22^{a}	13.6^{b}	19.0^{c}	18.6^{c}	17.3^{c}	31.35^{a}
	4	30.22^{a}	13.6^{b}	23.2^{c}	20.5^{c}	19.6^{c}	28.7^{a}
	8	30.22^{a}	13.6^{b}	25.9^{c}	23.0^{c}	22.8^{c}	27.65^{a}

[#]RD represents value at 0 h; Data represent mean (n=5/time point). RD = retinol deficient, FUCO = fucoxanthin, SFA = saturated fatty acids, MUFA = mono-unsaturated fatty acids, PUFA = poly-unsaturated fatty acids. Values represented as mean (n=5/time point). Values in a row between groups with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA and by Tukey's test.

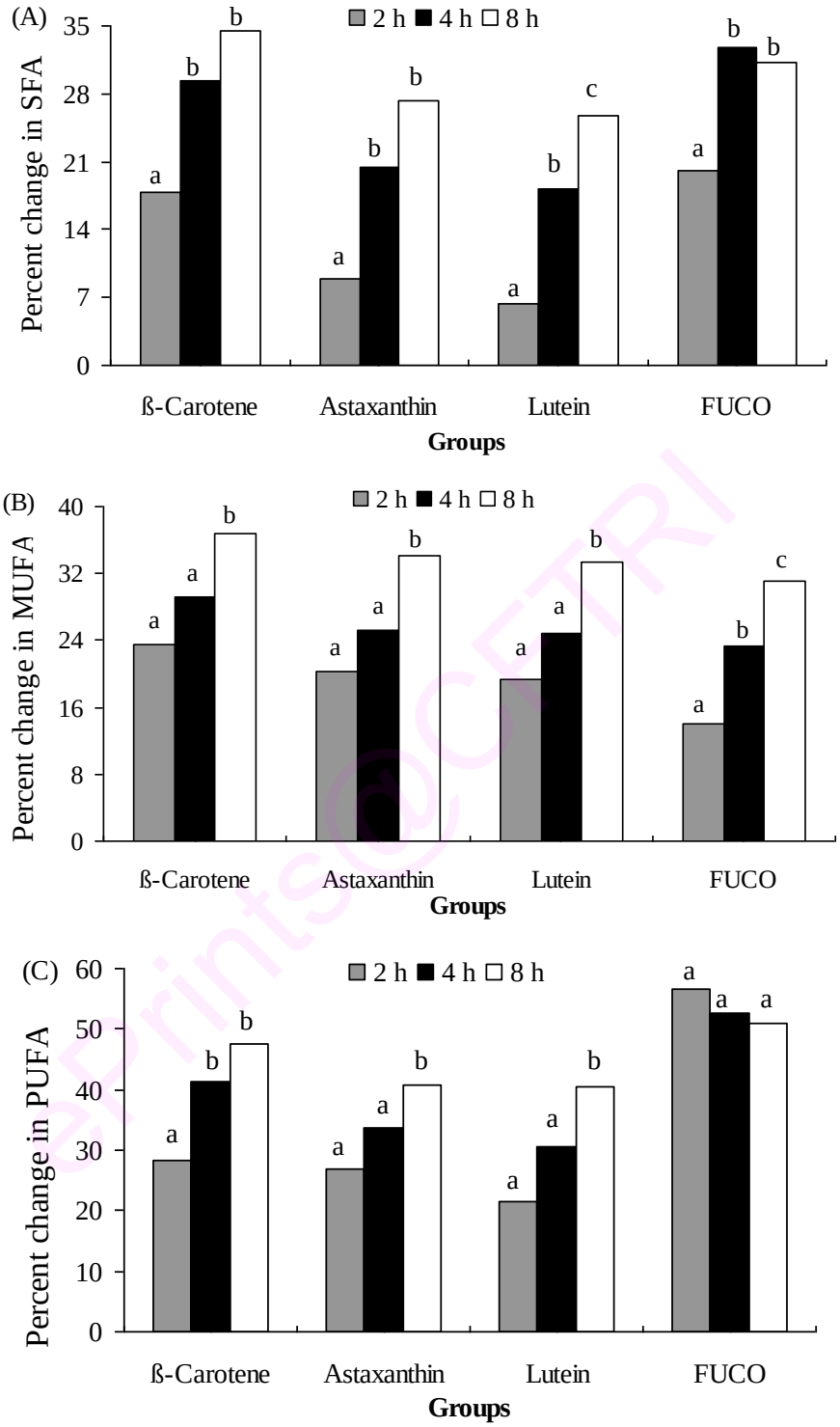


Figure 6.5 (A) Decrease (%) in saturated fatty acid (SFA) and increase (%) in (B) mono-unsaturated fatty acid (MUFA) and (C) poly-unsaturated fatty acid (PUFA) on feeding carotenoids as compared to RD group. Values are represented as mean (n=5/time point). Values within a carotenoid group at different time points with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test. FUCO = fucoxanthin.

Table 6.5. Blood lipid profile of retinol deficient (RD), control and RD rats fed on carotenoids.

Time (h)	Groups				
	RD[#]	β-Carotene	Astaxanthin	Lutein	FUCO
*Total cholesterol (mg/dl) **					
2 h	74.88 ± 10.5 [a]	82.55 ± 4.5 [a]	79.33 ± 2.9 [a]	78.95 ± 4.2 [a]	78.93 ± 4.9 [a]
4 h	74.88 ± 10.5 [a]	87.61 ± 5.2 [a]	81.72 ± 4.3 [a]	82.63 ± 5.2 [a]	80.88 ± 4.4 [a]
8 h	74.88 ± 10.5 [a]	85.92 ± 6.2 [a]	82.55 ± 3.8 [a]	81.99 ± 3.8 [a]	85.76 ± 5.6 [a]
*HDL-cholesterol (mg/dl)**					
2 h	36.92 ± 2.5 [a]	42.09 ± 3.1 [a]	45.89 ± 3.2 [a]	43.98 ± 2.6 [a]	40.91 ± 3.0 [a]
4 h	36.92 ± 2.5 [a]	43.82 ± 2.9 [a]	48.5 ± 3.9 [a]	46.67 ± 3.3 [a]	42.91 ± 1.8 [a]
8 h	36.92 ± 2.5 [a]	41.55 ± 3.2 [a]	44.66 ± 5.2 [a]	45.07 ± 2.9 [a]	44.72 ± 2.0 [a]
*LDL+VLDL-cholesterol**					
2 h	37.79 ± 1.9 [a]	40.12 ± 2.7 [a]	33.03 ± 2.0 [a]	34.18 ± 3.7 [a]	37.68 ± 2.3 [a]
4 h	37.79 ± 1.9 [a]	43.07 ± 2.2 [a]	32.82 ± 2.7 [a]	35.2 ± 2.2 [a]	37.35 ± 2.1 [a]
8 h	37.79 ± 1.9 [a]	43.92 ± 2.8 [a]	37.24 ± 2.3 [a]	36.01 ± 2.1 [a]	40.63 ± 3.2 [a]
*Phospholipids (mg/dl)**					
2 h	88.4 ± 1.8 [a]	108.35 ± 5.1 [a]	105.65 ± 2.9 [a]	104.98 ± 2.1 [a]	101.92 ± 1.3 [a]
4 h	88.4 ± 1.8 [a]	109.7 ± 3.5 [a]	106.91 ± 2.9 [a]	105.87 ± 3.1 [a]	103.75 ± 2.0 [a]
8 h	88.4 ± 1.8 [a]	107.67 ± 3.5 [a]	106.22 ± 3.4 [a]	103.37 ± 2.1 [a]	105.69 ± 3.1 [a]
*Triglycerides (mg/dl)***					
2 h	100.2 ± 5.9 [a]	155.6 ± 8.8 [a]	149.9 ± 10.3 [a]	135.7 ± 9.2 [a]	129.73 ± 5.6 [a]
4 h	100.2 ± 5.9 [a]	205.1 ± 10.5 [b]	199.5 ± 10.9 [b]	186.2 ± 8.9 [b]	142.90 ± 5.8 [c]
8 h	100.2 ± 5.9 [a]	160.9 ± 9.2 [b]	162.3 ± 9.7 [b]	145.8 ± 9.6 [c]	150.67 ± 8.0 [c]

[#]RD represents values at 0 h. Data represent mean ± SD (n=5/time point). Values in a row between groups with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test. FUCO = fucoxanthin. *Estimated in serum. ** Estimated in plasma

Blood lipid profile (mg/dl) of control group: total cholesterol= 93.1 ± 3.8, HDL-cholesterol= 48.29 ± 3.0, LDL+VLDL-cholesterol= 43.91 ± 1.9, phospholipids= 115.4 ± 1.0, triglycerides= 125.4 ± 7.7.

Table 6.6. Liver lipid profile (mg/g) of control and retinol deficient (RD) rats and RD rats fed on carotenoids.

Time (h)	Groups					
	Control[#]	RD[^]	β-Carotene	Astaxanthin	Lutein	FUCO
Total cholesterol (mg/g)						
2 h	14.5 ± 0.5 [a]	13.8 ± 1.0 [a]	14.2 ± 1.2 [a]	14.3 ± 1.1 [a]	14.0 ± 0.9 [a]	14.8 ± 1.1 [a]
4 h	14.5 ± 0.5 [a]	13.8 ± 1.0 [a]	14.9 ± 1.1 [a]	14.5 ± 2.0 [a]	14.3 ± 1.1 [a]	15.0 ± 1.0 [a]
8 h	14.5 ± 0.5 [a]	13.8 ± 1.0 [a]	15.5 ± 1.8 [a]	15.9 ± 1.9 [a]	15.0 ± 1.0 [a]	15.8 ± 0.9 [a]
Phospholipids (mg/g)						
2 h	20.8 ± 1.4 [a]	15.6 ± 0.8 [b]	18.6 ± 1.3 [a,b]	18.3 ± 1.2 [a,b]	17.8 ± 1.2 [a,b]	18.5 ± 1.3 [a,b]
4 h	20.8 ± 1.4 [a]	15.6 ± 0.8 [b]	19.8 ± 2.0 [a]	19.07 ± 1.1 [a]	18.3 ± 1.1 [a,b]	18.9 ± 1.1 [a]
8 h	20.8 ± 1.4 [a]	15.6 ± 0.8 [b]	21.03 ± 1.6 [a]	21.2 ± 1.8 [a]	19.7 ± 1.0 [a]	21.06 ± 1.0 [a]
Triglycerides (mg/g)						
2 h	19.5 ± 0.9 [a]	17.6 ± 0.9 [a]	20.1 ± 1.9 [a]	19.6 ± 1.7 [a]	19.3 ± 0.9 [a]	18.6 ± 0.8 [a]
4 h	19.5 ± 0.9 [a]	17.6 ± 0.9 [a]	22.8 ± 2.2 [a]	21.6 ± 1.5 [a]	20.0 ± 1.1 [a]	19.8 ± 0.9 [a]
8 h	19.5 ± 0.9 [a]	17.6 ± 0.9 [a]	25.9 ± 2.0 [b]	23.5 ± 1.8 [b]	21.8 ± 1.0 [a]	20.5 ± 1.1 [a]

[#]Group fed retinol sufficient diet; [^]Value at 0 h; Data represent mean ± SD (n=5/time point). Values in row between groups with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test. RD = retinol deficient, FUCO = fucoxanthin.

Retinol Binding Protein

Retinol binding protein (ng/ml serum) was estimated by ELISA and given in Table 6.7. The results revealed that RD resulted in nearly 380 fold lower ($p>0.05$) serum RBP concentration than control group. β-Carotene gavage to RD rats exhibited higher (39-77%) RBP concentrations (ng/dl) at 2h (230.6), 4h (612.4) and 8h (397.9) as compared to RD group (Figure 6.6) supporting the higher plasma retinol levels in β-carotene group (Chapter 4). In contrast, there was no change in the RBP levels in the astaxanthin and lutein groups over a period of 8 h when compared with RD group (140.5). The higher level of RBP in β-carotene group may be due to the newly formed retinol that was not true in the case of other carotenoid fed groups.

Table 6.7. Retinol Binding Protein concentration (ng/ml) over 8 hours in serum of retinol deficient (RD), control and RD rats gavaged with β-carotene, astaxanthin and lutein.

Group	2 h	4 h	8h
β-Carotene	230.55 ± 20.1[a]	612.43 ± 55.9[b]	397.89 ± 35.6[c]
Astaxanthin	145.92 ± 19.2[a]	130.66 ± 10.5[a]	140.37 ± 20.1[a]
Lutein	145.8 ± 15.5[a]	150.56 ± 20.0[a]	145.99 ± 17.2[a]

Data represent mean ± SD (n=5). Values within a group at different time points not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. RBP in Control = 53.35 μg/ml and RD group = 140.51 ± 13.2 ng/ml.

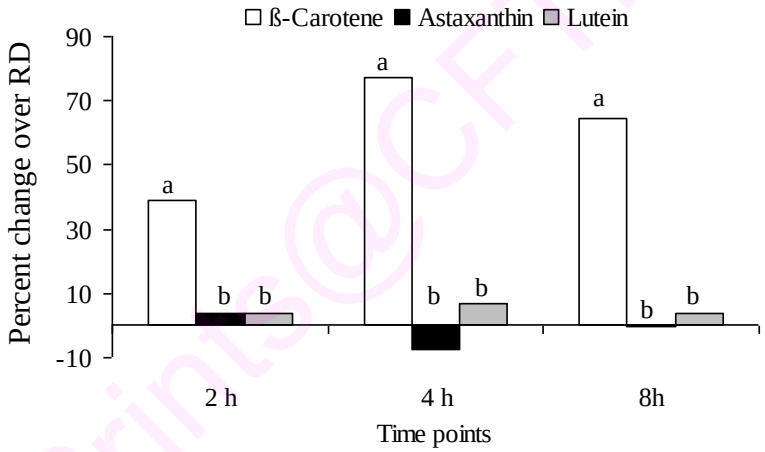


Figure 6.6. Percent change in retinol binding protein on carotenoids gavages compared with RD group. Values are represented as means (n=5/time point). Values within a time point, between carotenoid groups not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. RBP in RD group = 140.51 ± 13.2 ng/ml.

Repeated Dose Study

On feeding retinol deficient diet to rats, its deficiency was confirmed by plasma retinol level (0.3 μmol/l). RD rats were gavaged daily a dose of β-carotene, astaxanthin or lutein (0.1 mg/rat/day) for 7- and 15-days and sacrificed to collect blood and tissues for analysis of

antioxidant molecules, lipid peroxides, lipid profile and monooxygenase assay. The data thus obtained was compared with control and RD groups.

Activity of 15-15' β-carotene monooxygenase

Results on the activity of monooxygenase in the intestines of RD, control and rats intubated with carotenoids are given in Table 6.8. Results show an increased activity of monooxygenase (pmol retinal/h/mg protein) in rats gavaged with β-carotene (154.5) and astaxanthin (146) for 7-days by 69.5% and 67.7% respectively as compared to RD group (47.2). Similar to the 7-day results, an increased monooxygenase activity (72.1 and 68.6%) was observed after 15-days gavages with β-carotene (168.9 pmol retinal/h/mg protein) and astaxanthin (150.3 pmol retinal/h/mg protein) than RD group. In case of lutein fed group, no significant difference was found in the monooxygenase activity (pmol retinal/h/mg protein) after 7- and 15-days compared with RD and control groups (Figure 6.7). Increased monooxygenase activity and higher retinol levels in the plasma of the β-carotene and astaxanthin groups indicated their conversion to retinol. It is interesting to note that repeated dose but not single dose of astaxanthin resulted in its conversion to retinol in RD rats.

Table 6.8. Activity of monooxygenase (pmol retinal/h/mg protein) in intestine of RD, control and carotenoids intubated groups.

Duration (days)	Groups				
	Control[#]	RD*	β-Carotene	Astaxanthin	Lutein
Intestinal 15-15' β-carotene monooxygenase					
7	32.9±1.5[a]	47.2±1.1[a]	154.5±4.7[b]	146.0±6.7[b]	44.4±1.6[a]
15	32.9±1.5[a]	47.2±1.1[a]	168.9 ± 10.4[b]	150.3 ± 12.5[b]	47.25 ± 4.5[a]

Data represent mean ± SD (n=5/group). [#]Group fed retinol sufficient diet; *baseline value at 0 day. Values in a row between groups with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test. RD = retinol deficient.

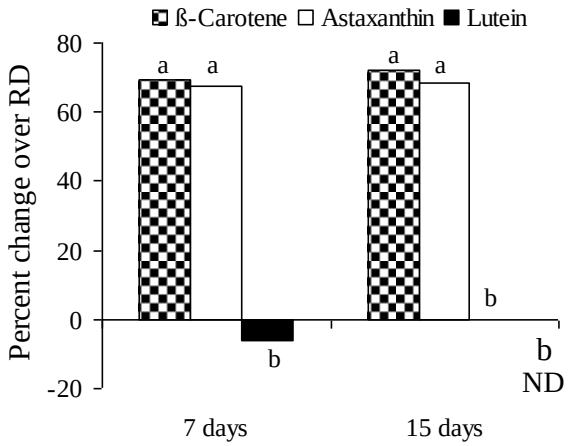


Figure 6.7. Percent change in the activity of intestinal monooxygenase in groups intubated with carotenoid over RD group. Values are represented as mean (n=5). Values between groups with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test.

Lipid peroxidation and activity of antioxidant enzymes and molecules

The lipid peroxide levels in the plasma and liver of RD (baseline), control and carotenoid intubated groups are presented in Figure 6.8 and 6.9. Retinol deficiency was found to raise the Lpx levels in plasma and liver by 67% and 76% respectively (Figure 6.10) as compared to control group (4.1 TMP nmol/ml, 32.3 TMP nmol/g). Results showed that after 7-days of β-carotene, astaxanthin and lutein intubations, Lpx was brought down by 40, 45, 42% (plasma) and 60, 59, 54 % (liver) when compared to RD group (Figure 6.10). Similarly, after 15-days gavage with β-carotene, astaxanthin and lutein resulted in decreased Lpx in plasma by 57, 88, and 68% respectively and in liver by 56, 77, and 61 % respectively, as compared to RD group (Figure 6.10).

Activity of SOD, CAT, GST and GSH in RD, control and carotenoid intubated rats is given in Table 6.9. Elevated Lpx in RD group due to retinol deficiency was associated with lowered activity of SOD, CAT and GSH in plasma by 66, 55, 55% respectively and SOD, CAT, GST and GSH in liver by 51, 63, 64% respectively as compared to control group (Figure 6.11). The comparative results between β-carotene, astaxanthin and lutein groups revealed elevated activity of SOD (44, 62, 55%), CAT (29, 47, 44%) and GSH (28, 46, 43%) in plasma and SOD (22, 47, 39%), CAT (29, 55, 41%), GST (37, 53, 42%) and GSH (30, 57, 48%) in liver after 7-days of gavage when compared with RD group (Table 6.10). Similarly, on repeated gavages with

β-carotene, astaxanthin and lutein for 15-days, elevated activity of SOD (46, 58, 69%), CAT (41, 67, 52%) and GSH (44, 55, 48%) was measured in the plasma as compared to RD group. As in the case of plasma, there was increase in the activity of SOD (13, 44, 29%), CAT (44, 69, 52%), GST (35, 56, 43%) and GSH (42, 64, 52%) in liver as compared to RD group.

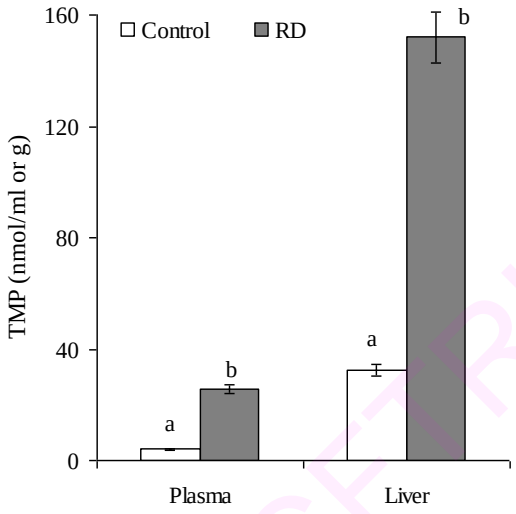


Figure 6.8. Lipid peroxide levels in plasma and liver of control and RD rats. Data represent mean ± SD (n=5/time point). Values between groups with similar superscript are not significantly different (p>0.05) as determined by one-way ANOVA followed by Tukey's test. RD = retinol deficient.

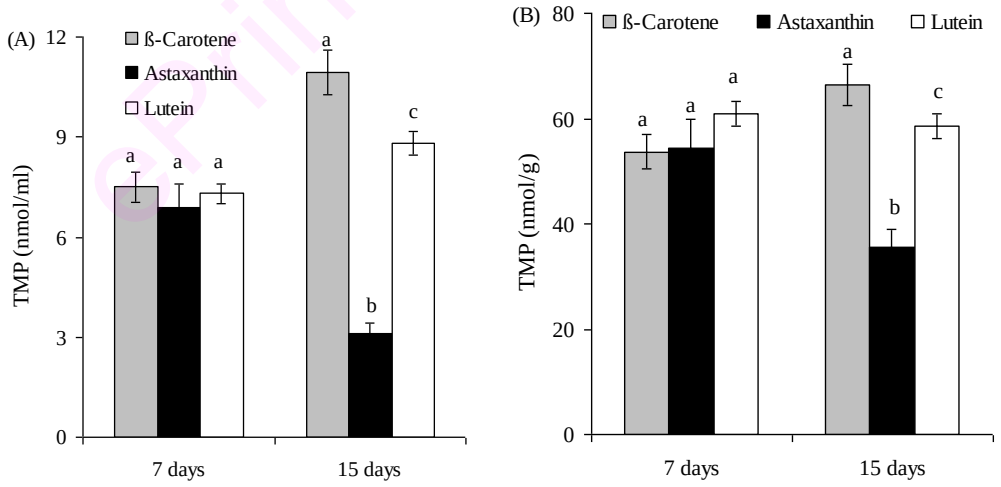


Figure 6.9. Lipid peroxide levels in plasma (A) and liver (B) of RD rats gavaged with carotenoids for 7- and 15-days. Data represent mean ± SD (n=5/time point). Values between groups with similar superscript are not significantly different (p>0.05) as determined by one-way ANOVA followed by Tukey's test.

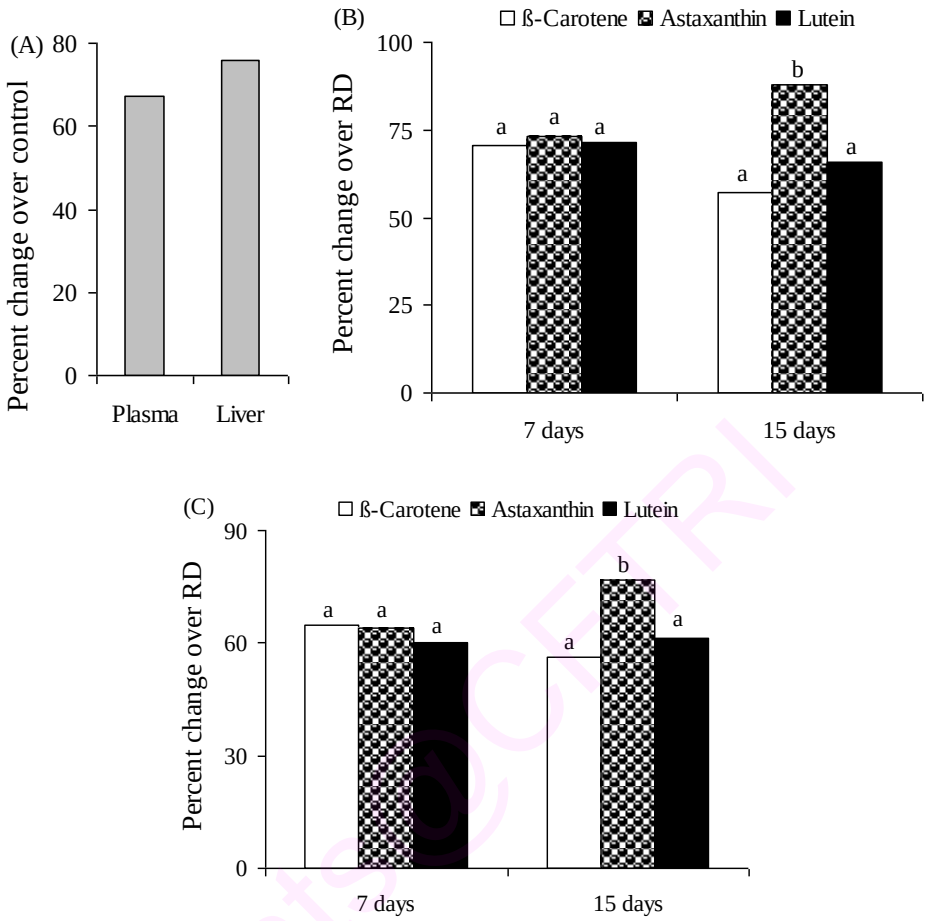


Figure 6.10. Percent increase in lipid peroxidation in plasma and liver of RD over control group (A), percent decrease in lipid peroxidation in carotenoid fed groups over RD group in plasma (B) and liver (C). RD= Retinol deficient. Values are represented as mean (n=5). Values between groups with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test.

It is seen from the results that the antioxidant property of astaxanthin was superior with respect to modulating the Lpx and antioxidant molecules than β-carotene and lutein. In addition, on comparison between the results of 7-days and 15-days, the ameliorative effect of carotenoids was found to be time dependent showing greater suppression of Lpx after 15-days, than 7-days of gavage, especially in the case of astaxanthin.

Table 6.9. Activity of antioxidant molecules in plasma and liver of retinol deficient (RD), control and RD rats intubated with carotenoids.

Duration (days)	Groups				
	Control	RD	β-Carotene	Astaxanthin	Lutein
Plasma Superoxide Dismutase (U/mg protein)					
7	6.5±0.7[a]	2.2±0.2[b]	3.9±0.5[c]	5.8±0.5[a]	4.9±0.7[c]
15	6.5±0.7[a]	2.2±0.2[b]	4.1 ± 0.4[c]	6.8 ± 0.5[a]	5.3 ± 0.5[d]
Liver Superoxide Dismutase (U/mg protein)					
7	7.2±0.9[a]	3.5±0.2[b]	4.5±0.6[c]	6.6±0.5[a]	5.7±0.2[c]
15	7.2±0.9[a]	3.5±0.2[b]	4.0 ± 0.4[c]	6.2 ± 0.6[c]	4.9 ± 0.5[c]
Plasma Catalase (μmol/min/mg protein)					
7	2.2±0.5[a]	1.0±0.1[b]	1.4±0.4[c]	1.9±0.2[a]	1.8±0.2[c]
15	2.2±0.5[a]	1.0±0.1[b]	1.7 ± 0.2[c]	3.0 ± 0.3[a]	2.1 ± 0.2[d]
Liver Catalase (μmol/min/mg protein)					
7	2.7±0.06[a]	1.0±0.02[b]	1.4±0.02[c]	2.2±0.04[a]	1.7±0.02[c]
15	2.7±0.06[a]	1.0±0.02[b]	1.8 ± 0.2[c]	3.2 ± 0.3[a]	2.1 ± 0.2[c]
Liver Glutathione Transferase (μmol/min/mg protein)					
7	18.0±1.8[a]	8.1±1.0[b]	12.8±2.8[c]	17.1±2.3[a]	14.0±2.5[c]
15	18.0±1.8[a]	8.1±1.0[b]	12.4 ± 1.2[c]	18.5 ± 1.5[a]	14.3 ± 1.2[c]
Plasma Glutathione (mg/ml)					
7	9.8±2.1[a]	4.4±0.3[b]	6.1±1.8[c]	8.2±1[a]	7.7±0.7[c]
15	9.8±2.1[a]	4.4±0.3[b]	7.9 ± 0.3[c]	9.8 ± 0.5[a]	8.4 ± 0.6[c]
Liver Glutathione (mg/ml)					
7	13.6±3.2[a]	4.9±0.8[b]	7.0±1.0[c]	11.4±2.2[a]	9.4±0.8[c]
15	13.6±3.2[a]	4.9±0.8[b]	8.5 ± 0.3[c]	13.6 ± 0.9[a]	10.2 ± 0.5[d]

Data represent mean ± SD (n=5/group). Values between groups in a row with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test.

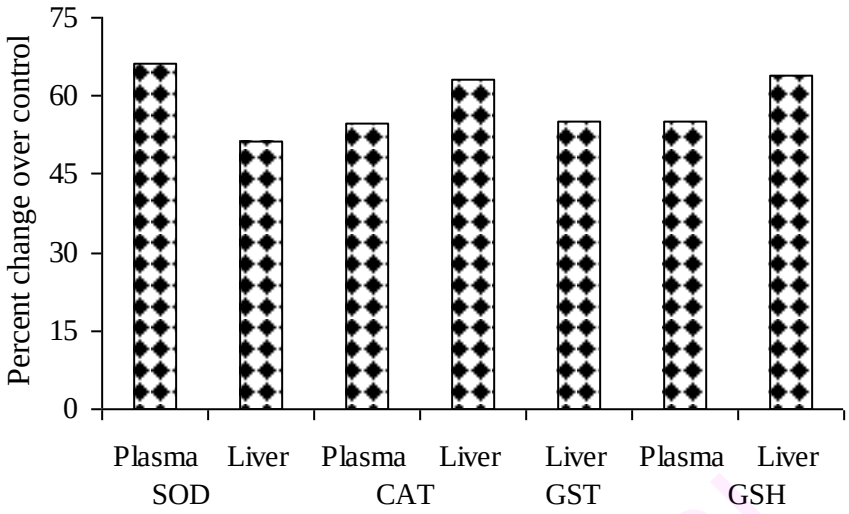


Figure 6.11. Percent decrease in the activity of antioxidant molecules over control in RD group.

Table 6.10. Percent increase in the activity of antioxidant molecules in rats fed carotenoids over RD group.

Duration (days)	Groups		
	β-Carotene	Astaxanthin	Lutein
Plasma Superoxide Dismutase			
7	43.59[a]	62.07[b]	55.10[c]
15	46.34 [a]	67.65[b]	58.49[c]
Liver Superoxide Dismutase			
7	22.22[a]	46.97[b]	38.60[c]
15	12.50[a]	43.55[b]	28.57[c]
Plasma Catalase			
7	28.57[a]	47.37[b]	44.44[b]
15	41.18[a]	66.67[b]	52.38[c]
Liver Catalase			
7	28.57[a]	54.55[b]	41.18[c]
15	44.44[a]	68.75[b]	52.38[c]
Liver Glutathione Transferase			
7	36.72[a]	52.63[b]	42.14[c]
15	34.68[a]	56.22[b]	43.36[c]
Plasma Glutathione			
7	27.87[a]	46.34[b]	42.86[b]
15	44.30[a]	55.10[b]	47.62[b]
Liver Glutathione			
7	30.00[a]	57.02[b]	47.87[c]
15	42.35[a]	63.97[b]	51.96[c]

Data represent mean (n=5/group). Values between groups in a row with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test.

Lipid Profile

The results of the fatty acid profile in plasma after 7- and 15-days of gavage of β-carotene, astaxanthin and lutein is given in Table 6.11. Results demonstrate that retinol deficiency resulted in elevated ($p<0.05$) saturated (SFA), mono- and poly-unsaturated (MUFA and PUFA) fatty acids by 50, 52 and 44% respectively (Figure 6.12). Whereas, gavages with β-carotene, astaxanthin and lutein for 7-days resulted in decrease in plasma SFA by 45, 43 and 37% and increase in MUFA and PUFA by 48, 47, 45% and 42, 38, 33% respectively (Figure 6.13). A similar result was obtained after gavages with β-carotene, astaxanthin and lutein for 15-days where there was decrease in plasma SFA (33.6, 34.9, 38.5 %) and increase in MUFA (30.1, 29.5, 28.2%) and PUFA (34.1, 32.4, 29.9%) (Figure 6.12).

Analysis of blood lipid parameters (Table 6.12) revealed that retinol deficiency significantly decreased ($p<0.05$) the total cholesterol, HDL-cholesterol, LDL+VLDL-cholesterol, phospholipids and triglycerides (75.03, 35.69, 39.29, 83.4, 101.6 mg/dl) as compared to control group (95.1, 50.02, 45.03, 135.9, 122.9 mg/dl). After 7-days of carotenoid gavage, total cholesterol level (mg/dl) was elevated for carotenoid fed groups in comparison with RD (75.03) group. Although the increase was statistically significant for β-carotene group (90.6), the increase in total cholesterol (mg/dl) was significantly ($p<0.05$) higher in β-carotene (95.8) and astaxanthin (92.5) groups after 15-days of gavage. As in the case of total cholesterol, the HDL-cholesterol and LDL+VLDL-cholesterol levels (mg/dl) were significantly ($p<0.05$) increased after 7-days gavages for β-carotene (48.1, 42.4) and astaxanthin (45.9, 42.3) groups as compared to RD (35.7, 39.3) group. Similarly, after 15-days of gavage, HDL cholesterol level (mg/dl) was significantly ($p<0.05$) increased in β-carotene (48.1), astaxanthin (49.7) and lutein (48) groups while the LDL+VLDL cholesterol (mg/dl) level was significantly ($p<0.05$) higher only in β-carotene (47.7) and astaxanthin (42.8) groups as compared to RD group. Intubatioṇ of β-Carotene, astaxanthin and lutein for 7-days significantly ($p<0.05$) increased the phospholipids (34.6, 29.1 and 17.3%) and triglycerides (37.6, 35.2, 26.2%) levels as compared to RD (83.4, 101.6 mg/dl) group. Similarly, phospholipids and triglycerides levels were significantly ($p<0.05$) elevated on β-carotene (38.3, 42.3%), astaxanthin (34.2, 40.2%) and lutein (25.2, 28.8%) intubation for 15-days as compared to RD group.

Retinol deficiency resulted in decreased the liver cholesterol, phospholipids and triglycerides levels (13.5, 15.1, 16.5 mg/dl) when compared to control (14.9, 21.8, 18.9 mg/dl) and the decrease was significant ($p<0.05$) for phospholipids (Table 6.13). Gavages with carotenoids for 7- and 15-days resulted in higher cholesterol levels (mg/g), than RD (13.5) group.

The cholesterol levels (mg/g) were significantly ($p>0.05$) higher in β-carotene group (16.3, 16.5) after 7- and 15-days and in astaxanthin (16.3) group after 15-days of gavage. Gavages for 7- and 15-days resulted in elevation ($p>0.05$) of phospholipid levels (mg/g) in β-carotene (22.6, 23.6), astaxanthin (21.6, 22.5) and lutein (20.03, 21.1) groups as compared to RD (15.1) group. Triglycerides level (mg/g) was significantly elevated ($p>0.05$) in β-carotene (26.3, 28.4), astaxanthin (23.9, 25) and lutein (21, 22) groups as compared to RD (16.5) group. Results revealed that the elevation of liver triglycerides was greater in β-carotene and astaxanthin groups than lutein group.

Table 611. Plasma fatty acid profile (%) of retinol deficient (RD), control and rats fed on carotenoids for 7- and 15-days.

Fatty acids	Control	RD	β-Carotene		Astaxanthin		Lutein	
			7 d	15 d	7 d	15 d	7 d	15 d
12:0	0.4	1.05	0.51	0.50	0.55	0.53	0.57	0.6
14:0	0.25	0.92	0.32	0.31	0.35	0.32	0.41	0.35
16:0	20.5	38.55	22.9	21.6	23.7	22.6	26.8	25.2
18:0	9.4	20.35	9.9	9.7	10.3	9.1	10.7	10.1
16:1	4.5	2.90	3.7	4.1	3.5	4.0	3.01	3.75
18:1	31.1	17.05	30.4	28.8	28.9	28.7	26.9	27.6
18:2	19.92	7.32	19.2	19.1	18.5	18.8	17.7	18.1
20:4	12.55	8.22	10.9	11.5	11.03	11.1	10.5	10.9
SFA	30.55^{a}	60.87^{b}	33.63^{a}	32.11^{a}	34.9^{a}	32.55^{a}	38.48^{c}	36.25
MUFA	32.47^{a}	15.54^{b}	30.1^{a}	32.9^{a}	29.53^{a}	32.7^{a}	28.2^{a}	31.35^{a}
PUFA	35.6^{a}	19.95^{b}	34.1^{a}	30.6^{c}	32.4^{a}	29.9^{c}	29.91^{b}	29.0^{c}

Data represent mean (n=5). SFA = saturated fatty acids, MUFA = mono-unsaturated fatty acids, PUFA = poly-unsaturated fatty acids, d = days. Values in a row between groups with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test.

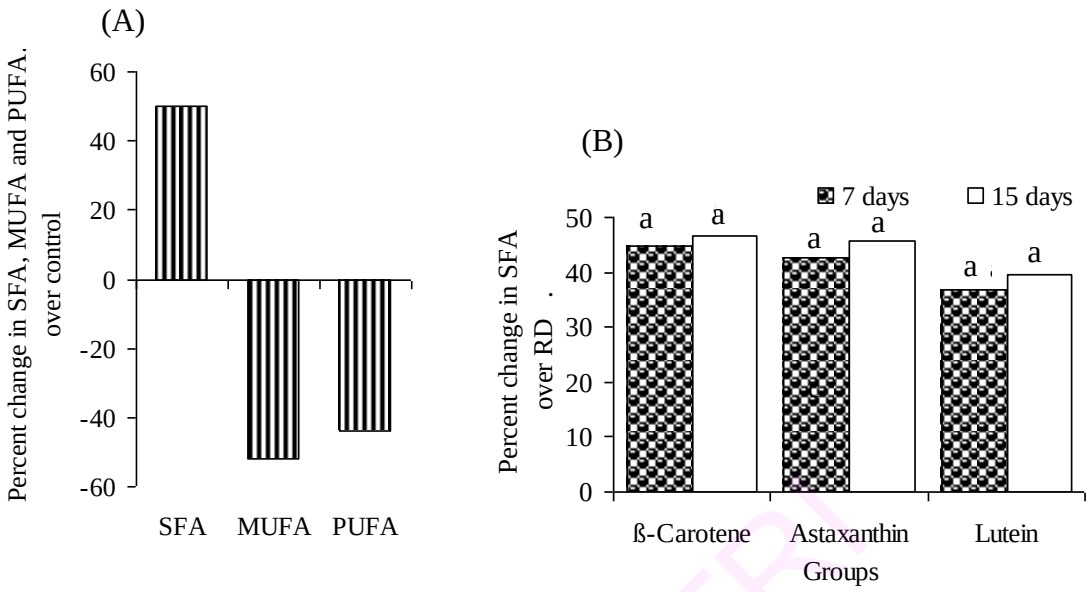


Figure 6.12. Percent change in saturated (SFA), mono-unsaturated (MUFA) and poly-unsaturated (PUFA) fatty acids in RD group as compared to control (A) and percent decrease in SFA in carotenoid fed groups as compared to RD group (B). Data represent mean (n=5/group). Values in a group between 7- and 15-days with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test.

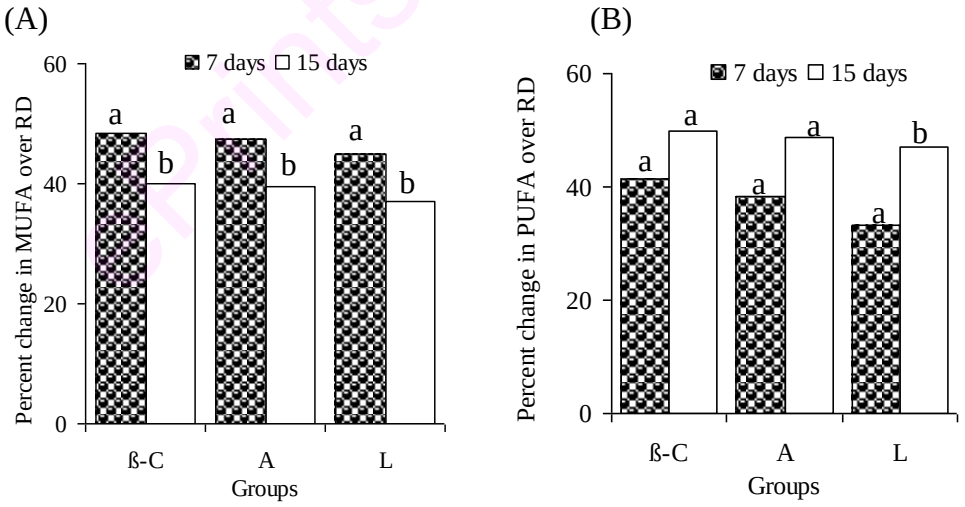


Figure 6.13. Percent increase in (A) mono-unsaturated fatty acid (MUFA) and (B) poly-unsaturated fatty acid (PUFA) in carotenoid fed groups as compared to RD group. Data represent mean (n=5/group). Values in a group between 7- and 15-days with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test. β-C=β-carotene, A=astaxanthin, L= lutein.

Table 6.12. Blood lipid profile of RD, control and rats fed carotenoids.

Duration (days)	Groups				
	Control[#]	RD[^]	β-Carotene	Astaxanthin	Lutein
*Total cholesterol (mg/dl)**					
7	95.1 ± 2.5 [a]	75.03 ± 5.2 [b]	90.55 ± 3.5 [a]	88.33 ± 2.4[a,b]	79.68 ± 3.2[a,b]
15	95.1 ± 2.5 [a]	75.03 ± 5.2 [b]	95.82 ± 3.2 [a]	92.53 ± 2.1 [a]	85.81 ± 4.2 [a,b]
*HDL-cholesterol (mg/dl) **					
7	50.02 ± 2.0 [a]	35.69 ± 2.1 [b]	48.09 ± 2.1 [a]	45.99 ± 3.5 [a]	44.76 ± 2.5 [a]
15	50.02 ± 2.0 [a]	35.69 ± 2.1 [b]	48.1 ± 2.0 [a]	49.73 ± 3.1 [a]	47.96 ± 2.9 [a]
*VLDL+LDL-cholesterol (mg/dl)**					
7	45.03 ± 1.5 [a]	39.29 ± 1.1 [b]	42.42 ± 2.1 [a]	42.3 ± 2.0 [a]	34.89 ± 2.6 [b]
15	45.03 ± 1.5 [a]	39.29 ± 1.1 [b]	47.72 ± 3.3 [a]	42.8 ± 2.1 [a]	37.85 ± 2.5 [b]
*Phospholipids (mg/dl)**					
7	135.9 ± 8.0 [a]	83.4 ± 7.5 [b]	127.53 ± 8.2 [a]	117.7 ± 4.9 [a]	100.96 ± 5.5 [c]
15	135.9 ± 8.0 [a]	83.4 ± 7.5 [b]	135.0 ± 7.3 [a]	126.75 ± 5.1 [a]	111.5 ± 4.5 [c]
*Triglycerides (mg/dl)***					
7	122.9 ± 4.3 [a]	101.6 ± 4.8 [b]	162.8 ± 6.4 [c]	156.9 ± 9.3 [c]	137.6 ± 7.3 [a]
15	122.9 ± 4.3 [a]	101.6 ± 4.8 [b]	175.9 ± 7.2 [c]	169.8 ± 4.3 [c]	142.7 ± 5.4 [a]

Data represent mean ± SD (n=5/time interval). Values in a row between groups with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test. [#]Group fed retinol sufficient diet, [^]baseline value, *estimated in serum, **estimated in plasma. RD = retinol deficient.

Table 6.13. Liver lipid profile of retinol deficient (RD), control and rats fed carotenoids.

Duration (days)	Groups				
	Control[#]	RD^	β-Carotene	Astaxanthin	Lutein
Total cholesterol (mg/g)					
7	14.9 ± 0.9 [a]	13.5 ± 1.3 [a]	16.3 ± 1.8 [b]	16.04 ± 1.2 [a]	15.8 ± 1.0 [a]
15	14.9 ± 0.9 [a]	13.5 ± 1.3 [a]	16.5 ± 1.1 [b]	16.3 ± 1.0 [b]	16.0 ± 1.8 [a]
Phospholipids (mg/g)					
7	21.8 ± 1.7 [a]	15.1 ± 0.7 [b]	22.6 ± 1.2 [a]	21.6 ± 1.5 [a]	20.03 ± 1.3 [a]
15	21.8 ± 1.7 [a]	15.1 ± 0.7 [b]	23.6 ± 1.0 [c]	22.5 ± 1.3 [a]	21.1 ± 1.8 [a]
Triglycerides (mg/g)					
7	18.9 ± 1.1 [a]	16.5 ± 1.0 [a]	26.3 ± 2.1 [b]	23.9 ± 1.0 [b]	21.04 ± 1.1 [c]
15	18.9 ± 1.1 [a]	16.5 ± 1.0 [a]	28.4 ± 2.0 [c]	25.0 ± 1.5 [c]	22.01 ± 1.8 [a]

Data represent mean ± SD (n=5/time point). Values in a row between groups with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test. [#] Group fed retinol sufficient diet, [^] baseline value. RD = retinol deficient.

Retinol Binding Protein

Retinol binding protein (RBP) concentration (ng/ml) estimated by ELISA is presented in Table 6.14. The level of RBP was significantly lower ($p<0.05$) in RD group (148.5 ng/ml) as compared to control (45.85 μg/ml) group. Gavage with β-carotene (250.1, 265.7) and astaxanthin (175.8, 192.4) for 7- and 15-days resulted in higher levels of RBP as compared to RD group (148.5), while no significant difference was found between lutein (150.2, 140.8) and RD group. The higher plasma retinol levels and intestinal monooxygenase activity (Table 6.8, Figure 6.6) found in β-carotene and astaxanthin groups further supports the conversion of these carotenoids to retinol (Chapter 4). On comparison between 7- and 15-days results, the RBP levels were marginally higher ($p>0.05$) in the 15-days samples of β-carotene and astaxanthin groups.

Table 6.14. Retinol Binding Protein (RBP) concentration (ng/ml) in serum of retinol deficient (RD), control and rats gavaged with β-Carotene, astaxanthin and lutein for 7- and 15-days.

Group	RBP	
	7 days	15 days
β-Carotene	250.13 ± 19.4^{a}	265.74 ± 19.8^{a}
Astaxanthin	175.84 ± 15.6^{b}	192.36 ± 12.3^{b}
Lutein	150.18 ± 16.6^{c}	140.81 ± 13.8^{c}
RD[#]	148.51 ± 12.8^{c}	148.51 ± 12.8^{c}

Data represent mean $\pm$ SD (n=5). Values within a column, between groups, not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. RBP in Control = 45.85 μg/ml. [#]RD represents baseline value.

Dietary Study

RD rats were fed diet supplemented with purified astaxanthin, carrot powder (β-carotene source) and dill leaf powder (lutein source) for a period of 20 days and the data obtained on various biochemical parameters was compared with the RD and control groups.

Activity of 15-15' β-carotene monooxygenase

Results on the activity of monooxygenase in the intestines of the experimental, control and RD groups are given in Table 6.15. The results show that groups fed on astaxanthin, carrot powder and dill leaf powder supplemented diet exhibited significantly ($p<0.05$) increased monooxygenase activity (73.3, 68.6, 72.6%) as compared to RD group (Figure 6.14) while no difference was noticed between control (50.2 pmol retinal/h/mg protein) and RD (48.8 pmol retinal/h/mg protein) group. This corresponded with increased retinol levels evidenced in the respective groups, indicating the cleavage of β-carotene and astaxanthin to retinol. The carrot powder supplemented diet (Chapter 2) contained β-, α-carotene and lutein while dill leaf powder diet contained β-carotene and lutein. Although astaxanthin supplemented diet did not contain any of the provitamin A carotenoids, an increased retinol level (Chapter 4) and monooxygenase activity (Table 6.15, Figure 6.14) observed in this group indicates that astaxanthin was converted

to retinol in RD rats. The possible pathway of retinol formation from astaxanthin is explained in Chapter 5.

Table 6.15. Activity of monooxygenase in intestine and antioxidant molecules in plasma and liver of RD, control and rats fed dietary carotenoids for 20 days.

Sample	Groups				
	Control	RD	Carrot powder	Astaxanthin	Dill leaf powder
15-15' β-carotene monooxygenase (pmol retinal/h/mg protein)					
Intestine	50.2 ± 3.2^{a}	48.8 ± 5.2^{a}	182.5 ± 10.1^{b}	155.3 ± 9.1^{c}	177.9 ± 14.6^{b}
Superoxide dismutase (U/mg protein)					
Plasma	6.4 ± 0.6^{a}	1.5 ± 0.2^{b}	3.9 ± 0.4^{c}	5.9 ± 0.5^{a}	4.5 ± 0.5^{d}
Liver	8.2 ± 0.8^{a}	1.9 ± 0.2^{b}	3.5 ± 0.4^{c}	7.1 ± 0.7^{a}	4.2 ± 0.3^{c}
Catalase (μmol/min/mg protein)					
Plasma	4.3 ± 0.3^{a}	1.0 ± 0.1^{b}	1.9 ± 0.2^{c}	3.8 ± 0.3^{a}	2.3 ± 0.2^{c}
Liver	4.5 ± 0.4^{a}	1.2 ± 0.1^{b}	2.2 ± 0.2^{c}	4.2 ± 0.4^{a}	2.9 ± 0.3^{c}
Glutathione Transferase (μmol/min/mg protein)					
Liver	25.2 ± 2.1^{a}	10.1 ± 1.3^{b}	17.4 ± 1.2^{c}	22.5 ± 1.9^{a}	18.3 ± 2.3^{c}
Glutathione (mg/ml)					
Plasma	12.5 ± 1.0^{a}	4.1 ± 0.3^{b}	8.6 ± 0.7^{c}	10.8 ± 0.9^{a}	8.5 ± 0.6^{c}
Liver	20.1 ± 2.0^{a}	4.5 ± 0.5^{b}	12.5 ± 0.9^{c}	18.8 ± 2.0^{a}	13.5 ± 1.5^{d}

Data represent mean ± SD (n=5). Values between groups in a row with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test.

Lipid peroxidation and activity of antioxidant enzymes and molecules

Lipid peroxides (Lpx) level in blood and liver of the groups fed diets supplemented with purified astaxanthin, carrot powder, dill leaf powder, control and RD groups are given in Table 6.16, while the activity of antioxidant enzymes and glutathione level is given in Table 6.15. It is seen that retinol deficiency resulted in increased Lpx in plasma and liver by 72 and 87.7% respectively as compared to control group while its level was lower by 49.8, 20.3, 42.8% (plasma) and 37.7, 59.8, 44.3% (liver) in astaxanthin, carrot powder and dill leaf powder supplemented groups when compared with RD group (Figure 6.15).

The increase in plasma and liver Lpx in RD group corresponded with decreased activity of SOD (1.5 U/mg protein), CAT (1μmol/min/mg protein) and GSH (4.1 mg/ml) in plasma and activity of SOD (1.9 U/mg protein), CAT (1.2 μmol/min/mg protein), GST (10.1 μmol/min/mg protein) and GSH (4.5 mg/g) in liver of RD group as compared to control (Figure 6.16). Whereas, on feeding carrot powder, astaxanthin and dill leaf powder supplemented diet, the plasma SOD (62, 75, 67%), CAT (47, 74, 57%) and GSH (52, 62, 52%) were increased compared with RD group (Table 6.17). Similarly, elevated activities of SOD (46, 73, 55%), CAT (46, 71, 59%), GST (42, 55, 45%) and GSH (64, 76, 67%) were observed in liver of astaxanthin, β-carotene and lutein fed groups.

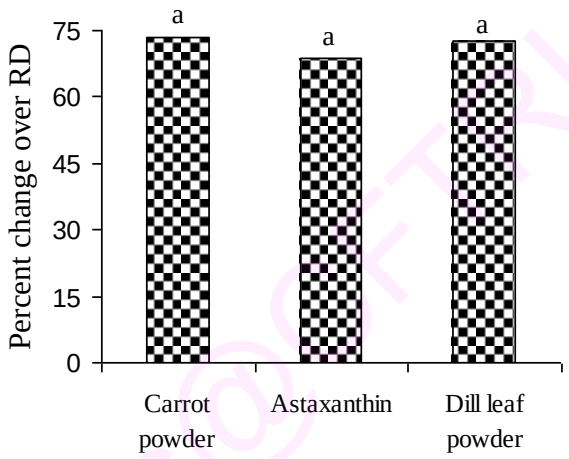


Figure 6.14. Percent increase in the activity of intestinal monooxygenase in carotenoid fed groups over RD group. Data represent mean ± SD (n=5). Values between groups with similar superscript are not significantly different (p>0.05) as determined by one-way ANOVA followed by Tukey's test.

Table 6.16. Lipid peroxide levels (TMP nmol/ml or g) in plasma and liver of RD, control and rats fed dietary carotenoids for 20 days.

Sample	Groups				
	Control	RD	Carrot	Astaxanthin	Dill leaf
Plasma	3.5 ± 0.33^{a}	27.63 ± 2.63^{b}	13.88 ± 1.6^{c}	22.01 ± 2.43^{a}	15.81 ± 1.89^{c}
Liver	16.5 ± 1.54^{a}	135.87 ± 15.43^{b}	84.59 ± 7.8^{c}	54.62 ± 5.32^{a}	75.71 ± 8.11^{c}

Data represent mean ± SD (n=5/time point). Values between groups in a row with similar superscript are not significantly different (p>0.05) as determined by one-way ANOVA followed by Tukey's test.

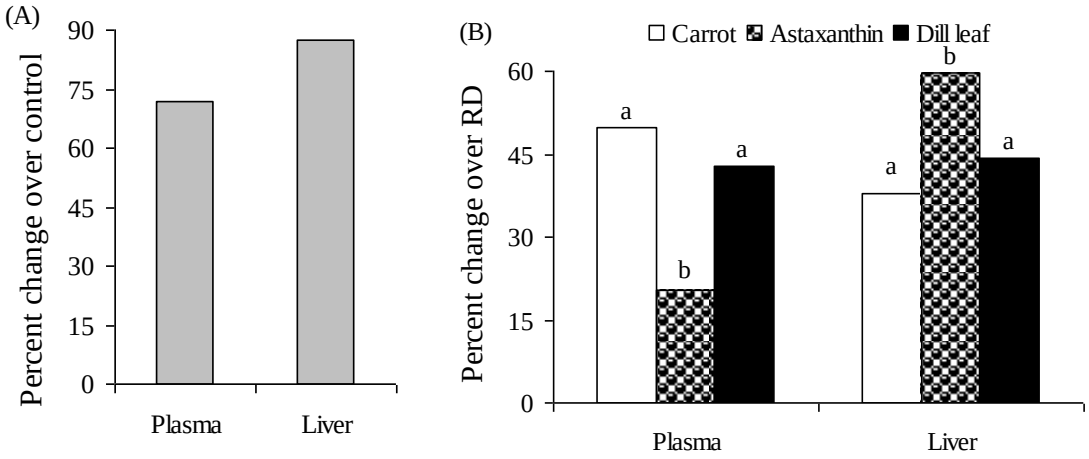


Figure 6.15. Percent increase in lipid peroxidation as a result of retinol deficiency over control group (A) and percent decrease in lipid peroxidation in carotenoid fed groups over RD group (B). Data represent mean (n=5). Values between groups with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test.

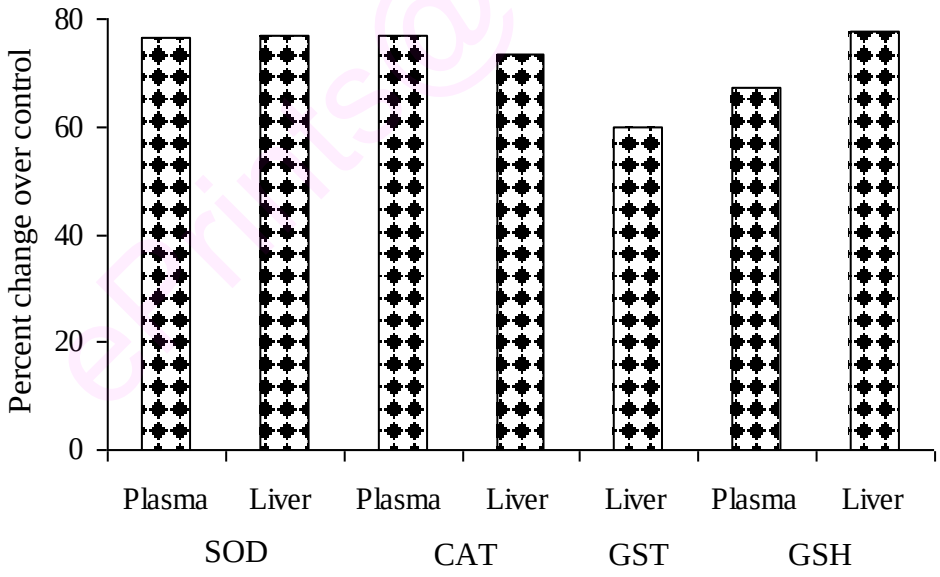


Figure 6.16. Percent decrease in the activity of antioxidant molecules in RD group over control.

Table 6.17. Percent increase in activity of antioxidant molecules in groups fed dietary carotenoids for 20 days over retinol deficient (RD) group.

Samples	Groups		
	Carrot powder	Astaxanthin	Dill leaf powder
Superoxide dismutase (%)			
Plasma	61.54	74.58	66.67
Liver	45.71	73.24	54.76
Catalase (%)			
Plasma	47.37	73.68	56.52
Liver	45.45	71.43	58.62
Glutathione Transferase (%)			
Liver	41.95	55.11	44.81
Glutathione (%)			
Plasma	52.33	62.04	51.76
Liver	64.00	76.06	66.67

Data represent mean (n=5/group).

Lipid Profile

The plasma fatty acid profile of control, RD and experimental groups is given in Table 6.18. It is seen from the results that retinol deficiency resulted in significantly increased ($p<0.05$) levels of saturated fatty acids (SFA) by 50% and decreased ($p<0.05$) mono- and poly-unsaturated fatty acids (MUFA, PUFA) by 44 and 55%, respectively over control (Figure 6.17). In contrast, feeding diet supplemented with carrot powder, astaxanthin and dill leaf powder showed a decrease in SFA by 47, 48, 47% and increase in MUFA by 41, 41, 42% and PUFA by 51, 51, 52% over RD group (Figure 6.18). Lipid parameters were analyzed in plasma and liver of rats and are presented in Tables 6.18 and 6.19. Retinol deficiency resulted in lowered levels (mg/dl) of total cholesterol (72.9), HDL-cholesterol (35), LDL+VLDL-cholesterol (37.7), phospholipid (81.8) and triglycerides (80.9) in blood as compared to control group (92.1, 46.5, 45.5, 133.5, 121) (Table 6.19). Total cholesterol, HDL-cholesterol, LDL+VLDL-cholesterol, phospholipids and triglycerides levels (mg/dl) were significantly higher ($p<0.05$) in carrot powder (93.6, 46.2, 47.1, 133.7, 150), astaxanthin (91.4, 48.8, 42.3, 125.5, 146.9) and dill leaf powder (95.7, 47.8, 47.7, 136.7, 157.7) supplemented diet fed groups than RD (72.9, 35, 37.7, 81.8, 80.9) group (Table 6.18).

Table 6.18. Plasma fatty acid profile of RD, control and carotenoid fed groups.

Fatty acids	Groups				
	Control	RD	Carrot powder	Astaxanthin	Dill leaf powder
12:0	0.51	1.2	0.55	0.57	0.50
14:0	0.27	1.01	0.33	0.25	0.30
16:0	21.1	37.6	21.5	22.3	22.7
18:0	8.5	21.03	9.6	8.7	9.01
16:1	4.3	2.73	4.3	4.1	4.1
18:1	30.0	16.59	28.6	28.5	29.4
18:2	19.52	7.02	19.2	18.9	18.9
20:4	12.9	7.65	11.0	11.3	11.8
SFA	30.38	60.84	31.98	31.82	32.51
MUFA	34.3	19.32	32.9	32.6	33.5
PUFA	32.42	14.67	30.2	30.2	30.7

Data represent mean (n=5/time point). SFA = saturated fatty acids, MUFA = mono-unsaturated fatty acids, PUFA = poly-unsaturated fatty acids.

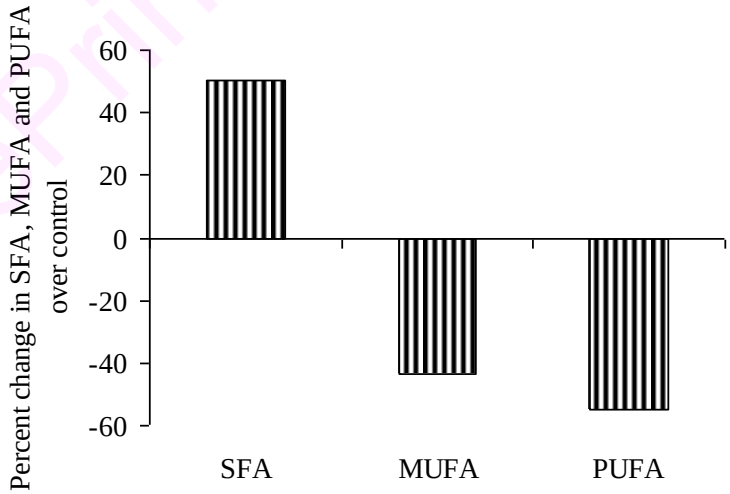


Figure 6.17. Percent increase in saturated fatty acids (SFA) and decrease in mono-unsaturated (MUFA) and poly-unsaturated (PUFA) fatty acid in RD group as compared to control.

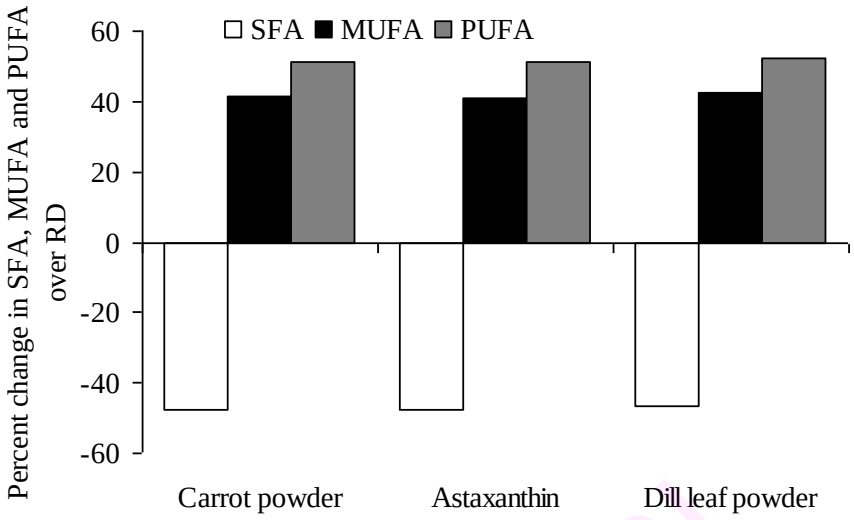


Figure 6.18. Percent decrease in saturated fatty acids (SFA) and increase in mono-unsaturated (MUFA) and poly-unsaturated (PUFA) fatty acids in carotenoid fed groups as compared to RD group.

Table 6.19. Blood lipid profile of retinol deficient (RD), control and rats fed carotenoids.

Parameter (mg/dl)	Groups				
	Control	RD	Carrot	Astaxanthin	Dill leaf
*Total cholesterol**	92.1 ± 1.0 [a]	72.9 ± 2.4 [b]	93.6 ± 5.3 [a]	91.43 ± 2.5 [a]	95.73 ± 3.3 [a]
*HDL-cholesterol**	46.5 ± 1.1 [a]	35.0 ± 1.1 [b]	46.2 ± 2.0 [a]	48.8 ± 2.11 [a]	47.8 ± 2.0 [a]
VLDL+LDL-cholesterol *	45.5 ± 1.6 [a]	37.7 ± 2.1 [b]	47.1 ± 3.3 [a]	42.3 ± 1.2 [a]	47.66 ± 2.1 [a]
*Phospholipids**	133.5 ± 3.1 [a]	81.8 ± 4.5 [b]	133.7 ± 5.3 [a]	125.5 ± 2.9 [a]	136.66 ± 4.4 [a]
*Triglycerides***	121.0 ± 3.1 [a]	80.9 ± 6.5 [b]	150.0 ± 6.3 [c]	146.95 ± 3.1 [c]	157.65 ± 5.5 [c]

Data represent mean ± SD (n=5/time point). Values between groups in a row with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test. *Estimated in serum, ** estimated in plasma.

Liver levels (mg/dl) of cholesterol (12.4), phospholipids (14.6) and triglycerides (15.8) were significantly lowered ($p<0.05$) in RD group as compared to control (Table 6.20). Cholesterol, phospholipids and triglycerides levels (mg/g) were elevated on feeding carrot

powder (16.1, 22.7, 25.9), astaxanthin (15.5, 21.2, 22.9) and dill leaf powder (16.8, 24, 26.3) supplemented diet as compared to RD (12.4, 14.6, 15.8) group.

Table 6.20. Liver lipid profile of retinol deficient (RD), control and rats fed carotenoids.

Parameter (mg/g)	Groups				
	Control	RD	Carrot	Astaxanthin	Dill leaf
Total cholesterol	15.01 ± 1.1^{a}	12.4 ± 0.9^{b}	16.1 ± 1.3^{a}	15.5 ± 1.1^{a}	16.8 ± 1.2^{a}
Phospholipids	21.7 ± 1.0^{a}	14.6 ± 0.9^{b}	22.7 ± 1.0^{a}	21.2 ± 1.2^{a}	24.0 ± 1.0^{a}
Triglycerides	19.1 ± 1.0^{a}	15.8 ± 1.1^{b}	25.9 ± 2.2^{c}	22.9 ± 1.3^{a}	26.34 ± 1.4^{c}

Data represent mean ± SD (n=5/time point). Values between groups in a row with similar superscript are not significantly different ($p>0.05$) as determined by one-way ANOVA followed by Tukey's test.

Retinol Binding Protein

Retinol binding protein (RBP) concentration in serum was estimated by ELISA is given in Table 6.21. Retinol deficiency resulted in lower ($p<0.05$) RBP levels (147 ng/ml) as compared to control (62.3 μg/ml). Whereas, the levels were increased significantly ($p<0.05$) on feeding carrot powder (259.8), astaxanthin (189.6) and dill leaf (245.7) powder when compared with RD (147.04) group, indicating higher plasma retinol levels (Chapter 4) in carotenoid fed groups. The native PAGE gel electrophoresis for serum RBP shows more prominent bands in control than the RD group (Figure 6.19). In addition, RBP standard was run alongside to identify the RBP in the samples and based on the electrophoresis and ELISA results it was highest in the control and amongst the carotenoid fed groups it was in the order of carrot fed group > dill leaf fed group > astaxanthin fed group.

Table 6.21. Retinol Binding Protein (RBP) concentration (ng/ml) in serum of retinol deficient (RD), control and rats fed carrot powder (β-carotene source), astaxanthin and dill leaf powder (lutein source) in diet.

Group	RBP
Carrot powder	259.76 ± 20.79^{a}
Astaxanthin	189.62 ± 15.6^{b}
Dill leaf powder	245.69 ± 21.7^{c}
RD	147.04 ± 11.6^{d}

Data represent mean ± SD (n=5). Values between the groups not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. RBP in Control = 62.3 μg/ml.

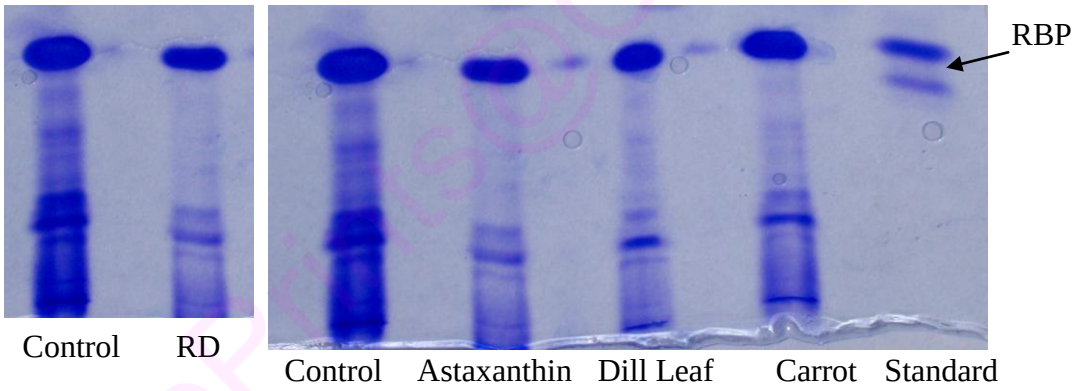


Figure 6.19. Native PAGE for RBP of control, retinol deficient (RD) groups and rats fed carotenoid supplemented diets.

Discussion

Carotenoid metabolizing enzyme

Intestinal monooxygenase is the β-carotene cleavage enzyme that cleaves provitamin A carotenoids to retinol (Olson, 1994). Apart from provitamin A carotenoids, under retinol deficiency, this enzyme may cleave nonprovitamin A carotenoids to retinol (Sangeetha and Baskaran, 2010b). Thus, the activity of the enzyme is higher in intestines of rats that have been

fed carotenes. In the present study, monooxygenase activity was significantly higher after a dose of β-carotene over 8h. However, no significant difference was noticed in monooxygenase activity in astaxanthin and lutein groups as compared with RD group. This indicates that β-carotene, but not astaxanthin and lutein, was cleaved to retinol by the enzyme. This corresponds well with the higher plasma retinol levels in the β-carotene group (Chapter 4), but not in astaxanthin and lutein groups. Raju and Baskaran (2009), Raju et al. (2006) and Barua and Olson (2000) have also reported enhanced monooxygenase activity in retinol sufficient and deficient rats fed β-carotene.

In contrast to the results of the single dose study, there was an increase in monooxygenase activity in astaxanthin as well as β-carotene groups after their repeated dose for 7- and 15-days. The retinol level in β-carotene and astaxanthin groups was 10 fold higher than RD and lutein group. This result was further supported by the presence of β-carotene in the astaxanthin group. The presence of β-carotene in the plasma and liver of astaxanthin group demonstrates that astaxanthin may be converted to β-carotene via echinenone (Chapter 5), which may be cleaved to retinol, resulting in the increased retinol levels and monooxygenase activity. Although the enzyme involved in the conversion of echinenone to retinol via β-carotene needs further studies, this is the first study to report the conversion of astaxanthin to echinenone and β-carotene in rats. Thus, the conversion of astaxanthin to β-carotene explains the increased monooxygenase activity of astaxanthin group. It is speculated that the activity of the enzyme may be more expressed after repeated astaxanthin feeding but not after single dose. In contrast to the results obtained for astaxanthin group, monooxygenase activity in lutein group was not significantly different from RD group indicating that lutein was not recognized as a substrate and thus was not cleaved to retinol. This was supported by the plasma retinol levels, which did not show any significant increase in its levels. A similar result was found on dietary feeding of astaxanthin that showed an increased intestinal monooxygenase activity and plasma retinol levels (Chapter 4) as compared to RD group. However, in comparison with astaxanthin group, the carrot powder and dill leaf powder supplemented diet groups exhibited higher monooxygenase activity and plasma retinol levels. This is may be due to the presence of β-carotene in carrot powder and dill leaves which may have cleaved to retinol.

Lipid peroxidation and activity of antioxidant molecules

Lipids are an integral part of tissues and cell membranes. Lpx is an established marker for oxidative stress by either pro-oxidants or nutrient deficiency. Due to the higher levels of unsaturated fats in membranes, they are highly susceptible to peroxidation by free radicals or

nutrient deficiency (Oliveros et al., 2000). Hence, antioxidants, which are effective in suppressing Lpx at the membrane level, are of significant importance. Carotenoids and retinoids being lipophilic in nature readily form an integral part of the cell membrane. Depending upon their polarity, structure and functional groups attached, they are localized in the membrane structure where they provide protection to the lipids from peroxidation (Woodall et al., 1997). Anzulovich et al. (2000) studied the effect of retinol deficiency on structural and functional alterations in liver of rats. They have suggested that modifications of the tissue components in retinol deficiency may predispose the membrane to peroxidation-induced damage. Halliwell and Guteridge (1989) correlated the Lpx at cellular level and pathological abnormalities in tissues. The present study revealed that retinol deficiency resulted in significantly elevated levels of lipid peroxides and decreased activity of SOD, CAT GST and GSH in plasma and liver. This corroborated well with earlier studies, which also showed elevated Lpx in retinol deficiency in liver and heart of rats (Anzulovich et al., 2000; Oliveros et al., 2000; Kaul and Krishnakantha, 1997). Whereas, on intubation with carotenoids, the levels of Lpx were significantly decreased and the effect was more pronounced in astaxanthin and FUCO groups as compared to lutein and β-carotene groups. The decrease in Lpx was correlated with the activity of SOD, CAT, GST and GSH that showed higher activity in carotenoid fed groups.

Vitamins and carotenoids are known to scavenge reactive oxygen species and up-regulate the activities of antioxidant enzymes (Fang et al., 2002). Amongst the carotenoids used in this study, the activity of antioxidant molecules was greater in astaxanthin group as compared to other groups. This difference was evident in single as well as repeated dose studies. In the dietary study, the antioxidant activity was higher in astaxanthin fed group than the group fed dill leaf powder, which in turn was higher than group fed carrot powder supplemented diet. The activity of antioxidant molecules was significantly higher in carotenoids fed groups when compared to RD. The suppressive effect of astaxanthin on Lpx was higher than dill leaf followed by carrot fed groups. The decrease in Lpx by carotenoids can be attributed to their antioxidant properties (McNulty et al., 2007; Sachindra et al., 2007). The differences in the antioxidant potential among the carotenoids may be due to their different chemical structures and thus different spatial arrangement in the membrane of the cells (Britton, 1995). While β-carotene is highly hydrophobic and lipophilic and thus resides in the lipid core, parallel to the membrane, xanthophylls may be arranged perpendicular to the membrane (McNulty et al., 2007; Woodall et al., 1997). Thus, β-carotene may participate in the antioxidant reactions in the lipid phase while xanthophylls may participate in reactions at both aqueous and the lipid phases. This may be the reason for the greater effectiveness of astaxanthin (with more number of polar functional groups-

2 keto and 2 hydroxyl groups on the β-ionone rings) and FUCO (2 hydroxyl groups, an epoxide and an acetyl group on the β-ionone rings and a keto group in the polyene chain), as compared to lutein (2 hydroxyl groups on the β-ionone rings) and β-carotene (no polar functional groups).

Lipid Profile

Retinol deficiency was found to affect lipid parameters in plasma and liver. In plasma of RD rats, the fatty acid profile was altered as compare to control with elevated levels of SFA and decreased levels of MUFA and PUFA. The total cholesterol, HDL-cholesterol and LDL+VLDL cholesterol, phospholipid and triglyceride levels were decreased as compared to control. On the other hand, there was amelioration in the fatty acid profile with decreased SFA and increase in MUFA and PUFA on feeding carotenoids as compared to RD. This was evidenced in single, 7- and 15-days repeated dose as well as the dietary studies. The alteration in saturated and unsaturated fatty acids in carotenoid administered groups can be attributed to the fact that carotenoids might inhibit desaturase activity and thereby increase levels of unsaturated fatty acids suggesting that the metabolism of fatty acids is affected (Grolier et al., 1995; Hamm et al., 1987). The total cholesterol, HDL-cholesterol and LDL+VLDL cholesterol, phospholipid and triglyceride levels were also ameliorated on feeding the carotenoids to RD rats. Murello (1992) has reported elevated cholesterol and HDL-cholesterol levels on feeding astaxanthin and canthaxanthin to rats. Murello (1992) however reported that β-carotene did not exhibit similar effect as astaxanthin and canthaxanthin in the rats. Whereas, in the present study, it was found that there was an alteration in the total cholesterol and HDL cholesterol on feeding β-carotene, astaxanthin, lutein and fucoxanthin. The ameliorative effect of carotenoids on plasma phospholipids as compared to RD may have been due to their protective effect against lipid oxidation. Oshima et al. (1993) have reported that astaxanthin and β-carotene protect the phospholipid layers in unilamellar liposomes against photosensitized oxidation. Asai et al. (1999) have reported lower phospholipid hydroperoxides in RBC, liver lipid peroxidizability, on feeding turmeric and capsicum extracts to mice. They have attributed these effects to the antioxidant property of the spices. In the present study, increased HDL-cholesterol and LDL+VLDL-cholesterol was observed on carotenoid feeding. However, on comparison, it was found that the change in HDL level was higher than LDL+VLDL levels in xanthophylls. Takahashi et al. (2004) have also observed incorporation of astaxanthin in HDL (70%). Further, they have suggested that since astaxanthin was incorporated in HDL and LDL to a greater extent, their secretion and levels may be higher in circulation leading to higher cholesterol levels on feeding carotenoids.

Retinol Binding Protein

Retinol is released from the liver combined with retinol-binding protein and enters to the blood circulates along with prealbumin. Their levels were reported to be lower in severely malnourished children (Venkataswamy et al., 1977). Thus, RBP is a biomarker for retinol status as its levels are proportional with that of circulating retinol. In the results, single, repeated and dietary studies show that RD group had significantly lowed RBP as compared to control, whereas, their levels were increased after the gavage of single dose of β-carotene to RD rats. Gamble et al. (2001) have also reported that RBP levels dropped in the serum of vitamin A deficient rats. In contrast, intubation of astaxanthin, lutein and fucoxanthin did not show alteration in RBP levels indicating that only β-carotene is cleaved to retinol. This was further supported by enhanced monooxygenase activity in β-carotene group unlike the other groups. However, as discussed earlier, enhanced retinol levels and monooxygenase activity was observed on repeated gavage of astaxanthin and. This was associated with higher RBP levels in astaxanthin and β-carotene groups. In the dietary study, RBP level was higher for carotenoid supplemented groups, however its level was greater in the case of carrot powder and dill leaf powder groups than astaxanthin group. This corresponded well with the increased retinol levels and monooxygenase activity in those groups. The probable reason for increased RBP in carrot and dill leaf powder fed groups may be due to the presence of β-carotene, which was cleaved to retinol. The conversion of astaxanthin to β-carotene was less efficient and therefore resulted in lower levels of retinol. Thus, the RBP levels confirm that retinol was formed in the astaxanthin group. Muto et al. (1972) have reported that the blood RBP was increased after a single oral dose of vitamin A. The present results and the available reports demonstrate that retinol deficiency results in alteration of various biochemical parameters in rats, which were ameliorated by feeding either purified or dietary. The ameliorative effect was in the order of astaxanthin > fucoxanthin > lutein > β-carotene.

CHAPTER 7: Effect of dietary carotenoids (β-carotene, astaxanthin, lutein and fucoxanthin) on vitamin A deficiency induced changes in biochemical constituents in tissue membrane of rats with respect to structure and function

Introduction

The main underlying cause of vitamin A deficiency (VAD) is a diet that is insufficient in vitamin A. VAD can lead to lower body stores of retinol. VAD causes disorders like xerophthalmia, the leading cause of preventable childhood blindness, anaemia, and weakened host resistance to infection, which can increase the severity of infectious diseases and risk of death. In addition, VAD can cause profound biochemical changes at the tissue membrane level as well. Retinol deficiency may provoke a general alteration in the physical characteristics of tissue membranes (Kon' IIa et al., 1990).

Retinol is essential for maintaining the structure and function of the membranes. Due to its antioxidant nature, its deficiency leads to alterations in membrane fluidity, thereby affects the membrane function (Kaul and Krishnakantha, 1997). Cellular and sub-cellular membranes are susceptible to lipid oxidation due to high concentration of polyunsaturated fatty acids and close proximity to oxygen, transition metals, and peroxidases. Highly reactive oxygen species such as superoxide anion radical [O_2-], hydroxyl radical [HO], hydrogen peroxide [H_2O_2], and singlet oxygen [$^1O_2$] are produced continuously in the course of normal aerobic cellular metabolism. However, these oxidative products can damage healthy cells if they are not eliminated (Chew, 1995). Lipid peroxidation (Lpx) is a highly destructive process that affects cell organelles and causes them lose biochemical function and/or structural integrity (Bhatia and Jain, 2004). Retinoids have been found to act effectively *in vitro* as antioxidants and radical scavengers (Palacios et al., 1996). Antioxidant potential of retinol has also been studied *in vivo*. Vitamin A inhibits doxorubicin-induced membrane Lpx in rat tissues (Ciaccio et al., 1993). Oliveros et al. (2000) have provided experimental evidence on the pro-oxidative effect of VAD in rats. VAD leads to a reduction in the essential fatty acids and increases Lpx in retinol deficient microsomal membranes of the liver, kidney, spleen and brain in rats (Kaul and Krishnakantha, 1997).

Membrane functions such as permeability, enzyme activity, hormone receptor interactions and efficiency of transport systems are influenced by membrane dynamics. The efficiency of nutrients transport is controlled by membrane fluidity. Membrane structural

determinants like lipid composition are known to affect the membrane fluidity (Kaul and Krishnakantha, 1997). It has been suggested that retinol could act as a physiological antioxidant as it is well integrated within the membrane milieu by virtue of its lipid solubility and hydrophobicity and could offer protection from Lpx. Retinol deficiency leads to loss of resistance to lipid peroxidation and failure to protect the unsaturated fatty acids in the membrane from peroxidation. This could, in turn, change the membrane fluidity. The unsaturation index of phospholipids, cholesterol: phospholipid ratios are important indicators of membrane fluidity (Kaul and Krishnakantha, 1997). Cholesterol and fatty acids plays an important role in regulating membrane fluidity. Thus, the cholesterol: phospholipid ratio, an index of the fluidity of the membrane, plays an important role in controlling the fluidity of the membrane. The ratio of these biomolecules is inversely proportional to membrane fluidity (Niranjan and Krishnakantha, 2001). Vitamin E, β-carotene and ascorbic acid are known non-enzymatic antioxidants, which quench the free radicals, inhibit lipid peroxidation and prevent DNA damage (Liu and Mori, 1993). Lipid bilayers of cell membranes are highly susceptible to peroxidation due to their polyunsaturated fatty acids. Free radical reactions in the lipid domain also result in damage to membrane proteins thus leading to alteration and impairment of membrane functions (Wiseman, 1996). Membrane properties of cells directly reflect membrane lipid composition. Fatty acids provide a lipid environment for membrane bound-enzymes involved in immunological responses. Changes in the membrane lipid composition of the immune cell may alter its receptor binding sites (Hummel, 1993). The variations in membrane fluidity, in turn, can modulate the activity of receptors, membrane-bound enzymes and ion channels involved in cell proliferation (Calder, 1996).

Membrane function is vital to normal cell processes and is modulated by a wide range of factors. Studies have shown the influence of dietary components on membrane fluidity, stability and susceptibility to oxidative damage (Wiseman, 1996). Fatty acids have many diverse functions in cells; their principal roles are as energy sources and membrane constituents. Changing the proportions of the fatty acids in cell membranes may alter the fluidity and integrity of the membrane (Kelly and Daundu, 1993). A number of membrane bound enzymes have been shown to be sensitive to their fatty acid environments. These include among others, Na^+K^+ ATPase, which is involved in monovalent of cation transport. It has been established that ATPases require a 'fluid' lipid environment for proper functioning (Stubbs and Smith, 1984). The above literature survey indicates that not many studies are available with regard to the effect of VAD and carotenoid feeding on membrane bound enzymes and lipid profile, which indicate change in membrane structure and function.

In view of the above, it was planned to study the effect of retinol deficiency and subsequent feeding of carotenoids on the membrane bound enzymes, lipid peroxidation, antioxidant molecules and lipid profile. Fatty acid composition and cholesterol content of the membrane affects its fluidity. Thus, the cholesterol: phospholipid ratio, which is an index of the fluidity of the membrane was estimated to demonstrate the effect of retinol deficiency and subsequent carotenoid feeding on membrane structure and function. RD rats were fed single dose (time-course), repeated dose (7- and 15-days) or dietary (20-days) source of β-carotene, astaxanthin, lutein or fucoxanthin. At the termination of experiments, liver and brain were sampled and used for preparation of microsomes. Activity of membrane bound enzymes in liver and brain microsomes, antioxidant molecules and lipid parameters in liver microsomes were estimated. In all experiments, a separate control group received retinol in diet and RD (baseline) were maintained throughout the experimental run.

Results

Single dose study

Retinol and carotenoids in liver microsomes

Feeding diet devoid of retinol resulted in its deficiency in plasma (0.38 μmol/l). The HPLC chromatogram of retinol and carotenoids extracted from the liver microsomes of the carotenoid fed rats is shown in Figure 7.1. Retinol deficiency resulted in its depleted ($p<0.05$) levels (1.6 nmol/ml) as compared to the control group (874 nmol/ml) in liver microsomes. On gavage with β-carotene, its level was increased in the liver microsomes over a period of 8 h (3.53-20.34 nmol/ml) as compared to RD, astaxanthin and lutein groups (Table 7.1). β-Carotene was detected (2.73 pmol/ml) 8h after intubation while astaxanthin, lutein and fucoxanthin were detected (pmol/ml) at 2h (0.36, 0.27, 0.1), 4h (1.68, 1.38, 0.6) and 8h (5.91, 4.76, 4.2) in liver microsomes. Comparison between the groups revealed that β-carotene was cleaved to retinol while astaxanthin, lutein and fucoxanthin were not converted to retinol. In addition to the intact lutein, there was a detectable level of its isomer, zeaxanthin. In contrast, no intact FUCO was detected, rather, its metabolites, FUOH and AAx were detected in the FUCO fed group.

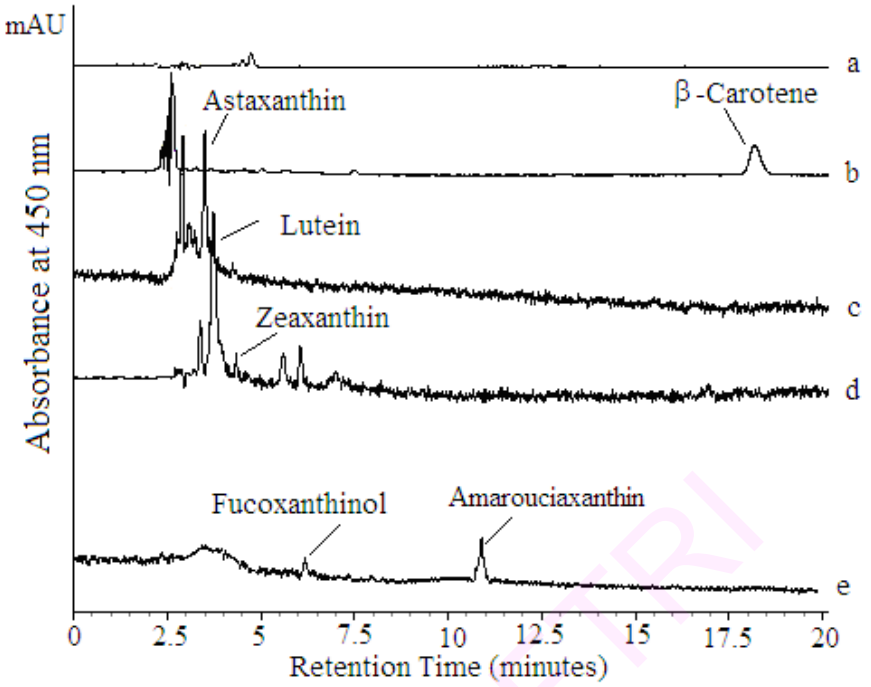


Figure 7.1. HPLC chromatograms of carotenoids extracted from liver microsomes (8 h sample) of retinol deficient (a), β-carotene (b), astaxanthin (c), lutein (d) and fucoxanthin (e) groups.

Table 7.1. Retinol levels (nmol/ml) in liver microsomes of rats gavaged with carotenoids.

Time (h)	Groups					
	Control	RD	β-Carotene	Astaxanthin	Lutein	Fucoxanthin
2	874 ± 28.79^{a}	1.6 ± 0.09^{b}	3.53 ± 0.34^{c}	1.44 ± 0.83^{b}	1.57 ± 0.23^{b}	1.58 ± 0.33^{b}
4	874 ± 28.79^{a}	1.6 ± 0.09^{b}	9.31 ± 0.71^{c}	1.56 ± 0.23^{b}	1.34 ± 0.32^{b}	1.59 ± 0.21^{b}
8	874 ± 28.79^{a}	1.6 ± 0.09^{b}	20.34 ± 2.20^{c}	1.26 ± 0.16^{b}	1.35 ± 0.13^{b}	1.56 ± 0.25^{b}

Data represent mean ± SD (n=5). Values in a row between groups not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

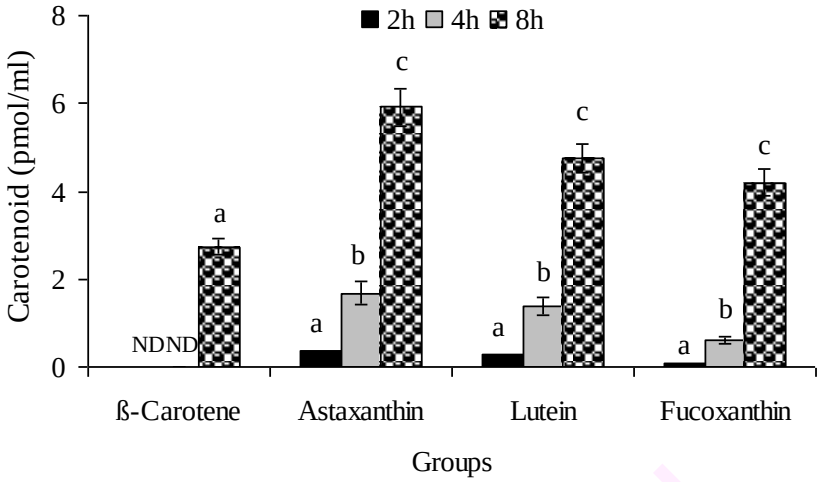


Figure 7.2. Carotenoid levels (pmol/ml) in liver microsomes of RD rats gavaged with β-carotene, astaxanthin, lutein and fucoxanthin. Data represent mean ± SD (n=5). Values in a group at different time points not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. ND = Not detected. The detectable limit under the conditions used = 1 pg. Fucoxanthin = FUOH + AAx, Lutein = lutein + zeaxanthin.

Membrane bound enzymes

Activity of membrane bound enzymes was estimated in the liver and brain microsomes of RD and carotenoid gavaged rats (Table 7.2). Results showed that retinol deficiency significantly ($p<0.05$) increased the activity of Na^+K^+-ATPase (75%), Ca^{2+}-ATPase (69%), Mg^{2+}-ATPase (51%) in liver microsomes and decreased the activity of acetylcholine esterase (AChE) in brain microsomes by 78% as compared to the control (Table 7.2, Figure 7.3). On gavage of β-carotene, astaxanthin, lutein and fucoxanthin over 8 h the activity of Na^+K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase were decreased in liver microsomes while the activity of AchE was increased in brain microsomes as compared to RD group. As compared to RD group, Na^+K^+-ATPase, Ca^{2+}-ATPase, Mg^{2+}-ATPase activities were decreased by 20-60, 26-57, 11-45% (β-carotene group), 9-36, 14-39, 9-33% (astaxanthin group), 4-33, 13-35, 3-26 % (lutein group) and 55-61, 14.8-38.3, 7.4-31% (fucoxanthin group). On the other hand, the activity of AChE was increased over a period of 8h in β-carotene (0-52%), astaxanthin (20-57.1%), lutein (7.7-50%) and fucoxanthin (14.3-52%) over RD group. On comparison of the results, it was observed that β-carotene was more effective in modulating the activities of the ATPases in liver microsomes,

followed by astaxanthin, fucoxanthin and lutein while astaxanthin was more effective than β-carotene, lutein and fucoxanthin in ameliorating the AChE activity in brain microsomes.

Table 7.2. Effect of retinol deficiency and subsequent gavage of carotenoids on the activities of membrane bound Na^+K^+, Ca^{2+}, Mg^{2+}-ATPases in liver microsomes and acetylcholine esterase (AChE) in brain microsomes.

Time (h)	Groups					
	Control	RD	β-Carotene	Astaxanthin	Lutein	Fucoxanthin
Na^+K^+ATPase (P_i/h/mg protein)						
2	6.8 ± 0.6 [a]	26.9 ± 2.0 [b]	21.7 ± 2.7 [c]	24.5 ± 2.5 [b]	26.0 ± 2.8 [b]	12.0 ± 0.5 [d]
4	6.8 ± 0.6 [a]	26.9 ± 2.0 [b]	17.8 ± 1.6 [c]	21.1 ± 2.0 [c]	21.9 ± 2.1 [c]	11.9 ± 0.9 [d]
8	6.8 ± 0.6 [a]	26.9 ± 2.0 [b]	10.8 ± 1.2 [c]	17.2 ± 1.6 [d]	18.1 ± 1.5 [d]	10.5 ± 0.1 [c]
Ca^{2+}ATPase (P_i/h/mg protein)						
2	4.9 ± 0.4 [a]	15.9 ± 1.4 [b]	11.7 ± 1.2 [b]	13.7 ± 1.5 [b]	13.9 ±1.5 [b]	13.5 ± 1.2[b]
4	4.9 ± 0.4 [a]	15.9 ± 1.4 [b]	9.9 ± 0.9 [b]	12.0 ± 1.2 [b]	12.0 ± 1.4 [b]	13.0 ± 1.1[b]
8	4.9 ± 0.4 [a]	15.9 ± 1.4 [b]	6.8 ± 0.7 [a]	9.8 ± 0.9 [b]	10.4 ± 1.5 [b]	9.8 ± 0.9[b]
Mg^{2+}ATPase (P_i/h/mg protein)						
2	11.3 ± 1.1 [a]	22.9 ± 1.9 [b]	20.3 ± 0.9 [b]	20.9 ± 2.0 [b]	22.3 ± 2.1 [b]	21.2 ± 2.0[b]
4	11.3 ± 1.1 [a]	22.9 ± 1.9 [b]	16.4 ± 0.8 [b]	17.3 ± 1.1 [b]	19.0 ± 1.5 [b]	18.2 ± 1.7[b]
8	11.3 ± 1.1 [a]	22.9 ± 1.9 [b]	12.7 ±1.2 [a]	15.3 ± 1.2 [c]	17.0 ± 1.1 [b]	15.8 ± 1.3 [b]
AChE (nmol/min/mg protein)						
2	0.6 ± 0.04 [a]	0.1 ± 0.01 [b]	0.1 ± 0.03 [b]	0.2 ± 0.04 [b]	0.1 ± 0.04 [b]	0.1 ± 0.01[b]
4	0.6 ± 0.04 [a]	0.1 ± 0.01 [b]	0.2 ± 0.04 [b]	0. 2± 0.05 [c]	0.2 ± 0.03 [b]	0.2 ± 0.01[c]
8	0.6 ± 0.04 [a]	0.1 ± 0.01 [b]	0.3 ± 0.03 [c]	0.3 ± 0.03 [c]	0.2 ± 0.04 [c]	0.3 ± 0.02[c]

Data represent mean ± SD (n=5). Values within a row between groups not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. RD = baseline (0h) group, control = group fed with retinol sufficient diet.

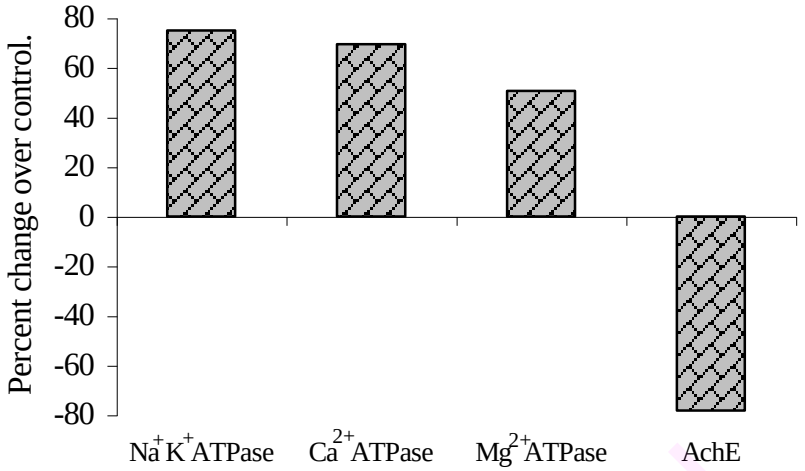


Figure 7.3. Percent increase in activities of membrane bound Na^+K^+, Ca^{2+}, Mg^{2+}-ATPases in liver microsomes and percent decrease in acetylcholine esterase (AChE) activity in brain microsomes in RD group as compared to control.

Table 7.3. Percent decrease in activities of membrane bound Na^+K^+, Ca^{2+}, Mg^{2+}-ATPases in liver microsomes and percent increase in acetylcholine esterase (AChE) activity in brain microsomes in carotenoid fed groups as compared to RD group.

Time (h)	Groups			
	β-Carotene	Astaxanthin	Lutein	FUCO
Na^+K^+ATPase (% decrease)				
2	19.5	9.2	3.6	55.4
4	34.1	21.8	18.8	55.9
8	60.0	36.2	33.0	61.2
Ca^{2+}ATPase (% decrease)				
2	26.1	13.9	12.7	14.8
4	37.9	24.3	24.4	18.0
8	57.2	38.6	34.5	38.3
Mg^{2+}ATPase (% decrease)				
2	11.2	8.7	2.6	7.4
4	28.3	24.3	17.0	20.5
8	44.7	33.3	25.7	31.0
AChE (% increase)				
2	0.0	20.0	7.7	14.3
4	20.0	40.0	29.4	33.3
8	52.0	57.1	50.0	52.0

Data represent mean (n=5).

Lipid peroxidation and antioxidant molecules

Results on the effect of VAD over control and subsequent gavages of carotenoids on the lipid peroxidation (Lpx) levels, reduced glutathione levels and activity of superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) in the liver microsomes are given in Figure 7.4 and Table 7.4. Results reveal that retinol deficiency resulted in increased Lpx (95%) and decreased SOD (69%), CAT (71%), GST (48%) and GSH (51%) as compared to control (Figure 7.5). Whereas, on gavage with β-carotene, astaxanthin, lutein and fucoxanthin, there was a decrease in Lpx by 12-33, 17-66, 16-45, 17-62% respectively (Figure 7.6) and increase in activities of SOD by 6.9-40, 0-25, 3.6-32.5, 3.6- 32.5 %, CAT by 11-38, 0-22, 4-32, 30-56 %, GST by 2-35, 0.4-23, 1-30, 1-44 % and GSH levels by 8.5-42.2, 1.1-27.7, 6.2-33.7, 5.2-43.8 % respectively over 8 h as compared to RD group (Table 7.5). On comparison between the groups, it is seen that the decrease in Lpx was higher by astaxanthin and fucoxanthin followed by lutein and β-carotene. Whereas, with respect to activity of antioxidant enzymes, β-carotene and fucoxanthin showed higher activity followed by lutein and astaxanthin.

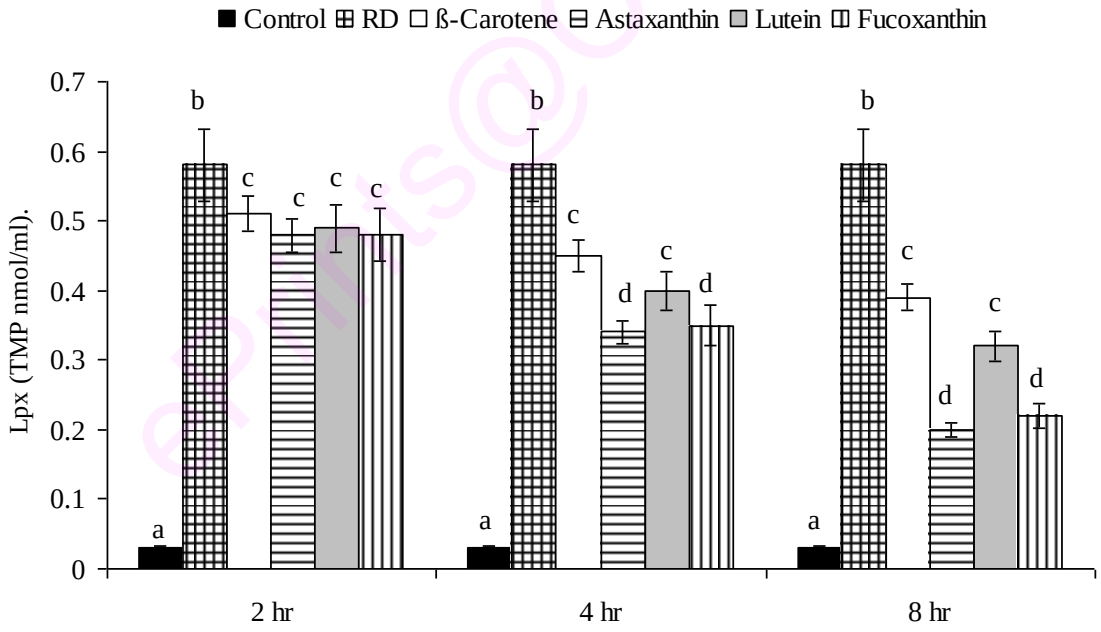


Figure 7.4. Effect of retinol deficiency and subsequent carotenoid gavage on lipid peroxide levels in liver microsomes of rats. Data represent mean ± SD (n=5). Values within each time point between groups not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. RD refers to baseline (0h) group while control refers to the group fed on diet containing retinol.

Table 7.4. Effect of retinol deficiency and subsequent carotenoid gavage on the activity of antioxidant enzymes and reduced glutathione levels in liver microsomes of rats.

Time (h)	Groups					
	Control	RD	β-Carotene	Astaxanthin	Lutein	Fucoxanthin
Superoxide Dismutase (U/mg protein)						
2	8.8 ± 0.5^{a}	2.7 ± 0.1^{b}	2.9 ± 0.1^{b}	2.7 ± 0.1^{b}	2.8 ± 0.2^{b}	2.8 ± 0.2^{b}
4			3.6 ± 0.2^{b}	3.0 ± 0.1^{b}	3.3 ± 0.3^{b}	3.4 ± 0.3^{b}
8			4.5 ± 0.3^{c}	3.9 ± 0.3^{c}	4.0 ± 0.3^{c}	4.2 ± 0.4^{c}
Catalase (μmol/min/mg protein)						
2	8.5 ± 0.7^{a}	2.5 ± 0.2^{b}	2.8 ± 0.1^{b}	2.5 ± 0.1^{b}	2.6 ± 0.2^{b}	3.55 ± 0.1^{c}
4			3.2 ± 0.2^{b}	2.8 ± 0.1^{b}	3.1 ± 0.1^{b}	5.78 ± 0.1^{c}
8			4.0 ± 0.2^{c}	3.2 ± 0.2^{b}	3.7 ± 0.2^{c}	5.7 ± 0.4^{d}
Glutathione Transferase (μmol/min/mg protein)						
2	50.2 ± 3.5^{a}	25.9 ± 2.1^{b}	26.3 ± 1.1^{b}	26.0 ± 1.5^{b}	26.1 ± 1.2^{b}	26.1 ± 1.4^{b}
4			32.7 ± 1.5^{b}	28.3 ± 1.9^{b}	30.5 ± 1.8^{b}	38.2 ± 3.1^{c}
8			40.1 ± 3.3^{c}	39.8 ± 2.1^{c}	37.2 ± 2.9^{c}	45.9 ± 3.4^{a}
Glutathione (mg/ml)						
2	52.5 ± 3.3^{a}	25.8 ± 1.9^{b}	28.2 ± 2.1^{b}	26.1 ± 2.0^{b}	27.5 ± 1.5^{b}	27.2 ± 2.1^{b}
4			37.4 ± 2.9^{c}	35.8 ± 2.1^{c}	33.9 ± 3.1^{b}	36.2 ± 3.2^{c}
8			44.6 ± 3.2^{c}	45.7 ± 2.9^{c}	38.9 ± 3.2^{c}	45.9 ± 4.1^{c}

Data represent mean ± SD (n=5). Values within a row between groups not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

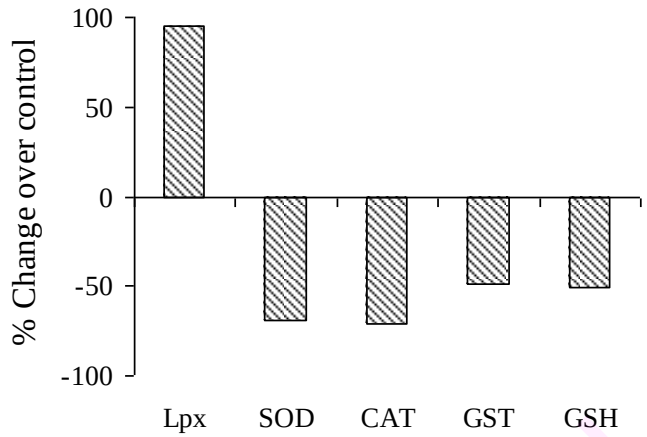


Figure 7.5. Percent increase in lipid peroxides level, decrease in the activity of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and reduced glutathione levels (GSH) in liver microsomes of RD group as compared to control group.

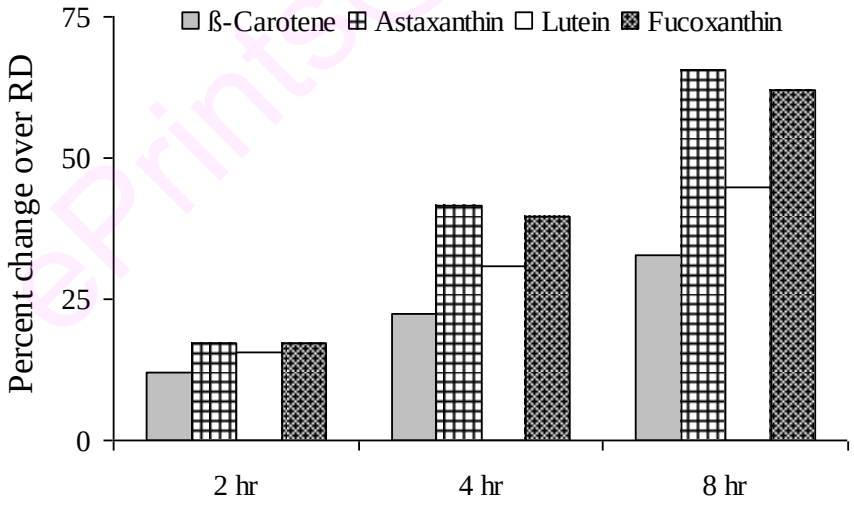


Figure 7.6. Percent decrease in lipid peroxides level in liver microsomes of rats gavaged with carotenoids as compared to RD group.

Table 7.5. Percent increase in the activity of antioxidant enzymes and reduced glutathione levels in liver microsomes of rats gavaged with carotenoids as compared to RD group.

Time (h)	β-Carotene	Astaxanthin	Lutein	Fucoxanthin
Superoxide Dismutase (U/mg protein)				
2	6.9	ND	3.6	3.6
4	25.0	10.0	18.2	20.6
8	40.0	25.0	32.5	35.7
Catalase (μmol/min/mg protein)				
2	10.7	ND	3.8	29.6
4	21.9	10.7	19.4	56.6
8	37.5	21.9	32.4	55.8
Glutathione Transferase (μmol/min/mg protein)				
2	1.5	0.4	0.8	0.8
4	20.8	8.5	15.1	32.2
8	35.4	23.4	30.4	43.6
Glutathione (mg/ml)				
2	8.5	1.1	6.2	5.2
4	31.0	16.2	23.9	28.7
8	42.2	27.7	33.7	43.8

Data represent mean (n=5), ND = not detected.

Lipid profile

Results on the effect of RD and subsequent gavages of carotenoids on the cholesterol, triglycerides and phospholipids level and fatty acid profile in liver microsomes of rats are given in Tables 7.6 and 7.7. As evidenced in plasma (Chapter 6), retinol deficiency exhibited increased saturated fatty acids (SFA, 20%) and decreased mono- (MUFA, 43%) and poly- (PUFA, 60%) unsaturated fatty acids as compared to control (SFA-66%, MUFA-17.9%, PUFA-5.3% of total fatty acid) (Figure 7.7). On gavage with β-carotene, astaxanthin, lutein and fucoxanthin, there was a decrease in SFA by 0.2-15, 0.4-27, 0.2-20, 20-26% and increase in MUFA by 6-21, 5-26, 5-24, 26-33 % and PUFA by 5-50, 16-57, 16-53, 28-53% (Table 7.8). Further, the cholesterol: phospholipid ratio (Table 7.7) was significantly ($p<0.05$) lower in control group (0.69) as compared to RD group (0.92). Whereas, on gavage with β-carotene, astaxanthin, lutein and fucoxanthin, the range of the decrease in the cholesterol: phospholipid ratio was ranged as 0.79-0.72, 0.89-0.78 and 0.94-0.79, 0.85-0.77 respectively over 8h as compared to RD group.

Table 7.6. Effect of retinol deficiency and subsequent carotenoid gavages on fatty acids (%) in liver microsomes of rats.

Group	Time (h)	12:0	13:0	14:0	15:0	16:0	18:0	16:1	18:1	18:2	SFA	MUFA	PUFA
Control	-	1.9	21.1	3.9	1.9	27.7	9.5	4.6	13.3	5.3	66.0 [a]	17.9 [a]	5.3 [a]
RD	0	3.5	30.1	4.9	2.6	30.8	10.3	2.1	8.2	2.1	82.2 [b]	10.3 [b]	2.1 [b]
β-Carotene	2	3.5	30.0	4.8	2.5	30.7	10.5	2.0	8.9	2.2	82.0 [b]	10.9 [b]	2.2 [b]
	4	3.2	28.5	4.6	2.4	28.5	10.1	2.5	9.4	3.0	77.3 [b]	11.9 [c]	3.0 [b]
	8	2.8	25.3	4.1	2.2	25.9	9.6	3.1	9.9	4.2	69.9 [a]	13.0 [c]	4.2 [c]
Astaxanthin	2	3.4	29.5	4.9	2.8	30.9	10.4	2.2	8.6	2.5	81.9 [b]	10.8 [b]	2.5 [b]
	4	3.0	26.1	4.2	2.5	25.7	9.6	3.0	9.5	3.5	71.1 [a]	12.5 [c]	3.5 [c]
	8	2.2	21.8	3.5	2.0	22.1	8.5	3.8	10.2	4.9	60.1 [a]	14 [c]	4.9
Lutein	2	3.5	29.9	4.8	2.7	30.8	10.3	2.0	8.8	2.5	82.0 [b]	10.8 [b]	2.5 [b]
	4	3.1	27.6	4.4	2.6	28.3	9.4	3.1	9.2	3.6	75.4 [b]	12.3 [c]	3.6 [c]
	8	2.5	23.8	3.9	2.2	24.9	8.8	3.5	10.0	4.5	66.1 [a]	13.5 [c]	4.5 [c]
Fucoxanthin	2	3.2	22.5	5.3	2.7	23.9	8.5	4.3	9.7	2.9	66.0 [a]	14.0 [c]	2.9 [b]
	4	2.9	21.7	4.3	2.0	28.3	9.3	2.8	12.5	4.5	68.5 [a]	15.3 [c]	4.5 [c]
	8	3.0	22.0	3.8	2.3	21.7	8.3	2.3	10.2	3.4	61.1 [a]	12.5 [c]	3.4 [c]

Data represents mean (n=5). Values within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. SFA = saturated fatty acids, MUFA = mono- unsaturated fatty acids, PUFA = poly-unsaturated fatty acids.

Table 7.7. Effect of retinol deficiency and carotenoid gavages on cholesterol, phospholipid and triglyceride levels and cholesterol: phospholipid ratio in liver microsomes of rats.

Time (h)	Groups					
	Control	RD	β-Carotene	Astaxanthin	Lutein	FUCO
Cholesterol (mg/ml)						
2h	1.5 ± 0.1^{a}	1.2 ± 0.1^{b}	1.5 ± 0.1^{a}	1.6 ± 0.1^{a}	1.7 ± 0.1^{a}	1.7 ± 0.2^{a}
4h			1.6 ± 0.1^{a}	1.8 ± 0.2^{c}	1.6 ± 0.1^{b}	1.9 ± 0.2^{d}
8h			1.8 ± 0.1^{c}	1.8 ± 0.1^{c}	1.9 ± 0.1^{d}	2.0 ± 0.2^{d}
Phospholipid (mg/ml)						
2h	2.2 ± 0.2^{a}	1.3 ± 0.1^{b}	1.9 ± 0.1^{a}	1.8 ± 0.1^{a}	2.1 ± 0.2^{a}	2.0 ± 0.1^{a}
4h			2.0 ± 0.1^{a}	2.1 ± 0.2^{a}	2.03 ± 0.1^{a}	2.3 ± 0.2^{a}
8h			2.5 ± 0.2^{a}	2.3 ± 0.2^{a}	2.4 ± 0.1^{a}	2.6 ± 0.2^{c}
Triglycerides (mg/ml)						
2h	1.9 ± 0.1^{a}	1.7 ± 0.1^{a}	1.8 ± 0.1^{a}	1.9 ± 0.2^{a}	1.8 ± 0.2^{a}	1.7 ± 0.1^{a}
4h			2.1 ± 0.1^{a}	2.3 ± 0.1^{a}	2.2 ± 0.1^{a}	2.2 ± 0.2^{a}
8h			2.5 ± 0.1^{b}	2.8 ± 0.2^{b}	2.6 ± 0.2^{b}	2.4 ± 0.2^{b}
Cholesterol: Phospholipid						
2h	0.69^{a}	0.92^{b}	0.79^{c}	0.89^{b}	0.94^{b}	0.85^{b}
4h			0.8^{c}	0.86^{b}	0.79^{c}	0.83^{c}
8h			0.72^{a}	0.78^{c}	0.79^{c}	0.77^{c}

Data represent mean $\pm$ SD (n=5). Values within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

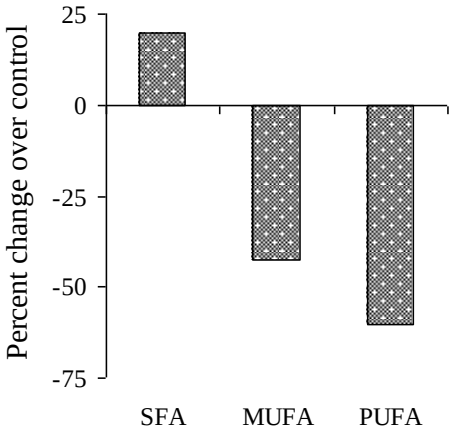


Figure 7.7. Percent increase in saturated fatty acids (SFA) and decrease in mono- and poly-unsaturated fatty acids (MUFA, PUFA) as a result of retinol deficiency over control.

Table 7.8. Percent decrease in saturated fatty acids (SFA) and increase in mono- and poly-unsaturated fatty acids (MUFA, PUFA) as a result of carotenoid gavages as compared to RD group.

Group	Time (h)	SFA	MUFA	PUFA
β-Carotene	2	0.2	5.5	4.5
	4	6.0	13.4	30.0
	8	15.0	20.8	50.0
Astaxanthin	2	0.4	4.6	16.0
	4	13.5	17.6	40.0
	8	26.9	26.4	57.1
Lutein	2	0.2	4.6	16.0
	4	8.3	16.3	41.7
	8	19.6	23.7	53.3
Fucoxanthin	2	19.7	26.4	27.6
	4	16.7	32.5	53.3
	8	25.7	17.3	38.2

Data represent mean (n=5).

Repeated dose study

The typical HPLC chromatograms of carotenoids extracted from liver microsomes of rats intubated with carotenoids for 15-days are shown in Figure 7.8. The retinol levels in the liver microsomes of are given in Table 7.9. The results showed that the retinol levels in liver microsomes were significantly lower ($p<0.05$) in RD group (0.8 nmol/ml) as compared to control (66.5 nmol/ml). Retinol level in microsomes was higher ($p<0.05$) after gavages with β-carotene and astaxanthin for 7-days (31, 5.5 nmol/ml) and 15-days (93.4, 15.4 nmol/ml) respectively as compared to RD group. Whereas, no significant difference ($p>0.05$) was found in retinol levels in microsomes of lutein group (1, 2.1 nmol/ml) as compared to RD (Table 7.9).

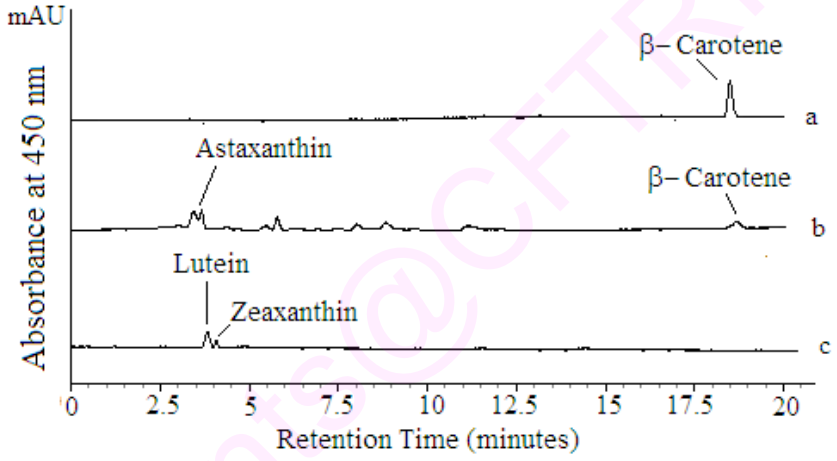


Figure 7.8. HPLC chromatograms of carotenoids extracted from liver microsomes of RD rats gavaged with β-carotene (a), astaxanthin (b) and lutein (c) for 15-days.

The levels of β-carotene, astaxanthin and lutein in liver microsomes are presented in Table 7.10. The HPLC results revealed that after 7- and 15-days gavages, β-carotene levels were 0.12 and 0.75 nmol/ml. The astaxanthin and lutein levels were 0.27, 0.86 nmol/ml and 0.47 and 0.91 nmol/ml respectively. Further, interestingly, the results showed that there was quantifiable level of β-carotene in astaxanthin group (0.094 nmol/ml) after 15-days of intubation (Table 7.10) indicating its conversion to β-carotene. This corresponded with increased retinol level in astaxanthin group.

Table 7.9. Effect of retinol deficiency and subsequent gavages with carotenoids on retinol levels (nmol/ml) in liver microsomes of rats.

Duration (days)	Groups				
	Control	RD (baseline)	β-Carotene	Astaxanthin	Lutein
7	66.5 ± 2.9^{a}	0.8 ± 0.01^{b}	31 ± 1.2^{c}	5.5 ± 0.3^{d}	1 ± 0.1^{b}
15	66.5 ± 2.9^{a}	0.8 ± 0.01^{b}	93.4 ± 10.1^{c}	15.4 ± 1.8^{d}	2.1 ± 0.1^{e}

Data represent mean $\pm$ SD (n=5). Values within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

Table 7.10. Effect of retinol deficiency and carotenoid gavages on the carotenoid levels (nmol/ml) in liver microsomes of rats

Duration (days)	Groups				
	Control	RD (baseline)	β-Carotene	Astaxanthin	Lutein
7	ND	ND	0.12 ± 0.01^{a}	$0.27 \pm 0.01^{*a}$	0.47 ± 0.02^{a}
15	ND	ND	0.75 ± 0.08^{b}	$0.86 \pm 0.09^{**b}$	0.91 ± 0.1^{b}

ND= Not detected. * β-Carotene = Not detected, ** β-Carotene = 0.094 nmol/ml

Data represent mean $\pm$ SD (n=5). Values within a column not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

Membrane bound enzymes

Results on the effects of retinol deficiency and subsequent gavages of carotenoids on the activity of membrane bound enzymes are given in Table 7.11. Activity of $Na^{+}K^{+}$-ATPase was measured for the 7-day samples while $Na^{+}K^{+}$-ATPase, Ca^{2+}-ATPase, Mg^{2+}-ATPase and acetylcholine esterase (AChE) was measured for the 15-day samples. Results revealed that $Na^{+}K^{+}$-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase were significantly higher by 69, 74 and 59 % respectively, while AChE was significantly lower by 84% as result of retinol deficiency as compared to the control group (Figure 7.9). Whereas, gavages with β-carotene, astaxanthin and lutein for 7-days brought down the $Na^{+}K^{+}$-ATPase activity by 51, 30, 11 % respectively (Figure 7.10). As in the case of 7-day intubations, gavages of β-carotene, astaxanthin and lutein for 15-

days, decreased the activity of Na^+K^+-ATPase by 60, 69, 35 %, Ca^{2+}-ATPase by 48, 65, 21%, and Mg^{2+}-ATPase by 49, 55, 42%, respectively and increased the activity of AChE by 83, 82, 76% respectively as compared to RD group (Figure 7.10).

Table 7.11. Effect of retinol deficiency and carotenoid gavages on the activity of membrane bound enzymes in liver microsomes of rats.

Duration (days)	Groups				
	Control	RD	β-Carotene	Astaxanthin	Lutein
Na^+K^+-ATPase (P_i/h/mg protein)					
7	6.8±1.4^a	22.1±1.5^b	10.8±2.4^c	15.5±3.4^d	19.6±2^b
15	6.8±1.4^a	22.1±1.5^b	10.99 ± 1.5^c	8.53 ± 0.8^a	17.89 ± 1.6^d
Ca^{2+}-ATPase (P_i/h/mg protein)					
15	4.47 ± 0.4^a	16.87 ± 1.5^b	8.75 ± 0.9^c	5.87 ± 0.6^a	13.39 ± 1.2^d
Mg^{2+}-ATPase (P_i/h/mg protein)					
15	10.29 ± 0.9^a	24.82 ± 2.4^b	12.66 ± 1.1^a	11.24 ± 0.9^a	14.31 ± 1.5^c
AChE(nmol/min/mg protein)					
15	0.62 ± 0.5^a	0.10 ± 0.01^b	0.58 ± 0.1^a	0.56 ± 0.1^a	0.42 ± 0.1^c

Data represent mean ± SD (n=5). Values within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

The data further demonstrates that the protective effect of carotenoids in ameliorating the activity of Na^+K^+ATPase was higher after 15-days than 7-days gavages. Further, the effect was more pronounced in astaxanthin (45%) and lutein (9%) groups than β-carotene (1.2%) group. In contrast, no difference was found in the activity of the enzyme between 7- and 15-days gavage of β-carotene indicating the protective effect was evidenced within 7-days unlike 15-days for astaxanthin and lutein. Thus, β-carotene was more effective in modulating the enzyme activities that had been altered by retinol deficiency. Further, astaxanthin was found to be superior to lutein in ameliorating the enzyme activities altered by retinol deficiency. Therefore, the order of effectiveness of the carotenoids on the membrane bound enzymes after gavage was β-carotene>astaxanthin>lutein.

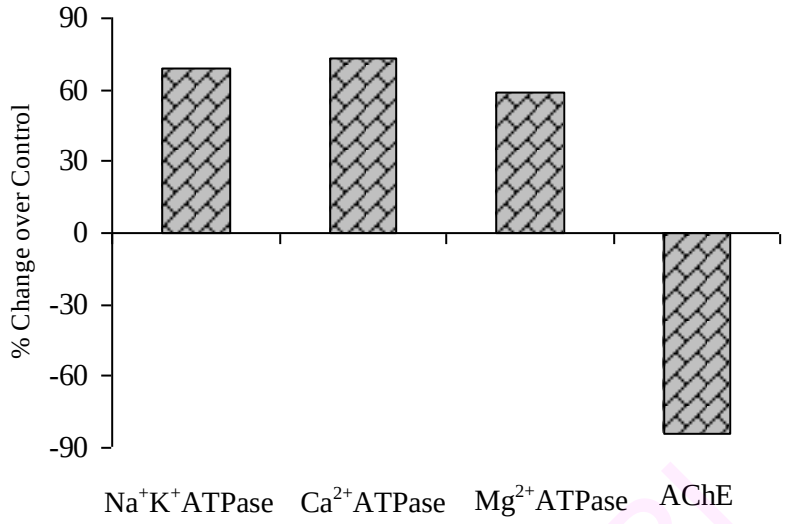


Figure 7.9. Percent increase in Na^+K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase in liver microsomes and decrease in acetylcholine esterase (AChE) in brain microsomes of RD group over control group.

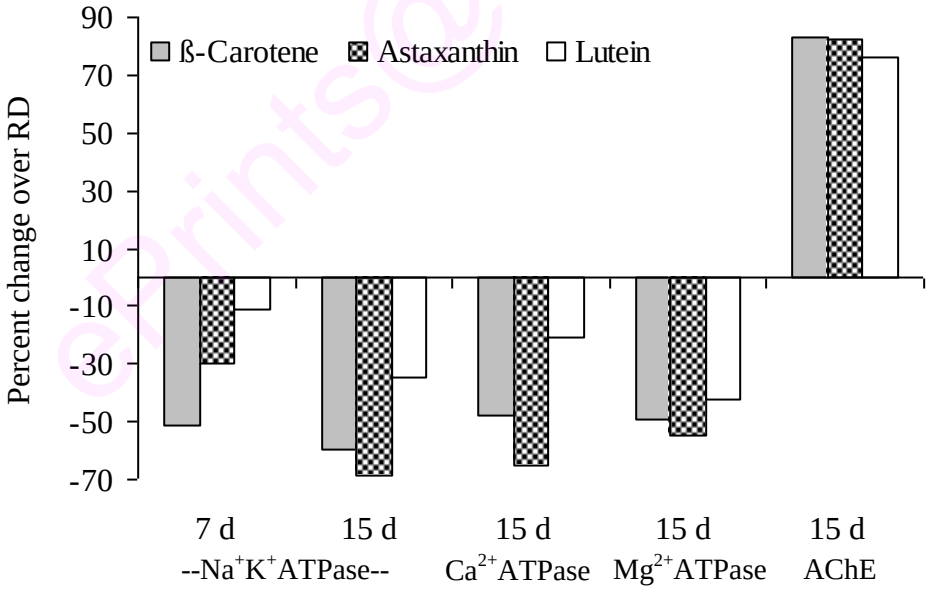


Figure 7.10. Percent decrease in Na^+K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase in liver microsomes and increase in acetylcholine esterase (AChE) in brain microsomes after gavages of carotenoids for 7-days (7 d) and 15-days (15 d) of carotenoids over retinol deficient (RD) group.

Lipid peroxidation and antioxidant molecules

Results on the effect of retinol deficiency and subsequent gavages of carotenoids on the lipid peroxidation (Lpx) and the activity of antioxidant molecules in rats are given in Figure 7.11 and Table 7.12. Results show that retinol deficiency resulted in increased Lpx by 84% and this was associated with decreased activity of superoxide dismutase (SOD) by 70%, catalase (CAT) by 73%, glutathione-S-transferase (GST) by 52% and reduced glutathione (GSH) by 62% levels as compared to control (0.07 TMP nmol/ml, 8.3 U/mg protein, 8.5 μmol/min/mg protein, 48.8 μmol/min/mg protein, 60.4 mg/ml) (Figure 7.12). Whereas, the Lpx level was decreased on intubation of β-carotene (77 and 51%), astaxanthin (84 and 86%) and lutein (77 and 58%) after 7- and 15-days (Figure 7.13).

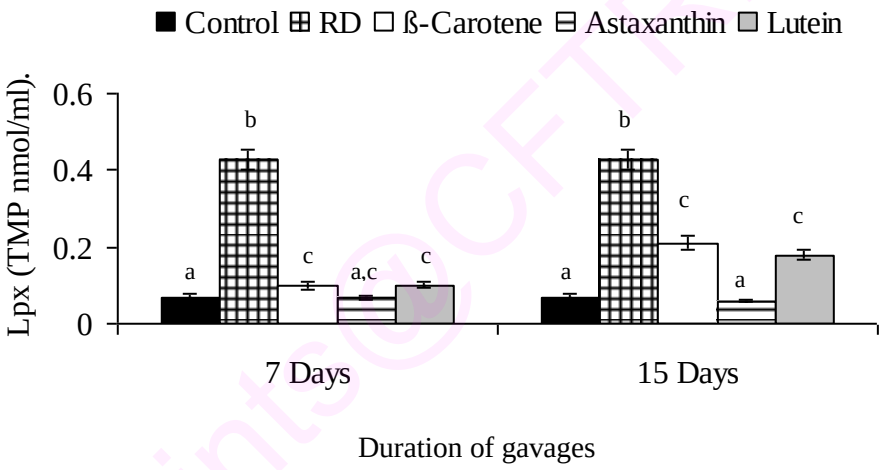


Figure 7.11. Effect of retinol deficiency and carotenoid gavage on lipid peroxides level in liver microsomes of rats. Data represent mean ± SD (n=5). Values between groups at 7- and 15-days not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

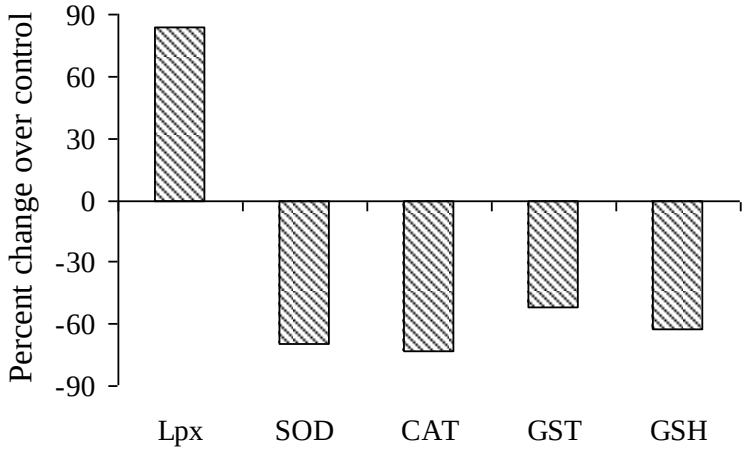


Figure 7.12. Percent increase in lipid peroxidation levels (Lpx) and decrease in the activity of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and reduced glutathione levels (GSH) in the liver microsomes of RD group as compared to control group.

Table 7.12. Effect of retinol deficiency and carotenoid gavage on the activity of antioxidant enzymes and reduced glutathione levels in liver microsomes of rats.

Duration (days)	Groups				
	Control	RD	β-Carotene	Astaxanthin	Lutein
Superoxide Dismutase (U/mg protein)					
7	8.3±0.9^{a}	2.5 ± 0.3^{b}	4.8±0.6^{c}	7.5±0.5^{a}	6.4±0.2^{d}
15	8.3±0.9^{a}	2.5 ± 0.3^{b}	5.2 ± 0.5^{c}	7.9 ± 0.7^{a}	5.8 ± 0.6^{c}
Catalase (µmol/min/mg protein)					
7	8.5 ± 0.7^{a}	2.3 ± 0.02^{b}	6.1±1^{c}	8.7±1.9^{a}	8.9±1.7^{a}
15	8.5 ± 0.7^{a}	2.3 ± 0.02^{b}	6.0 ± 0.6^{c}	7.9 ± 0.8^{a}	6.8 ± 0.7^{c}
Glutathione –S– transferase (µmol/min/mg protein)					
7	48.8±1.1^{a}	23.4 ± 2.1^{b}	43.8±1.8^{c}	48.6±2.2^{a}	47.9±2.1^{a}
15	48.8±1.1^{a}	23.4 ± 2.1^{b}	33.8 ± 3.2^{c}	48.6 ± 3.5^{a}	36.2 ± 3.1^{c}
Glutathione (mg/ml or g)					
7	60.4 ± 3.2^{a}	22.9 ± 1.1^{b}	42.3±7.3^{c}	61±13.4^{a}	62.4±12.3^{a}
15	60.4 ± 3.2^{a}	22.9 ± 1.1^{b}	36.4 ± 2.2^{c}	56.2 ± 2.9^{a}	45.5 ± 3.5^{d}

Data represent mean ± SD (n=5). Values within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test.

The decrease in Lpx after β-carotene, astaxanthin and lutein gavages for 7-days corresponded with increased activities of SOD (48, 67, 61%), CAT (62, 74, 74%), GST (47, 52, 51%) and GSH levels (46, 63, 63%) as compared to RD group (Figure 7.14). Similar to 7-days, β-carotene, astaxanthin and lutein gavages for 15-days resulted in increased activity of SOD (52, 68, 57%), CAT (62, 71, 66%), GST (31, 52, 36%) and GSH levels (37, 59, 50%) as compared to RD group (Figure 7.14). From the results it is seen that, activity of antioxidant enzymes was similar in astaxanthin and lutein groups after 7-days of gavage, and greater than β-carotene group. However, the results of the 15-day gavage study show significantly higher activity in astaxanthin group followed by lutein and β-carotene groups. This corresponded with the higher suppression of Lpx by astaxanthin, followed by lutein and β-carotene. Thus, the protective effect of the carotenoids was found to be in the order of astaxanthin>lutein>β-carotene.

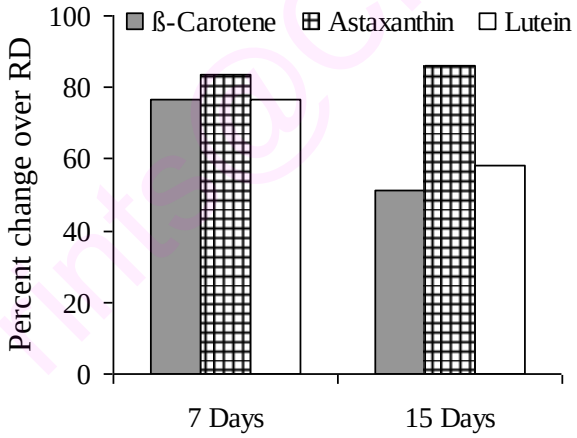


Figure 7.13. Percent decrease in lipid peroxidation levels in liver microsomes of rats gavaged with carotenoids as compared to RD group.

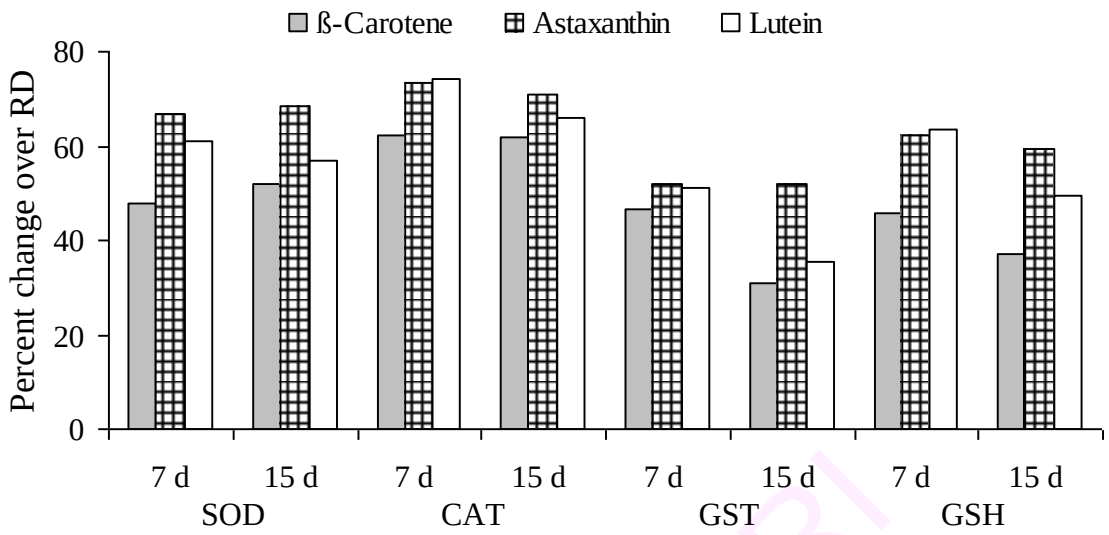


Figure 7.14. Percent increase in the activity of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and reduced glutathione levels (GSH) in the liver microsomes as a result of gavages with carotenoids for 7-days (7D) and 15-days (15 D) over retinol deficient (RD) group.

Lipid Profile

The effect of retinol deficiency and carotenoid gavages (7- and 15-days) on fatty acid profile, cholesterol, phospholipid and triglycerides levels in liver microsomes are given in Table 7.12 and 7.13. As evidenced in the single dose study, retinol deficiency increased saturated fatty acids (SFA, 27%) and decreased mono-unsaturated fatty acids (MUFA, 56%) and poly-unsaturated fatty acids (PUFA, 70%) after 7- and 15-days carotenoid gavages over control (Table 7.12, Figure 7.15). Whereas, SFA was decreased after 7- and 15-days gavages of β-carotene (8, 19%), astaxanthin (17, 26%) and lutein (5, 10%) as compared to RD group. In contrast, MUFA was increased after gavages for 7- and 15-days with β-carotene (22, 30%), astaxanthin (41, 50%) and lutein (30, 39%) as compared to RD group (Figure 7.16). Similar to MUFA, PUFA was also increased after 7- and 15-days gavages of β-carotene (46, 60%), astaxanthin (63, 69%) and lutein (46, 54%) over RD group.

The cholesterol, phospholipid and triglycerides levels in the liver microsomes are given in Table 7.13. Comparison between RD and control groups showed significantly lowered levels of cholesterol (1.1 mg/dl), phospholipids (1.2 mg/dl) and cholesterol: phospholipid ratio (0.92) in

RD group as compared to control group (1.4, 2, 1.8 mg/dl, 0.7). The results show that although 7-days gavages with β-carotene, astaxanthin and lutein exhibited increased cholesterol (1.3, 1.4, 1.4 mg/dl), phospholipid (1.8, 1.9, 1.6 mg/dl) and triglycerides (1.9, 2, 1.7 mg/dl) levels, this difference was not statistically significant. Similar to 7-days, 15-days gavage of β-carotene, astaxanthin and lutein increased cholesterol (1.4, 1.5, 1.6 mg/dl), phospholipid (2, 2.1, 2 mg/dl) and triglycerides (2.2, 2.2, 2.1 mg/dl) levels as compared to RD group, but the difference was not significant ($p>0.05$). The cholesterol: phospholipid ratio showed a significantly higher ($p<0.05$) value for the RD group (0.92) as compared to the control (0.7) group. The cholesterol: phospholipid ratio values were significantly lowered ($p<0.05$) on repeated gavages for 7- and 15-days with β-carotene (0.72, 0.7) and astaxanthin (0.74, 0.71), unlike lutein (0.88, 0.8).

Table 7.13. Effect of retinol deficiency and carotenoid gavages for 7- and 15-days on fatty acids in liver microsomes of rats.

Fatty acid (%)	Control	RD	β-Carotene		Astaxanthin		Lutein	
			7d	15d	7d	15d	7d	15d
12:0	2.0	4.9	3.5	3.0	2.9	2.2	3.8	3.5
13:0	25.0	32.0	30.5	27.5	26.1	24.3	31.1	29.9
14:0	3.5	5.2	4.1	3.3	3.8	3.2	4.5	3.1
15:0	1.5	2.7	2.5	2.1	2.1	1.8	2.2	2.3
16:0	20.0	29.0	27.3	23.0	25.1	21.1	28.2	27.6
18:0	9.0	10.1	9.4	9.2	9.7	9.5	9.7	9.2
16:1	6.1	2.0	3.9	4.5	4.9	5.9	4.5	5.1
18:1	14.2	6.9	7.5	8.2	10.2	11.9	8.1	9.5
18:2	9.3	2.8	5.2	7.0	7.5	9.1	5.2	6.1
SFA	61.0^{a}	83.86^{b}	77.3^{c}	68.1^{a}	69.7^{a}	62.1^{a}	79.5^{c}	75.6^{c}
MUFA	20.3^{a}	8.85^{b}	11.4^{c}	12.7^{c}	15.1^{c}	17.8^{a}	12.6^{c}	14.6^{c}
PUFA	9.3^{a}	2.8^{b}	5.2^{c}	7.0^{a}	7.5^{a}	9.1^{a}	5.2^{c}	6.1^{c}

Data represent mean (n=5). Values within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. d = days, RD = retinol deficient. SFA = saturated fatty acids, MUFA = mono-unsaturated fatty acids, PUFA = poly-unsaturated fatty acids.

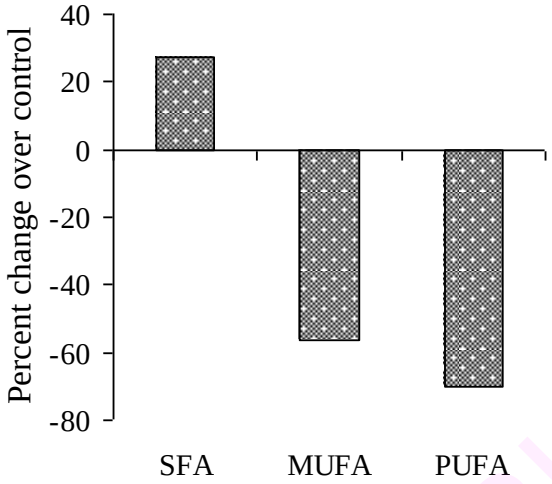


Figure 7.15. Percent increase in saturated fatty acids (SFA) and decrease in mono- and poly-unsaturated fatty acids (MUFA, PUFA) as a result of retinol deficiency over control.

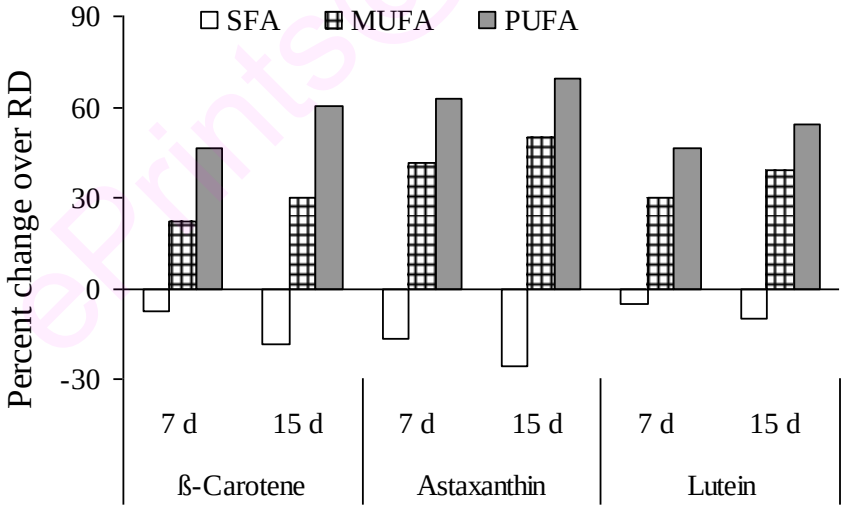


Figure 7.16. Percent decrease in saturated fatty acids (SFA) and increase in mono- and poly-unsaturated fatty acids (MUFA, PUFA) as a result of carotenoid gavage for 7-days (7 d) and 15-days (15 d) as compared to retinol deficient (RD) group.

Table 7.14. Effect of retinol deficiency and carotenoid gavages on cholesterol, phospholipid and triglyceride levels in liver microsomes of rats.

Duration (days)	Groups				
	Control	RD	β-Carotene	Astaxanthin	Lutein
Cholesterol (mg/ml)					
7	1.4 ± 0.1 [a]	1.1 ± 0.11 [b]	1.3 ± 0.10 [a]	1.4 ± 0.13 [a]	1.4 ± 0.12 [a]
15	1.4 ± 0.1 [a]	1.1 ± 0.11 [b]	1.4 ± 0.10 [a]	1.5 ± 0.15 [a]	1.6 ± 0.13 [a]
Phospholipid (mg/ml)					
7	2.0 ± 0.2 [a]	1.2 ± 0.10 [b]	1.8 ± 0.12 [a]	1.9 ± 0.11 [a]	1.6 ± 0.18 [a]
15	2.0 ± 0.2 [a]	1.2 ± 0.10 [b]	2.0 ± 0.11 [a]	2.1 ± 0.15 [a]	2.0 ± 0.1 [a]
Triglycerides (mg/ml)					
7	1.8 ± 0.1 [a]	1.5 ± 0.12 [a]	1.9 ± 0.12 [a]	2.0 ± 0.15 [a]	1.7 ± 0.15 [a]
15	1.8 ± 0.1 [a]	1.5 ± 0.12 [a]	2.2 ± 0.11 [a]	2.2 ± 0.13 [a]	2.1 ± 0.14 [a]
Cholesterol : Phospholipid ratio					
7	0.70 [a]	0.92 [b]	0.72 [a]	0.74 [a]	0.88 [b]
15	0.70 [a]	0.92 [b]	0.70 [a]	0.71 [c]	0.80 [b]

Data represent mean ± SD (n=5). Values within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. RD = retinol deficient.

Dietary Study

Typical HPLC chromatograms of carotenoids extracted from liver microsomes of carrot powder, astaxanthin and dill leaf powder fed groups are shown in Figure 7.17. Retinol and carotenoid levels in liver microsomes are given in Figure 7.18 and Table 7.14. Data shows that retinol deficiency resulted in depleted ($p<0.05$) retinol levels (nmol/ml) in liver microsomes of RD group (2.01) as compared to control (602.7) group. Whereas, feeding carrot powder, astaxanthin and dill leaf powder, resulted in significant ($p<0.05$) increase in retinol levels (70.2, 5.7. 50.3 nmol/ml) when compared to RD group. Amongst the groups, carrot powder fed group showed higher levels of retinol followed by dill leaf powder and astaxanthin fed group. The carotenoid profiles of liver microsomes of control and RD groups did not show any carotenoids unlike experimental groups, confirming that the carotenoids present were resultant of the diet fed with carotenoids. The liver microsomes of carrot powder fed group showed detectable levels of β-, α-carotene and lutein (95.5, 34.4, 47.7 pmol/ml) while astaxanthin fed group showed presence

of astaxanthin and β-carotene (69.3, 2.5 pmol/ml) and dill leaf fed group had β-carotene and lutein (23.7, 78.7 pmol/ml).

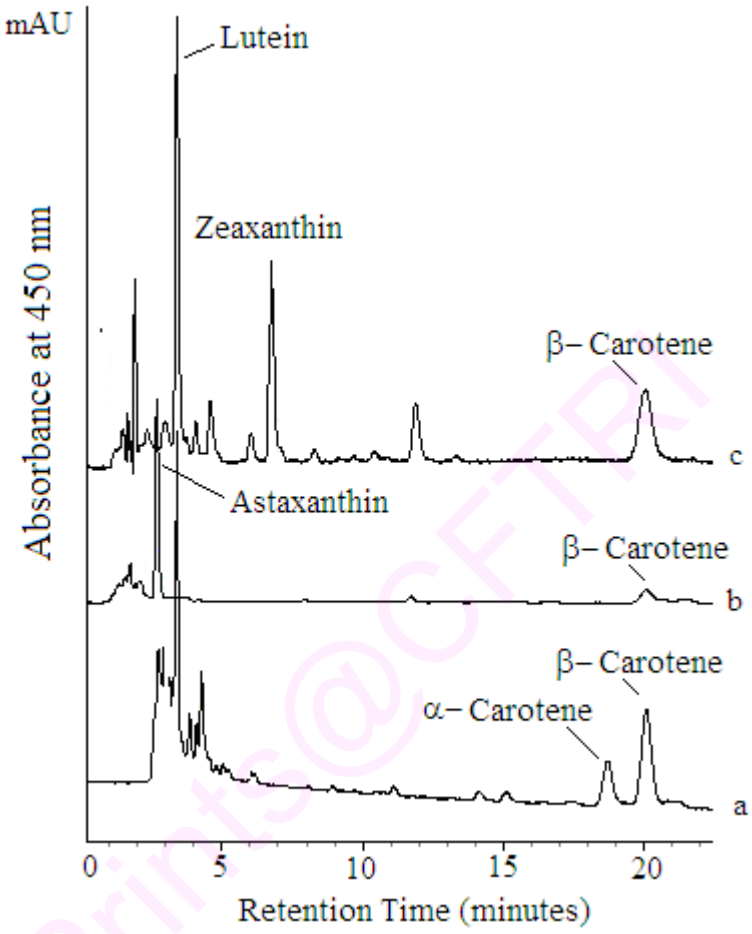


Figure 7.17. HPLC chromatograms of carotenoids extracted from the liver microsomes of RD rats fed diet supplemented with carrot powder (a), astaxanthin (b) and dill leaf powder (c) for 20 days. The presence of β-carotene in dill leaf powder fed group may be from the dietary source and in the case of astaxanthin fed group, it may be metabolized from astaxanthin.

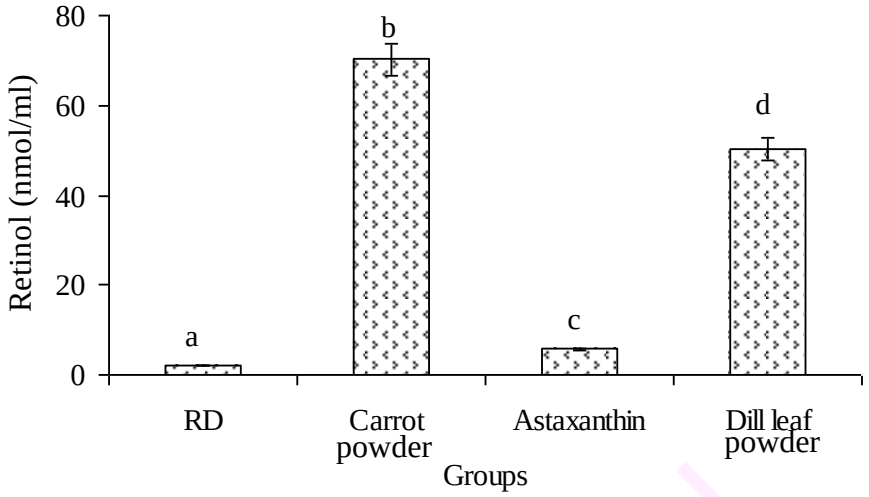


Figure 7.18. Effect of retinol deficiency and feeding carrot powder, astaxanthin or dill leaf powder supplemented diet for 20 days on retinol levels (nmol/ml) in liver microsomes of rats. Data represent mean ± SD (n=5). Values not sharing a common letter among the groups are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. Retinol level in control group = 602.73 ± 10.5 nmol/ml. RD = Retinol deficient group (baseline).

Table 7.15. Effect of retinol deficiency and feeding carrot powder, astaxanthin or dill leaf powder supplemented diet for 20 days on carotenoid levels (pmol/ml) in liver microsomes of rats.

Carotenoid detected	Groups				
	Control	RD (baseline)	Carrot	Astaxanthin	Dill leaf
β-Carotene	ND	ND	95.5 ± 2.4	2.5 ± 0.1	23.7 ± 1.6
α-Carotene	ND	ND	34.4 ± 1.2	ND	ND
Astaxanthin	ND	ND	ND	69.29 ± 7.2	ND
Lutein	ND	ND	47.7 ± 1.3	ND	78.7 ± 3.1

Data represent mean ± SD (n=5). ND = Not detected. Detectable limit under the HPLC conditions used = 1 pg.

Membrane bound enzymes

Results on the membrane bound $Na^{+}K^{+}$-ATPase, Ca^{2+}-ATPase, Mg^{2+}-ATPase in liver microsomes of RD rats fed diet containing carrot powder, astaxanthin and dill leaf powder and

acetylcholine esterase (AChE) in brain microsomes are given in Table 7.15. The results show that retinol deficiency resulted in increased ($p<0.05$) activity of Na^+K^+-ATPase (76%), Ca^{2+}-ATPase (75%), Mg^{2+}-ATPase (67%) in liver microsomes and decreased activity of AChE (73%) in brain microsomes as compared to control group (5.8, 4.6, 8.4 P_i/h/mg protein, 0.75 nmol/min/mg protein) (Table 7.15, Figure 7.19). Feeding diet supplemented with carrot powder, astaxanthin and dill leaf powder, decreased the activities of Na^+K^+-ATPase (73, 50, 70%), Ca^{2+}-ATPase (73, 41, 70%), Mg^{2+}-ATPase (63, 45, 61%) and increased the activity of AChE by 72, 60, 71% (Figure 7.20). Results showed that feeding carotenoid sources in the diet resulted in significantly decreased activities of the ATPases and increased AChE activity as compared to RD group. Amongst the experimental groups, the decrease in the activities of ATPases and increase in AChE activity was superior in carrot powder (β-carotene) and dill leaf powder (lutein) than astaxanthin fed group ($p<0.05$).

Table 7.16. Effect of retinol deficiency and feeding diet supplemented with carrot powder, astaxanthin and dill leaf powder for 20 days on the activity of membrane bound ATPases (P_i/h/mg protein) in liver microsomes and acetylcholine esterase (AChE, nmol/min/mg protein) in brain microsomes of rats.

Enzyme	Control	RD	Carrot	Astaxanthin	Dill leaf
Na^+K^+-ATPase	5.8 ± 1.2^a	23.9 ± 1.8^b	6.5 ± 0.4^a	11.2 ± 1.0^c	7.2 ± 0.5^a
Ca^{2+}-ATPase	4.6 ± 0.6^a	18.03 ± 1.1^b	4.9 ± 0.9^a	9.6 ± 0.6^c	5.2 ± 1.3^a
Mg^{2+}-ATPase	8.4 ± 0.7^a	25.6 ± 2.1^b	9.4 ± 0.6^a	14.2 ± 1.1^c	10.1 ± 1.0^a
AChE	0.75 ± 0.4^a	0.2 ± 0.01^b	0.71 ± 0.04^a	0.50 ± 0.01^c	0.68 ± 0.03^a

Data represent mean $\pm$ SD (n=5). Values within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. RD= retinol deficient.

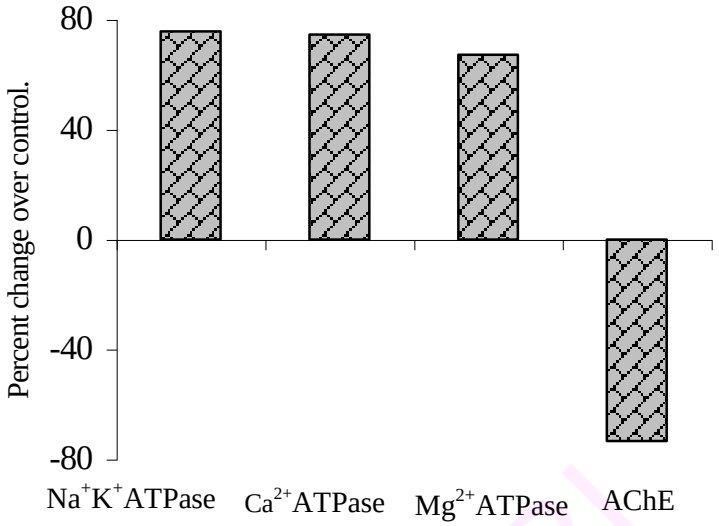


Figure 7.19. Percent increase in Na^+K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase in liver microsomes and decrease in acetylcholine esterase (AChE) in brain microsomes of retinol deficient group over control group.

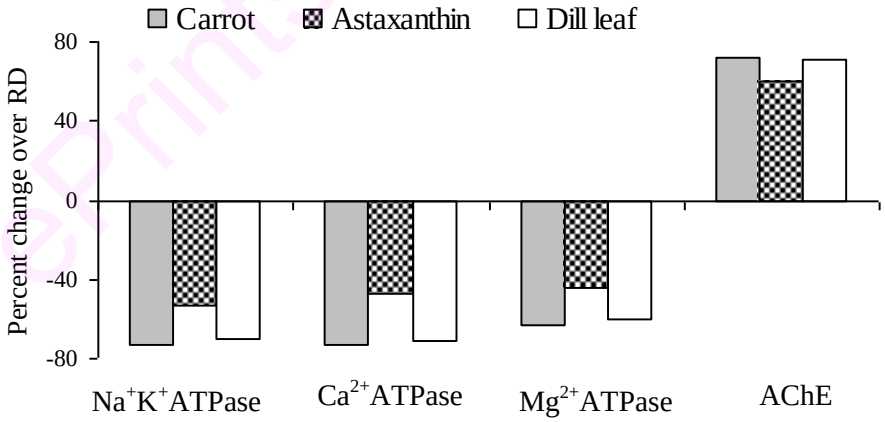


Figure 7.20. Percent decrease in Na^+K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase in liver microsomes and increase in acetylcholine esterase (AChE) in brain microsomes after feeding diet supplemented with carrot powder, astaxanthin and dill leaf powder for 20 days over retinol deficient (RD) group.

Lipid peroxides and antioxidant molecules

Results on the lipid peroxides (Lpx) level and activities of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione (GSH) levels in the experimental, control and RD groups are given in Table 7.16. Retinol deficiency was found to significantly increase ($p<0.05$) the Lpx by 86% and decrease ($p<0.05$) the activity of SOD (61%), CAT (70%), GST (55%) and GSH levels (63%) as compared to control (0.08 TMP nmol/ml, 8 U/mg protein, 8.2, 45.6 μmol/min/mg protein and 56.5 mg/ml) (Table 7.16, Figure 7.21). In contrast, on feeding diet supplemented with carrot powder, astaxanthin and dill leaf powder for 20 days, there was a decrease ($p<0.05$) in the Lpx by 84, 86, and 86 % over RD group (Table 7.16, Figure 7.22). In addition, increased ($p<0.05$) activities of SOD (61, 64, 61%), CAT (69, 71, 72%), GST (55, 57, 56%) and GSH levels (63, 65, 66%) were observed on feeding carrot powder, astaxanthin, and dill leaf powder supplemented diets (Figure 7.22) for 20 days. On comparing the results, it is seen that retinol deficiency resulted in significantly ($p<0.05$) higher Lpx and lower activities of SOD, CAT, GST and GSH levels. Feeding diets supplemented with carrot powder, astaxanthin and dill leaf powder resulted in decreased ($p<0.05$) Lpx and increased ($p<0.05$) activities of SOD, CAT, GST and GSH levels as compared to RD group. The effect of carrot powder, astaxanthin and dill leaf powder was similar.

Table 7.17. Effect of retinol deficiency and feeding diet supplemented with carrot powder, astaxanthin and dill leaf powder for 20 days on lipid peroxides level, activity of antioxidant enzymes and glutathione levels in liver microsomes of rats.

Control	RD (baseline)	Carrot powder	Astaxanthin	Dill leaf powder
Lipid peroxidation TMP nmol/ml				
0.08 ± 0.01^{a}	0.56 ± 0.03^{b}	0.09 ± 0.01^{a}	0.08 ± 0.01^{a}	0.08 ± 0.01^{a}
Superoxide dismutase (U/mg protein)				
8.0 ± 0.7^{a}	3.1 ± 0.2^{b}	7.9 ± 0.8^{a}	8.5 ± 0.6^{a}	8.0 ± 0.8^{a}
Catalase (μmol/min/mg protein)				
8.2 ± 0.7^{a}	2.5 ± 0.2^{b}	8.0 ± 0.5^{a}	8.5 ± 0.5^{a}	8.8 ± 0.2^{a}
Glutathione-S-transferase (μmol/min/mg protein)				
45.6 ± 3.2^{a}	20.5 ± 2.0^{b}	45.9 ± 2.6^{a}	47.4 ± 3.4^{a}	46.9 ± 3.5^{a}
Glutathione (mg/ml)				
56.5 ± 5.2^{a}	21.1 ± 1.4^{b}	57.3 ± 5.3^{a}	60.1 ± 2.8^{a}	61.8 ± 6.2^{a}

Data represent mean $\pm$ SD (n=5). Values within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. RD = retinol deficient.

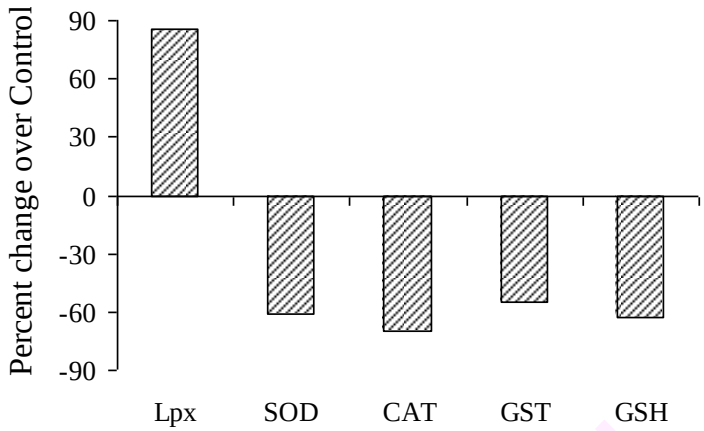


Figure 7.21. Percent increase in lipid peroxidation (Lpx) and decrease in activity of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione (GSH) levels in liver microsomes of rats as a result of retinol deficiency as compared to control group.

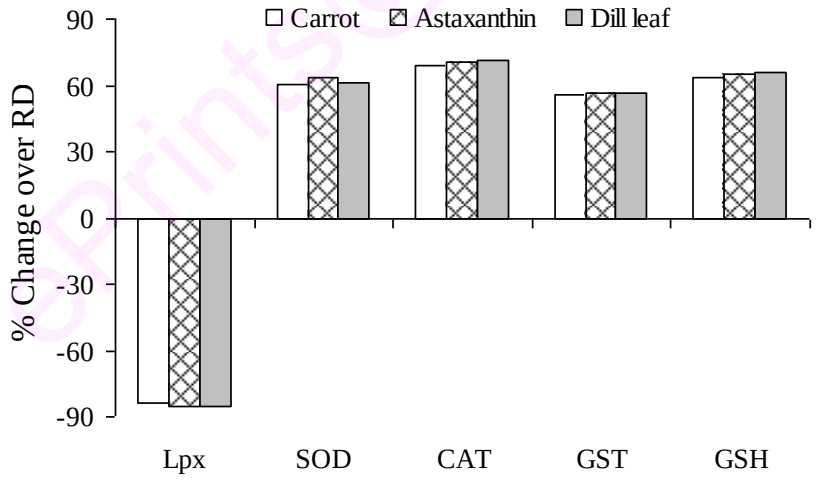


Figure 7.22. Percent decrease in lipid peroxidation (Lpx) and increase in activity of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione (GSH) levels in liver microsomes as a result of feeding carrot, astaxanthin and dill leaf for 20 days as compared to retinol deficient (RD) rats.

Lipid profile

Fatty acid profile, cholesterol, phospholipid and triglycerides in liver microsomes of the control, RD and experimental groups are presented in Table 7.17 and 7.18. The results showed that retinol deficiency increased ($p<0.05$) the saturated fatty acids (SFA) by 33% and decreased the mono- and poly-unsaturated (MUFA and PUFA) fatty acids by 60 and 68% respectively over control (58.6, 21.7, 9.6% of total fatty acids) group (Table 7.17, Figure 7.23). Whereas, feeding carrot powder, astaxanthin and dill leaf powder supplemented diets for 20 days ameliorated the effects of retinol deficiency with decreased ($p<0.05$) SFA (32, 29, 32%) and increased MUFA (60, 58, 59%) and PUFA (67, 66, 66%) (Table 7.17, Figure 7.24).

Table 7.18. Effect of retinol deficiency and feeding carrot powder, astaxanthin and dill leaf powder on fatty acid profile of liver microsomes of rats.

Fatty acid	Groups				
	Control	RD	Carrot powder	Astaxanthin	Dill leaf powder
12:0	2.5	5.1	2.6	2.8	2.5
13:0	22.0	33.2	21.8	22.2	22.0
14:0	3.1	5.5	3.0	3.4	3.2
15:0	1.4	2.9	1.5	1.8	1.4
16:0	19.5	28.9	20.1	20.7	20.0
18:0	10.1	11.5	10.2	10.8	10.2
16:1	7.2	2.3	7.5	7.0	7.1
18:1	14.5	6.5	14.5	14.0	14.2
18:2	9.6	3.1	9.5	9.0	9.1
SFA	58.6	87.1	59.2	61.7	59.3
MUFA	21.7	8.8	22	21	21.3
PUFA	9.6	3.1	9.5	9.0	9.1

Data represent mean $\pm$ SD (n=5). RD= retinol deficient, SFA= saturated fatty acids, MUFA=mono-unsaturated fatty acids, PUF= poly-unsaturated fatty acids.

Retinol deficiency also decreased the cholesterol (1.0 mg/ml) and phospholipid (1.1 mg/ml) levels than the control group (1.5, 2.2 mg/ml) while triglycerides level (mg/ml) was not significantly different ($p>0.05$) between RD (1.2) and control (1.2) groups (Table 7.18). The lipid parameters showed increased levels (mg/ml) of cholesterol, phospholipids and triglycerides over RD group as a result of feeding carrot (1.7, 2.4, 2.2), astaxanthin (1.4, 1.9, 2.0) and dill leaf (1.6, 2.21, 2.1) for 20 days, however, these differences were not significant ($p>0.05$). In contrast, the cholesterol-phospholipid ratio was significantly higher ($p<0.05$) in RD (0.91) as compared to control (0.69) group and this was ameliorated on feeding carrot (0.71), astaxanthin (0.74) and dill leaf (0.72) supplemented diets for 20 days. The results show that retinol deficiency resulted in alteration in the lipid parameters and feeding diet containing carotenoid sources ameliorated these effects.

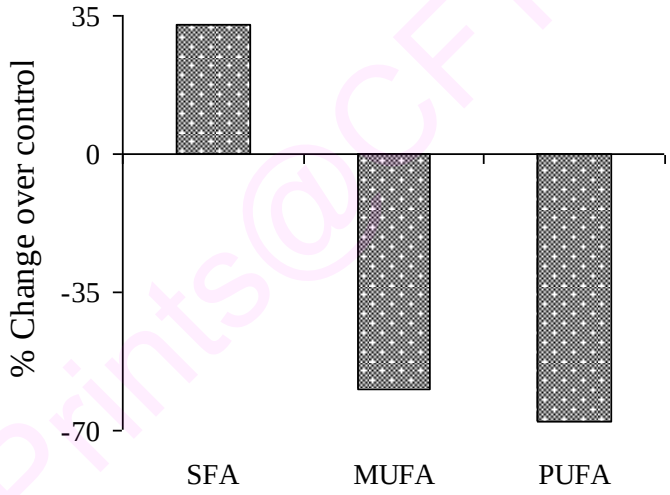


Figure 7.23. Percent increase in saturated fatty acids (SFA) and decrease in mono- and poly-unsaturated fatty acids (MUFA, PUFA) as a result of retinol deficiency over control.

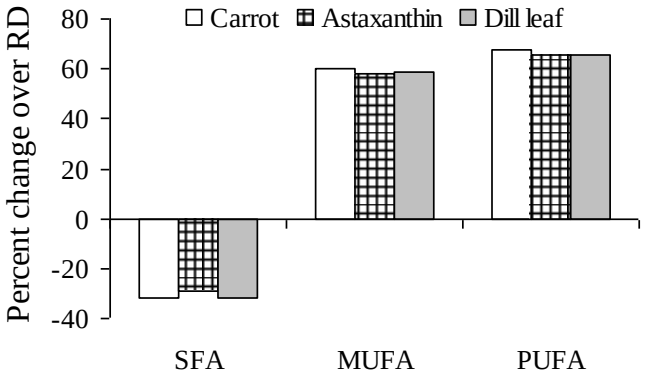


Figure 7.24. Percent decrease in saturated fatty acids (SFA) and increase in mono- and poly-unsaturated fatty acids (MUFA, PUFA) as a result of feeding carrot, astaxanthin and dill leaf supplemented diet as compared to retinol deficient (RD) group.

Table 7.19. Effect of retinol deficiency and feeding carrot powder, astaxanthin and dill leaf powder supplemented diet on cholesterol, phospholipid and triglyceride levels in liver microsomes of rats.

Control	RD	Carrot	Astaxanthin	Dill leaf
Cholesterol (mg/ml)				
1.5 ± 0.2^{a}	1.0 ± 0.1^{b}	1.7 ± 0.1^{a}	1.4 ± 0.1^{a}	1.6 ± 0.1^{a}
Phospholipid (mg/ml)				
2.16 ± 0.2^{a}	1.1 ± 0.1^{b}	2.4 ± 0.2^{a}	1.9 ± 0.1^{a}	2.21 ± 0.1^{a}
Triglyceride (mg/ml)				
1.6 ± 0.1^{a}	1.2 ± 0.1^{a}	2.2 ± 0.2^{a}	2.0 ± 0.1^{a}	2.1 ± 0.1^{a}
Cholesterol : Phospholipid ratio				
0.69^{a}	0.91^{b}	0.71^{a}	0.74^{a}	0.72^{a}

Data represent mean $\pm$ SD (n=5). Values within a row not sharing a common letter are significantly different ($p<0.05$) as determined by one-way ANOVA, followed by Tukey's test. RD= retinol deficient.

Discussion

Retinol is an essential vitamin, an integral part of the membrane of all cells and organelles and is involved in regulation of various membrane structure and functions. Therefore, its deficiency leads to altered integrity and function of the membrane. Results of this study show that retinol deficiency was associated with significant depletion of retinol levels in the liver microsomes of rats. In comparison with control, RD rats had >80 fold lower retinol levels. Gavage with single dose of β-carotene resulted in increased retinol levels while such a change was not observed in astaxanthin and lutein groups. This corresponded with elevated levels of retinol in plasma and liver (Chapter 4) and increased activity of the intestinal monooxygenase (Chapter 6) that cleaves β-carotene to retinol, in β-carotene fed group but not in astaxanthin and lutein groups. Repeated gavages of β-carotene and astaxanthin resulted in increased retinol levels in liver microsomes while lutein did not show any significant change ($p>0.05$) in retinol levels. The increased retinol levels in liver microsomes of β-carotene and astaxanthin group after repeated gavages for 7- and 15-days was associated with increased retinol in plasma and liver (Chapter 4) and increased monooxygenase activity (Chapter 6) of these groups. Dietary feeding of purified astaxanthin, carrot as a source of β-carotene and dill leaf as a source of lutein resulted in increased retinol levels in liver microsomes of all the groups, with carrot and dill leaf fed groups showing significantly higher level of retinol as compared to astaxanthin. The reason for the increase in retinol levels of dill leaf group may have been due to the presence of β-carotene in dill leaf. Whereas, no retinol was formed when purified lutein was gavaged to RD rats, indicating that lutein is not converted to retinol. The plasma and liver retinol levels of rats fed dietary carotenoids showed a corresponding increase (Chapter 4) with the increase in its level in the liver microsomes and monooxygenase activity (Chapter 6). Therefore, it is clear from the results that retinol deficiency resulted in depleted retinol levels in the microsomes. No carotenoids were detected in the liver microsomes of control and RD (baseline) groups indicating that the carotenoids detected in the experimental groups were absorbed from the ingested carotenoids.

The results of the single dose study showed that the level of astaxanthin was higher in the microsomes, followed by lutein, fucoxanthin and β-carotene. β-Carotene was detected only in the 8h after gavage of a single dose. This probably was due to its conversion to retinol resulting in higher retinol levels in the liver microsomes. Results of the repeated dose study, on the other hand, showed higher levels of intact lutein followed by astaxanthin and β-carotene. The increased retinol levels and monooxygenase activity in the β-carotene and astaxanthin groups indicates that

they may be converted to retinol by the action of monooxygenase. This may be the reason for lower levels of β-carotene and astaxanthin in plasma, liver and microsomes. Further, results show that astaxanthin was converted to β-carotene in RD rats (Chapter 5). This may be the reason for the presence of β-carotene in the microsomes of RD rats gavaged with astaxanthin. The conversion of astaxanthin to retinol via β-carotene may explain the lower levels of astaxanthin incorporated in the microsomes. Liver microsomes of rats fed carrot powder showed α-, β-carotene and lutein while astaxanthin fed group had astaxanthin and β-carotene. In the case of dill leaf fed group, lutein and β-carotene were found to be present in the liver microsomes indicating that the ingested carotenoids are incorporated into the membrane. Lakshminarayana et al. (2008) reported no β-carotene in plasma and liver of lutein deficient rats fed fenugreek leaves that contained lutein and β-carotene. In the present study absorption of β-carotene from the dill leaf may have been efficient due to its requirement for retinol in RD rats.

Membrane bound enzymes are very sensitive to changes in the structure and integrity of the cell membrane. They are considered as indicators of the functional aspects of the membrane under stress conditions like retinol deficiency (Kaul and Krishnakantha, 1997). In this study, it was found that retinol deficiency resulted in >3 fold increase in the activity of membrane bound Na^+K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase in the liver microsomes of the RD rats as compared to control group, whereas, acetylcholine esterase (AChE) activity in the brain microsomes was significantly decreased as compared to control. Kaul and Krishnakantha (1997, 1994 and 1993) have reported increased Na^+K^+-ATPase activity in the liver microsomes of RD rats. Fujiyara et al. (1992) have also reported alteration in the membrane bound enzymes as a result of retinol deficiency in cultured rat hepatocytes. On gavages with a single dose of carotenoids, it was seen that β-carotene and fucoxanthin decreased the activity of Na^+K^+-ATPase. Astaxanthin and lutein also lowered the Na^+K^+-ATPase activity, however comparison of results showed that the effect was lower than β-carotene and fucoxanthin. Similar to the results of the single dose study, repeated gavages with β-carotene, astaxanthin and lutein resulted in decreased Na^+K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase as compared to RD with β-carotene and astaxanthin showing greater suppression of the activity of the ATPases. On the other hand, AChE activity was increased in brain microsomes on feeding carotenoids as compared to RD. The results of single and repeated dose study demonstrate that the effect of carotenoids on modulation of membrane bound enzymes that were altered by retinol deficiency was almost similar. Whereas, the ameliorative effect of dietary feeding of carotenoids (carrot and dill leaf) for 20 days on membrane bound enzymes was higher than in single and repeated dose studies and comparable

with control. The reason for lower activity of ATPases and higher activity of AchE may be due to the synergistic effect of carotenoids absorbed from dietary sources as well as the newly formed retinol from β-carotene. Whereas, astaxanthin group exhibited comparatively lower effect on modulation of the membrane bound enzymes than carrot and dill leaf. This may be due to lower retinol levels in the microsomes of astaxanthin group. Thus, retinol deficiency resulted in increased activity of the ATPases, in particular Na^+K^+-ATPase, and decreased activity of AChE. This effect was ameliorated on feeding carotenoids. The effect on the modulation of the membrane bound enzymes was higher for β-carotene (decrease in Na^+K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase and increase in AChE activity). Kaul and Krishnakantha (1997, 1994) and Jessie and Krishnakantha (2005) reported a similar result by feeding curcumin/ turmeric and saffron to retinol deficient and sufficient rats. They have reported that although curcumin may not mimic the effects of retinol, it can protect the membranes against oxidative stress caused by retinol deficiency (Kaul and Krishnakantha, 1997). Therefore, besides retinol, carotenoids may provide protection to membranes and their structure and function by positively altering their anti-oxidant molecules.

Results show that retinol deficiency resulted in increased Lpx in the liver microsomes of rats. Anzulovich et al. (2000), Oliveros et al. (2000) and Kaul and Krishnakantha (1997, 1994) have also reported increased Lpx at membrane level as a result of retinol deficiency in rats. Anzulovich et al. (2000) have reported that retinol being an integral part of the membrane acts as an antioxidant and its depletion results in increased oxidative stress. The suppression of Lpx by carotenoids in the present study is further corroborated by the enhanced activity of antioxidant molecules. Vitamins and carotenoids are known to scavenge peroxides through up-regulation of the activities of antioxidant enzymes (Fang et al. 2002). The increased Lpx was associated with decreased antioxidant enzyme activities and GSH levels in retinol deficiency. Feeding single or repeated doses and dietary carotenoids resulted in decreased Lpx in the liver microsomes. In these experiments, β-carotene was found to cleave to retinol and its feeding was associated with decreased Lpx and increased activity of SOD, CAT, GST and GSH levels. Astaxanthin was also converted to retinol resulting in its higher levels in liver microsomes after repeated dose and dietary studies. Although astaxanthin was not converted to retinol after a single dose, it was found to suppress Lpx and increase activities of antioxidant enzymes. On comparison between the carotenoids, it was found that astaxanthin exerted higher antioxidant potential. This was followed by fucoxanthin and lutein. Other researchers have also reported that astaxanthin was more effective as an antioxidant *in vitro* as compared to other carotenoids (McNulty et al., 2007; Woodall et al., 1997).

The different antioxidant potential of carotenoids may be due to differences in their chemical structures. The number of double bonds in the polyene chain and functional groups present determine the antioxidant capacity of carotenoids (Britton, 1995). It has been reported that since xanthophylls possess polar groups on the β-ionone rings, their orientation in the membrane is different from the non-polar carotenes (McNulty et al., 2007). Carotenes are highly non-polar due to absence of polar functional groups and are aligned in the lipophilic core, parallel to the length of the membrane. Whereas, xanthophylls are arranged perpendicular or at an angle to the phospholipid bi-layer, making it possible for the xanthophylls to participate in antioxidant reactions and free radical scavenging in both the aqueous and lipid phase (McNulty et al. 2007; Gruszecki and Strzalka, 2005; Woodall et al., 1997). This may explain the greater effectiveness of the xanthophylls in suppressing Lpx and enhancing the activity of antioxidant enzymes. Amongst the xanthophylls, astaxanthin and fucoxanthin were found to possess higher antioxidant potential as compared to lutein. This may be attributed to the more number of polar functional groups in these carotenoids (2 hydroxyl and 2 keto groups in astaxanthin, 2 hydroxyl, 1 epoxide and 1 keto group).

Cellular and sub-cellular membranes are susceptible to lipid oxidation due to high concentration of polyunsaturated fatty acids and close proximity to oxygen, transition metals and peroxidases (Oliveros et al., 2000). Retinol deficiency did not alter cholesterol, phospholipid and triglycerides in the liver microsomes when compared to control. However, retinol deficiency altered the levels of saturated (SFA), mono- and poly-unsaturated (MUFA and PUFA) fatty acids. There was a significant increase in the SFA and decrease in MUFA and PUFA of RD group when compared to control. In the absence of retinol, resistance to lipid peroxidation may be diminished and hence the unsaturated fatty acid component of the membrane may be peroxidised easily. This could in turn change the membrane fluidity (Kaul and Krishnakantha, 1997). Kang et al. (2007) have reported vitamin A deficiency altered the lipid catabolic pathways in rodents. They specifically reported decreased expression of genes encoding enzymes of mitochondrial and peroxisomal-oxidation. Further, they have stated that genes involved in peroxisomal fatty acid-oxidation and fatty acid transport are differentially expressed, due to changes in fatty acid metabolism. Similar to the results of the present study, Kaul and Krishnakantha (1997) have reported decreased MUFA and PUFA as a result of retinol deficiency in rats. They have also reported in specific that fatty acids C18:1 (33.9%), C18:2 (12.1%) and C20:4 (35.3%) were decreased as a result of vitamin A deficiency. In the present study, feeding carotenoids by gavages or through diet resulted in the amelioration of altered fatty acid profile with decrease in SFA and increase in MUFA and PUFA as compared to RD group. This corresponds well with the

reduced Lpx, elevated activity of antioxidant enzymes and membrane bound enzymes. These may have been the reason for protective effect of carotenoids to the membrane components. In addition, the decrease in saturates and increase in unsaturates in carotenoids administered groups can be attributed to the inhibitory effect of carotenoids on desaturase activity resulting in increase in the content of α-linoleic acid, suggesting that the metabolism of fatty acids is affected (Grolier et al., 1991). Kaul and Krishnakantha (1997) have found a similar result with the administration of curcumin to RD rats. In the present study, gavaging and feeding carotenoid sources resulted in a non-significant increase in the levels of cholesterol, phospholipid and triglyceride both in plasma and liver microsomes as compared to RD group. However, the cholesterol-phospholipid ratio was found to be higher in RD rats as compared to control.

Fatty acid composition and cholesterol content of the membrane affects its fluidity (Kempaiah and Srinivasan, 2005). Thus, the cholesterol: phospholipid ratio, which is an index of the fluidity of the membrane, plays an important role in controlling the membrane structure and function. The cholesterol: phospholipid ratio is inversely proportional to the membrane fluidity. It was found in this study that retinol deficiency significantly increased the cholesterol: phospholipid ratio, which may affect the membrane fluidity and the activity of membrane bound enzymes. A number of membrane bound enzymes have been shown to be sensitive to changes in their fatty acid environments. It has been established that ATPases require a 'fluid' lipid environment for proper functioning (Stubbs and Smith, 1984). Decreased cholesterol-phospholipid ratio was observed on feeding either purified or dietary carotenoids. The protective effect was more pronounced in β-carotene group followed by astaxanthin and lutein groups. Niranjan and Krishnakantha (2001) have observed decreased membrane fluidity in the oxidized ghee fed rats and have speculated that it may have been because of the increased cholesterol: phospholipid ratio or increased saturated fatty acid/unsaturated fatty acid ratio, or increased lipid peroxidation which leads to increased cross-linked products resulting in reduced membrane fluidity. They have also reported that higher cholesterol to phospholipid ratio decreases membrane fluidity, as the cholesterol sterically prevents the large motion of phospholipid fatty acyl chains.

The present results suggest that retinol deficiency alters the structure and function of the membranes by altering the activity of membrane bound enzymes, antioxidant molecules, fatty acid profile, cholesterol-phospholipid ratio and increasing the lipid peroxidation in microsomes. Feeding either purified or dietary carotenoids ameliorated the biochemical changes that were caused by retinol deficiency. The beneficial effect of carotenoids with respect to membrane bound biochemical constituents was in the order of astaxanthin = fucoxanthin > lutein > β-

carotene while modulation of membrane bound enzymes was in the order β-carotene > astaxanthin > lutein. Thus, carotenoids can protect the membrane structure and function by positively modulating the membrane bound biochemical constituents.

CHAPTER 8: General Discussion and Summary

Vitamin A or retinol is an essential micronutrient that performs a multitude of physiological functions in the body including vision, cell growth and differentiation, cell mediated and non-specific defense, gene expression and transcription, glycoprotein and glycosaminoglycan synthesis, haemopoiesis, fertility and embryogenesis, bone, skeletal and teeth development, gap junction communication and antioxidant defense. Retinoids (group of compounds showing structural and functional similarity to retinol such as retinal, retinoic acid, etc.) exert a variety of effects on basic biological processes such as growth, differentiation, development and malignant transformation, in addition to receptor-induced signal transduction. In addition to preventing blindness, vitamin A is essential for maintaining the structural and functional integrity of epithelial cells and is ubiquitous in all cells, throughout the body (McLarsen and Frigg, 2001).

Vitamin A deficiency (VAD) has a profound effect on various physiological functions and its deficiency leads to anomalies and pathologies. The most apparent and immediate effect of VAD is vision impairment where retinol plays a critical role. The various pathological alterations in the eyes and vision have been classified under the collective term of Xerophthalmia that includes night blindness, conjunctival xerosis, bitot's spots, corneal xerosis, corneal ulceration/ keratomalacia, corneal scar and xerophthalmic fundus. VAD also affects other physiological attributes such as immunity, fertility, growth, oxidative stress, cell membrane structure and function. VAD results in the atrophy of sweat and mucus glands, and cause dryness of the epidermis. These effects eventually lead to decreased elasticity, cracking, ulcerations of the skin, and greatly increase the risk of infection (Ross, 1999). Retinol deficiency has pro-oxidative effect and increases the oxidative stress in rats (Anzulovich et al., 2000; Oliveros et al., 2000; Kaul and Krishnakantha 1997). Cellular and sub-cellular membranes are susceptible to lipid oxidation due to high concentration of polyunsaturated fatty acids and close proximity to oxygen, transition metals, and peroxidases. Increased lipid peroxidation due to VAD is reported to be indicative of damage to cell membrane structure-function, in turn contributing to pathological abnormalities in tissues as well (Anzulovich et al., 2000). Membrane bound ATPases and antioxidant molecules are intimately associated with the regulation of ions across the membrane and defense activities (Selvendiran and Sakthisekaran, 2004). Retinol deficiency induced oxidative stress leads to an elevated activity of Na^+K^+-ATPase and alters membrane fluidity in rat liver and kidney (Kaul and Krishnakantha, 1997). Anzulovich et al. (2000) and Oliveros et al. (2000) have reported the

change in liver histoarchitecture and heart tissue as well as elevated oxidative stress as a result of retinol deprivation. VAD was shown to affect the developmental stages of the brain (Arfaoui et al., 2009; Bavik et al. 1996). Halilagic et al. (2007) have reported that retinoids control anterior and dorsal properties in the developing forebrain of quails.

VAD has been declared as a public health problem in many countries including India (WHO, 2009), which requires immediate attention and high priority for its prevention. The Governments of various afflicted countries have undertaken preventive measures by administering synthetic vitamin A to the affected populations. The Government of India started the National Prophylaxis Programme for prevention of blindness due to VAD for infants and children. In addition, the Integrated Child Development Services (ICDS) provides supplementary nutrition with vitamin A, iron, folic acid to infants, pregnant and lactating mothers and also covers immunization for prevention of VAD caused by measles. The Reproductive and Child Health (RCH) programme deals with infants and women and provides vitamin A supplementation to children aged 6-36 months. The Kishore Shakti Yojana addresses the problems of anaemia, goitre and VAD and immunization for adolescent girls. Although these programmes have been implemented successfully, their lasting effect is limited. Hence, the recent strategy for prevention of VAD is to create awareness regarding its causes, preventive measures, inclusion of foods containing preformed vitamin A in the diet and consumption of provitamin A carotenoids. These provitamin A carotenoids are abundantly found in vegetables, leafy greens and fruits. Moreover, carotenoids have the added advantage of having other beneficial effects in addition to their role as precursors of vitamin A. Hence it is suggested to find alternative sources of vitamin A to aid in the prevention of VAD and its related disorders.

As discussed earlier, consumption of carotenoid-rich foods for prevention of VAD is recommended. This requires determination of carotenoid composition and vitamin A activity of agri-horticultural produce. In this regard, this study evaluated the carotenoid composition and vitamin A activity (as retinol equivalent (RE)) of leafy greens, medicinal plants, vegetables and algae. Green leafy vegetables were analyzed in this study and it was found that the carotenoid composition was similar with respect to the type of carotenoids, however, the concentrations of the respective carotenoids were varied. In addition, α-carotene was present in few of the leafy greens. The highest values for β-carotene were found in lamb's quarters (*C. album*), prickly amaranth (*A. spinosus*), jio (*C. benghalensis*), Indian dill leaf (*P.* sowa), colocasia leaves (*C. anti-quorum*) and amaranth leaves (keerai). The highest values for lutein content were found in lamb's quarters, jio, dill leaf, chilli leaves (*C. annuum*), *I. pestigridis,* prickly amaranth and colocasia leaves. Further, lamb's quarters, jio, dill leaf, prickly amaranth and colocasia leaves are rich

sources of both β-carotene and lutein. Concentration (mg/100g dry weight) of β-carotene and lutein in green leafy vegetables ranged between 1.5 to 120.2 and 11.7 to 185.2 respectively. Total carotenoids (xanthophylls + hydrocarbon carotenoids) ranged from 0.62 to 450.93 mg/100g dry weight with lamb's quarters having maximum concentration (450.9) and green cabbage (*B. oleracea var capitata*) having minimum level (0.62). RE values (in mg) were highest for Lamb's quarters (20) followed by jio (19), prickly amaranth (16), amaranth (keerai, 11), dill leaf (10), colocasia leaves (10) and lowest for green cabbage (0.01) and red cabbage (0.03). Aizawa and Inakuma (2007) have previously reported the β-carotene content (3.64 mg/100g fresh weight) of *A. tuberosum.* Rajyalakshmi et al. (2001) and Singh et al. (2001) reported 10.1 and 5.4 mg/100g fresh weight respectively for amaranth leaves. β-carotene content of *C. album* (120.2 mg) and *C. benghalensis* (95.7) in our study were comparable with those reported by Raju et al. (2007). Lutein content of *C. album* and *C. benghalensis* recorded in this study were comparable with those reported by Raju et al. (2007) whereas it was higher (54.13 mg/100g dry weight) in *B. diffusa.* Total provitamin A carotenoids and total xanthophylls were higher than those reported by Chanwitheesuk et al. (2005) for *A. graveolens* and *T. indicus.* The difference in the concentration of carotenoids in greens is most likely related to difference in species and variety. Differences in carotenoid levels have been reported even within the species and these can be attributed to cultivar, climate, growing conditions, seasonal changes (Aizawa and Inakuma, 2007) variety and stage of maturity of the samples used for analysis (Kimura and Rodriguez-Amaya, 2003).

In medicinal plants, the highest levels of β-carotene were recorded in butterfly pea (*C. ternatea*), thyme-leaved gratiola (*B. monnieri*) and holy basil (*O. sanctum*). The highest values for lutein content were found in butterfly pea, conch grass (*C. dactylon*) and hoary basil (*O. canum*). Thus, butterfly pea is the richest source of both lutein and β-carotene among the medicinal plants analyzed. α-Carotene mg/100g dry weight) was detected in 25 among 33 medicinal plants analyzed (0.1-15.7) and was highest in Indian borage (*C.aromaticus,* 15.73). RE values (in mg) were highest for thyme-leaved gratiola (5.7) followed by butterfly pea (5.3) and holy basil (5.3) and lowest for ginger leaf (*Z. officianale,* 0.4), bitter gourd leaf (*M. charantia,* 0.5) and Chinese lantern (*P. alkekengi,* 0.7). There is very limited literature on the carotenoid composition of plants used in Ayurvedic medicine. β-Carotene content in *C. asiatica* corroborates well with Raju et al. (2007), while the lutein value reported by them was lower (15.9 mg/100g dry weight) compared to the present result. β-carotene levels for *L. aspera* (2.3 mg/100g fresh weight) of the present study are in agreement with values (2.3 mg/100g fresh weight) reported by Rajyalakshmi et al. (2001). β-Carotene level in *O. sanctum* (10 mg/100g fresh weight) was

similar to those reported by Aizawa and Inakuma (2007) and Bhaskarachary et al. (1995). Lutein level of *O. sanctum* (15.4 mg/100g fresh weight) found in the present study was almost twice the amount reported by Aizawa and Inakuma (2007).

β-Carotene and α-carotene were the predominant provitamin-A carotenoids detected in vegetables except for β-cryptoxanthin, a provitamin-A xanthophyll that was detected only in bitter orange (*C. aurantium*). Kenaf (*H. cannabius*), red/green lettuce (*L. sativa*), carrot (*D. carota*) and yellow zuchchini (*C. pepo*) were found to be better sources of β-carotene (> 1 mg/100g dry weight) while carrot, yellow zuchchini, tomato (*L. esculentum*), and kenaf contain appreciable amounts of α-carotene (> 1). Yellow zuchchini, kenaf and carrot contain higher levels (mg/100g dry weight) of lutein (>10). Kenaf, contained highest levels of zeaxanthin (> 0.1). Lycopene was detected only in tomato. RE values (in mg) were highest for carrot (26.8) followed by kenaf (8.6), yellow zucchini (2.0) and tomato (1.5) and lowest (<0.01) for beetroot (*B. vulgaris*), white colocasia (*C. esculanta var. schott*), gerkhin (*C. anguria*), onion (*A. cepa*) and turnip. El-Qudah (2009) and the USDA (2007) reported higher concentration (>14 fold) of β-carotene in zucchini as compared to the present result. However, they do not report α-carotene as found in this study. They have also reported higher (>14 fold) concentrations of lutein and zeaxanthin in green zucchini as compared to the present study. Aizawa and Inakuma (2007) have reported 4 fold higher levels of β-carotene and 5 fold lower lutein content in yellow zucchini, than the present study. Moreover, a higher level of α-carotene was detected in yellow zucchini in the present study. Contrasting reports on provitamin-A carotenes in carrot are available. USDA (2007) reported double the amount of β-carotene than α-carotene while equal concentrations of both and no lutein were reported by El-Qudah (2009) in carrot. Whereas, the present study shows almost twice the amount of α-carotene in carrot than β-carotene and the values are higher than those reported by the above mentioned researchers. Niizu and Rodriguez-Amaya (2005) have reported lower level of β-carotene and lutein in carrots while Singh et al. (2001) have reported comparable concentration of β-carotene in carrot. β-Carotene levels reported by Bhaskarachary et al. (1995) for carrot corroborates well with the present study while the total carotenoids observed in this study are 2 fold higher. Aizawa and Inakuma (2007) have reported β-carotene level as 0.18 mg/100g fresh weight in green cabbage. The level of β-carotene and α-carotene reported by them for green and red cabbage was 20 fold higher than the values found in this study. In comparison, lutein level was higher in the present study for red cabbage, whereas, it was lower for green cabbage, green and red lettuce along with the presence of zeaxanthin in both lettuce varieties. El-Qudah (2009) has reported lower level of (>4 fold) β-carotene and lutein in tomato while no β-

carotene was detected in the present study. On the other hand, Aizawa and Inakuma (2007) have reported α-, β -carotene and lutein in tomato, whereas, α-carotene level was lower by 44 fold than the present study. Niizu and Rodriguez-Amaya (2005) have also reported the presence of β-carotene and >190 fold lower values for lutein in tomato. Lutein values reported by the USDA (2007) for tomato are comparable with the results of the present study.

This study reports lower ß-carotene level (0.01 mg/100g dry weight) in turnip than the report of Aizawa and Inakuma (2007) where higher levels of ß-carotene and lutein (3.3 and 3.11 mg/100g fresh weight) was reported. Whereas, Muller (1997) has reported lower lutein and zeaxanthin (0.1 and 0.016 mg/100 g fresh weight) in turnip. Niizu and Rodriguez-Amaya (2005) have reported lower β-carotene and lutein levels (270 and 770 μg/100 g fresh weight) and no α-carotene and zeaxanthin in green pepper as compared to the present results. Similar to the present results, only lutein was reported in onion (0.17) by Muller (1997). In addition, the author has reported similar result for lutein in potato and higher lutein concentration in onion (9 fold) and zeaxanthin in potato (>240 fold) as compared to the present results. Bhaskarachary et al. (1995) have reported higher values for β-carotene and total carotenoids (1.87 and 2.23 mg/100 g fresh weight) in sweet potato as compared to the present study, however they have not reported the presence of other carotenoids.

Amongst algae, highest levels of β-carotene (mg/100 g dry weight) were found in *Enteromorpha* (18.9), *C. racemosa* (16.9), *T. connoides* (15.5) and Palmaria (15.3). The highest values for lutein (mg/100 g dry weight) were found in *S. cristaefolium* (2.9), *H. stipulacia* (1.42) and *Enteromorpha* (1.33) while *D. dichromata*, *S. tenerrium*, *S. cristaefolim* and *P. tetrastromatica* are better sources of fucoxanthin (3.6-9.2 mg/100g dry weight). *Enteromorpha* and *H. pinifoliata* contained highest levels of zeaxanthin (17.1 and 2.6 mg/100g dry weight). Thus, *Enteromorpha* is a good source of lutein, zeaxanthin and β-carotene among the algae analyzed. α-Carotene mg/100g dry weight) was detected in 6 out of 18 algae (0.04 to 7.1) and was highest in *C.sertularciocles* (7.07). RE values (in mg) were highest for *Enteromorpha* (3.2) followed by *C. racemosa* (2.9), *T. connoides* (2.6) and *Palmaria* (2.6) and lowest for *H.* pinifoliata (0.01), *Acanthophora* (0.02), *E. kappaphycus* (0.3), *G. corticata* (0.3), *Ulva* (0.04) and *P. tetrastromatica* (0.07). Yoshii et al. (2004) have studied the carotenoid composition of the fresh water green alga *Aegagrophila linnaei*, *Ulvophyceae* and *Chlorophyta* and reported the presence of siphonoxanthin, 9-cis neoxanthin, loroxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin and β-carotene while Schubert et al. (2006) have studied many varieties of red algae and reported violaxanthin, antheraxanthin, zeaxanthin, β-cryptoxanthin and β-carotene in

Gracilaria gracilis and α-carotene as well in *G. textorii*. Esteban et al. (2009) have demonstrated the presence of lutein, β- and α-carotene in *Palmaria palmata* while Remias et al. (2005) detected astaxanthin, neoxanthin, lutein, β-carotene and pool of xanthophyll pigments (violaxanthin, antheraxanthin, zeaxanthin) in *Chlamydomonas nivalis*. Haugan and Jensen (1994) have reported β-carotene, zeaxanthin, luteoxanthin, violaxanthin, fucoxanthin, neochrome, neoxanthin and fucoxanthinol as the major carotenoids in 6 brown algae (*Fercus serratus, F. vesiculosis, Pelvetia canaliculata, Ascophyllum nodosum, Laminaria digitalia, L. saccharina*). Thus, we are the first to report the carotenoid composition of many of the Indian algae analyzed in this study.

The bioavailability and bioefficacy of β-carotene, astaxanthin, lutein and fucoxanthin was studied in retinol deficient (RD) rats in three separate studies (single, repeated and dietary). Feeding RD diet for 8 weeks resulted in significant depletion of the retinol levels in plasma and liver as compared to control (fed retinol sufficient diet). According to Olson (1984), plasma retinol levels <0.7μmol/l may be considered as deficient. RD rats in all the three studies had depleted plasma and liver retinol levels and none of the above mentioned carotenoids were detected in plasma and liver of the RD and control rats. β-Carotene was not detected in the plasma. This corresponded with increased retinol levels from 2 h to 8 h, indicating that the β-carotene was converted to retinol, which may explain why it was not detected in the plasma. The influx of β-carotene was further confirmed with the corresponding plasma triglycerides (TG) level (AUC of 1369 mg/dl/8h) indicating that bioavailability of β-carotene. The increase in retinol levels was supported by the enhanced intestinal monooxygenase activity over 8 h (56-70 %) as compared to RD group. In comparison, astaxanthin, lutein and fucoxanthin groups did not exhibit enhanced intestinal monooxygenase activity. In addition, no increase in retinol levels was observed in these groups in plasma and liver as compared to RD group (0.38 μmol/l, 3.05, 2.99 nmol/g). Raju and Baskaran (2009), Raju et al. (2006), Parvin and Sivakumar (2000) observed increase in retinol levels and higher intestinal monooxygenase activity on feeding β-carotene to RD rats. β-Carotene was detected in the liver after 8 h while it was not detected in plasma upto 8 h after a dose of β-carotene indicating conversion of absorbed β-carotene to retinol to meet its requirement in retinol deficiency. In contrast, Raju et al. (2009) reported presence of intact β-carotene in plasma after a dose of micellar β-carotene. Efficient conversion of β-carotene to retinol in RD rats may be the reason for increase in retinol levels and non-detection of intact β-carotene and other metabolites in this study. Previous reports are available where β-carotene conversion to retinol was more efficient in retinol deficiency than in normal condition (Barua and

Olson, 2000; Ribaya-Mercado et al., 2000; Grolier et al., 1995) and it is speculated that it may be due to a feedback mechanism.

Astaxanthin was detected in its intact form 2 h (28.8 pmol/ml) after gavage in plasma, reached a maximum after 4 h (81.6 pmol/ml) and remained detectable in the 8 h group (27.03 pmol/ml). Similarly, lutein was detected 2 h (12.8 pmol/ml) after gavage, reached maximum at 4 h (36.8 pmol/ml) and was detected in 8 h samples (10.9 pmol/ml). Fucoxanthin was not detected in its intact form and was quantified by measuring the fucoxanthinol and amarouciaxanthin content. Fucoxanthin metabolites were detected in 2 h samples in plasma and liver (0.13 pmol/ml, 0.93 pmol/g), however, unlike astaxanthin and lutein that peaked at 4 h after gavage, fucoxanthin reached maximum level 6 h after gavage (1.1 pmol/ml). β-Carotene alone exhibited provitamin A activity after a dose of carotenoids resulting higher retinol levels in plasma and liver over 8 h. Increased monooxygenase activity in his group further supports the above results. Raju and Baskaran (2009), Olson and Barua (2000) and Parvin and Sivakumar (2000) also reported similar association between increased activity of monooxygenase and plasma retinol levels. The absorption kinetics of astaxanthin and lutein in plasma reveal that both the carotenoids take the same time to reach maximum concentration, for absorption and clearance from plasma. While maximum absorption was observed for lutein at 4 h in the present study, Lakshminarayana et al. (2006) and Baskaran et al. (2003) have reported maximum absorption of lutein at 2h in rats and mice respectively. Lutein was not converted to retinol, moreover, there was no change in the activity of monooxygenase. Hence higher levels of lutein were detected in plasma (8.36 nmol/ml) and liver (15.7 nmol/g). Matsuno (1991) and Moren et al. (2003) have reported formation of retinol from xanthophylls via reductive pathways with the formation of 3-dehydroretinol in fish. However, no such intermediate compounds as seen in their proposed pathways were detected in this study.

Similar to the single dose study, gavages for 7- and 15-days with β-carotene resulted in increased retinol levels in plasma (5.7, 8.8 μmol/l) and liver (12.6, 45.8 nmol/g) as compared to RD group (0.3 mmol/l, 2 nmol/g). β-Carotene was not detected in plasma after 7-days of intubation, but was detectable in plasma (0.37 μmol/l) and liver (1.32 μmol/g) after 15- days of repeated gavage. Results indicate that at least 7 days of feeding β-carotene is required to stabilize circulating retinol levels that may be attained by the complete conversion of β-carotene to retinol. Feeding β-carotene more than a week resulted in its presence in the plasma indicating saturation of retinol formation. In contrast, Parvin and Sivakumar (2000) and Barua and Olson (2000) reported intact β-carotene in plasma of retinol sufficient and deficient rats after administration of

a single dose of β-carotene, which may have been due to the species and gender difference in rats. Further, they have studied β-carotene conversion in male (WNIN) and female (Sprague-Dawley) rats.

Interestingly, higher retinol levels were found in plasma (>16 fold) and liver (>2 fold) of astaxanthin group after gavages for 7- and 15-days as compared to RD group (0.3 μmol/l, 2 nmol/g). Additionally, β-carotene was also detected in the plasma and liver in 7- and 15-day groups. Repeated gavages of astaxanthin may have led the RD rats to make use of nonprovitamin-A astaxanthin as a source of retinol. It is speculated that this may have been the reason for the provitamin-A activity of astaxanthin, which has been reported earlier in lower vertebrates such as fish (Moren et al., 2002, Matsuno, 1991, Gross and Budowski, 1966). The elevated intestinal monooxygenase activity in astaxanthin group (68%), similar to β-carotene group (70%) observed in the present study further supports the conversion of astaxanthin to β-carotene and then to retinol. This may be the reason for the presence of β-carotene in astaxanthin group, which may have formed by reduction of astaxanthin to β-carotene via echinenone. Whereas, Matsuno et al. (1991) proposed a metabolic pathway for the formation of retinol from astaxanthin through zeaxanthin and 3-dehydroretinol in rats. They have suggested that 3-dehydroretinol formed from astaxanthin may give rise to retinol. In this study, lutein gavages did not show significant difference in retinol levels in plasma and liver from the RD group. This is in contrast with the findings of Matsuno (1991) who reported formation of 3-dehydroretinol from lutein by reduction reactions in fish and rats.

Bioavailability of β-carotene in plasma and liver from dietary feeding of carrot powder was 29.9 ng/ml and 89.1 ng/g respectively. There was increased retinol level (96, 97 and 95%) in plasma, liver and intestine after 20 days feeding as compared to RD group. Raju (2007) reported relatively lower bioavailability of β-carotene in plasma (42.6 nmol/l) and liver (189 pmol/g) of RD rats from diet supplemented with *C. album* leaves. The difference in bioavailability of b-carotene between these studies may be attributed to the difference in source and concentration of β-carotene. However, the percent increase in retinol levels found in the present study is comparable (93.6%) with the report of Raju (2007). In the present study, similar to the result obtained in repeated dose study, β-carotene was also detected in plasma (4.3 ng/ml) and liver (10.1 ng/g) of RD rats after 20 days of astaxanthin feeding indicating its conversion to β-carotene. The increase in retinol levels in plasma (>2 fold) and liver (>1.5 fold) in astaxanthin group as compared to RD group (0.4 μmol/l, 3 nmol/g) indicates that retinol was formed from astaxanthin via β-carotene resulting in its higher levels. Amar et al. (2004), Bell et al. (2000) and

Bjerkeng et al. (2000) have reported bioavailability of astaxanthin from feed in fish and its accumulation in liver, muscle, heart, brain and eye. Similar to the present results, Moren et al. (2002) have reported formation of retinol via 3-dehydroretinol from dietary astaxanthin by reductive pathways whereas; this study reports formation of retinol from astaxanthin via β-carotene and echinenone.

Bioavailability of lutein in plasma (43.9 ng/ml) and liver (159.8 ng/g) from dill leaf powder fed for 20 days to RD rats found in this study was higher (>2 fold) than that reported by Lakshminarayana et al. (2009) in retinol sufficient but lutein deficient rats. The difference in bioavailability may be due to the lutein source and the vitamin A status of the rats. Feeding diet supplemented with dill leaf powder for 20 days resulted in increased retinol levels in plasma, liver and intestine by 67, 91 and 95 % respectively. The reason for the increase in retinol levels of dill leaf group may have been due to the presence of β-carotene in dill leaf. Whereas, no retinol was formed when purified lutein was gavaged to RD rats, indicating that lutein is not converted to retinol. Rather, zeaxanthin was identified as a prime metabolite of lutein in plasma and liver of rats fed dill leaf powder as evidenced by Lakshminarayana et al. (2008) who fed fenugreek leaves as lutein source. Moreover, reports are available on bioavailability of carotenoids from vegetable and fruit sources are more bioavailable and a better source of retinol than leafy greens (van Het Hof et al., 2000; Castenmiller et al., 1999; de Pee et al., 1998; de Pee et al., 1995), indicating differences in the bioavailability and bioefficacy of carotenoids from different types of vegetables.

The identification and characterization of metabolites of carotenoids fed to RD rats may help in better understanding of the metabolic fates of carotenoids *in vivo*. Thus, the metabolites formed on feeding β-carotene, astaxanthin, lutein and fucoxanthin were characterized by HPLC and LC-MS. No carotenoid or their metabolites were detected in the baseline and control groups. This indicated that on feeding carotenoids to RD rats, they were either cleaved or oxidized or fragmented (metabolites). Retinol was the predominant metabolite on single and repeated gavages of β-carotene. Intact β-carotene was detected in plasma after 15-days gavages. Similar to single dose study, repeated gavage of β-carotene to RD rats resulted in a rise in plasma and liver retinol levels which corresponded with a lower level of β-carotene in plasma indicating its conversion to retinol. This is in agreement with several other reports that also reported the formation of retinol from β-carotene (Lemke et al., 2003; Bachmann et al., 2002, Barua and Olson, 2000). However, no apo-carotenal were detected in this study. Either the β-carotene or apo-carotenal were rapidly degraded to retinol or else the centric cleavage of β-carotene may have been predominant

resulting primarily in the formation of retinol. Barua and Olson (2000) have reported very less apo-carotenal formation on feeding of β-carotene to vitamin A deficient and sufficient rats.

Interestingly, astaxanthin gavages to RD rats also showed increased plasma and liver retinol levels. The presence of β-carotene in the plasma of astaxanthin group further supports the speculation that retinol may have been formed by the bioconversion of astaxanthin to β-carotene via reductive pathways as reported in lower vertebrates (Moren et al., 2002; Matsuno, 1991; Gross and Budowski, 1966). In fact, the biosynthesis process of carotenoids in micro algae indicates that astaxanthin is derived from β-carotene (Rodriguez-Amaya, 2001). In contrast, in RD rats, astaxanthin seems to be converted to β-carotene and subsequently to retinol in the present study. The newly formed retinol in β-carotene and astaxanthin groups in the present study is further supported by the increased activity of monooxygenase (73, 70%) compared to RD rats. This corroborates well with Raju and Baskaran (2009) who have also reported increased monooxygenase activity on feeding β-carotene to RD rats. Matsuno (1991) and Moren et al. (2002) have reported formation of retinol from xanthophylls such as astaxanthin, canthaxanthin, lutein, zeaxanthin and tunaxanthin via reductive pathways with the formation of zeaxanthin and 3-dehydroretinol in fish. Whereas, the present results did not indicate the presence of these intermediate compounds in rats. On the contrary, LC-MS analysis of liver samples revealed a compound whose mass corresponds with echinenone (m/z 551 $(M+H)^+$). The identity of the compound was further confirmed by its characteristic UV-Vis spectrum, recorded by the PDA detector. It is predicted that astaxanthin and its hydroxyl derivatives may undergo reduction reactions leading to dehydroxylation with the removal of two hydroxyl-groups and one keto-group to form echinenone by the action of reducing enzymes such as reductase, dehydrogenase and dehydroxylase. It is probable that the intermediate compounds of these reactions are unstable and short-lived and therefore not detected. Echinenone in turn may have been reduced to β-carotene, which may be cleaved by the monooxygenase to retinal. The newly formed retinal may be further very rapidly converted to retinol in the body. Reactions suggesting the probable mechanism of astaxanthin metabolism in RD rats have been depicted in Scheme 2 (Chapter 5). Matsuno (1991) have observed the formation of echinenone from canthaxanthin, while astaxanthin resulted in formation of zeaxanthin and 3-dehydroretinol in their study. It is suggested that due to the clinical retinol deficient condition of the rats in the present study, in contrast to previous reports, astaxanthin may have been reduced to echinenone and β-carotene to meet the requirement of retinol under RD condition. Such reaction was not evidenced in retinol sufficient rats fed with astaxanthin. Thus, this is the first study to report the formation of echinenone and β-

carotene from astaxanthin by reductive pathways, demonstrating the provitamin A activity of astaxanthin in RD rats.

Lutein was not cleaved to retinol as indicated by low retinol levels in the liver and plasma of RD rats. However, other metabolites of lutein were identified in the samples including zeaxanthin, anhydrolutein and diepoxylutein. Only few reports are available on epoxy-carotenoids in plasma (Lakshminarayana et al. 2008; Asai et al., 1999; Barua and Olson, 1998). The present results are in agreement with the observations of Barua and Olson (2001) who also reported epoxy carotenoids in humans. Lutein along with zeaxanthin, the commonly found isomer of lutein was also detected and identified. Lutein oxidation in liver may have resulted in the formation of the metabolites, lutein diepoxide and 5,6 epoxy-3 hydroxy-12'-β,ε-carotene-12'al (Chapter 5). Fragmentation of anhydrolutein may have resulted in the formation of the metabolites (2*E*, 4E)-3-methyl-5- (2,6,6-trimethylcyclohexa-2, 4-dien-1-yl) penta-2, 4-dien-1-ylium and 2,6,6-trimethylcyclohex-2-ene-1, 4-bis (ylium). Thus lutein and its diepoxides were identified in liver and plasma. Khachik et al. (2002) have suggested that the transport and the metabolic inter-conversion between lutein and zeaxanthin in the eyes are most probably induced by photooxidation due to sunlight as evidenced *in vitro* (Lakshminarayana et al., 2008). Hartmann et al. (2004) reported that dosing zeaxanthin to human volunteers resulted in considerable accumulation of *all-E*-3-dehyro-lutein in plasma and postulated that since lutein concentration remains unaffected by zeaxanthin dosing, the increase in *all-E*-3-dehydro-lutein might have been derived from zeaxanthin. Thurman et al. (2005) demonstrated that long-term intake of lutein resulted in an accumulation of 3`dehydro-lutein in human plasma. Lutein may be converted into several metabolites *in vivo*. In liver and plasma, oxidized molecules may be formed due to oxidative reactions, which indicate that lutein is involved in the chain breaking peroxyl- radical or quenching of the singlet oxygen (Stratton and Liebler, 1997; Yamauchi et al., 1998). Fragmentation of anhydrolutein may also have resulted in the formation of some of the metabolites.

Fucoxanthin (FUCO) gavage resulted in the formation of fucoxanthinol and amarouciaxanthin that were confirmed by their UV-VIS spectra as reported by Sugawara et al. (2002). Fucoxanthinol was the de-acetylation product of fucoxanthin and its mass spectra produced the similar UV-Vis spectrum and positive m/z ions at 617 which corresponds to $(M+1)^+$ for fucoxanthinol. Thus, fucoxanthinol was identified as a prime metabolite of FUCO in rats in this study and mice (Sugawara et al., 2002; Asai et al., 2004). Whereas, amarouciaxanthin had a different UV-VIS and mass spectra than fucoxanthinol with molecular ion $(M+1)^+$, at *m/z* 615 as reported by Asai et al. (2004). Interestingly, amarouciaxanthin was a major metabolite of

fucoxanthin in liver while in plasma it was fucoxanthinol, demonstrating that liver enzymes may play a role in hydrolyzing the fucoxanthinol to amarouciaxanthin (Asai et al., 2004). Similar to earlier reports in mice model (Asai et al., 2004), the conversion of fucoxanthin to fucoxanthinol and/or amarouciaxanthin was higher in rat liver, which may be due to dehydrogenation and/or isomerization of 5,6-epoxy-3-hydroxy-2,4-dihydro-β end group of fucoxanthinol to 6-hydroxy-3-oxo-ε end group of amarouciaxanthin by liver dehydrogenase (Sugawara et al., 2002; Asai et al., 2004). These metabolites may act as functional molecules *in vivo*, similar to lutein metabolites (Bhosale et al., 2007). Fucoxanthin seems to be rapidly hydrolyzed in the intestine to fucoxanthinol, amarouciaxanthin, fucoxanthinol-H_20 and amarouciaxanthin-H_20. Strand et al. (1998) reported that fucoxanthinol but not fucoxanthin was transported to egg yolk of laying hens fed with a diet containing 15% brown algal meal. Interestingly, metabolites bearing molecular mass at *m/z* 600 and *m/z* 367 (Chapter 5) were found in both plasma and liver samples. Some of the metabolites found in plasma and liver could have resulted from either fucoxanthinol or amarouciaxanthin by removal of water molecules due to enzymatic reactions involving oxygenases (Olson, 1994). Other metabolites could partly be due to enzymatic retroaldol cleavage (Bhosale et al., 2007) or enzymatic demethylation of fucoxanthinol and amarouciaxanthin.

Biochemical parameters at the plasma and tissue level were analyzed to determine the effect of retinol deficiency and subsequent gavages with carotenoids. The activity of the carotenoid cleavage enzyme, β-carotene, 15-15' monooxygenase was elevated in the β-carotene group over 8 h (56-70%) and this corresponded with elevated retinol levels (1.6-4.6 fold) in the β-carotene group as compared with RD group. Whereas, astaxanthin, lutein and fucoxanthin groups neither had elevated monooxygenase activity, nor raised retinol levels as compared to RD group. Whereas, in the repeated gavage study, β-carotene and astaxanthin groups had increased levels of retinol in plasma and liver (>2.6 fold) and had correspondingly elevated activity of the monooxygenase (>67.6%) as compared to RD group. However, repeated gavages of lutein did not result in any significant change in the retinol levels and monooxygenase activity as compared to RD group. In the dietary feeding study, carrot powder (β-carotene source), astaxanthin and dill leaf (lutein source), retinol levels were increased in all groups and this was associated with enhanced monooxygenase activity (>68%) as compared to RD group. Carrot powder and dill leaf contained provitamin A carotenoids and this explains the rise in retinol levels in these two groups. Interestingly, astaxanthin was converted to β-carotene and thereafter cleaved to retinol in the RD rats, similar to the repeated gavage study. Raju and Baskaran (2009), Raju et al. (2006) and Barua

and Olson (2000) have also reported enhanced monooxygenase activity in retinol sufficient and deficient rats fed β-carotene.

Lipid peroxidation in plasma and liver was significantly higher in the RD group (67-89%) as compared to control indicating that retinol deficiency induced oxidative stress. Corresponding to the increased levels of lipid peroxides, activity of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione (GSH) levels was significantly lower (> 54%) in retinol deficiency. Feeding carotenoids reversed the effect of retinol deficiency with decreased lipid peroxidation and increased the activities of SOD, CAT, GST and GSH levels as compared to RD group. In the repeated dose study, astaxanthin was more effective than lutein and β-carotene in suppressing the lipid peroxidation and enhancing the protective effect of antioxidant molecules. These results corroborated well with earlier studies, which also showed elevated Lpx in retinol deficiency in liver and heart of rats (Anzulovich et al., 2000; Oliveros et al., 2000; Kaul and Krishnakantha, 1997). Anzulovich et al. (2000) have suggested that retinol deficiency induces modifications of the tissue components, which leads to peroxidation-induced damage to the membrane. Halliwell and Guteridge (1989) correlated the Lpx at cellular level and pathological abnormalities in tissues. Depending upon their polarity, structure and functional groups attached, carotenoids are aligned in the membrane structure where they provide protection to the lipids from peroxidation (Woodall et al., 1997). Xanthophylls including astaxanthin and lutein possessing polar functional groups are aligned perpendicular or at an angle to the phospholipid bi-layer of the membrane offering protection at both lipid and aqueous phases while carotenes are highly lipophillic and are parallel to the membrane, affording protection to the membrane lipids (McNulty et al., 2007; Gruszecki and Strzalka, 2005). This may explain the better suppression of Lpx by astaxanthin (with more number of polar functional groups-2 keto and 2 hydroxyl groups on the β-ionone rings) and FUCO (2 hydroxyl groups, an epoxide and an acetyl group on the β-ionone rings and a keto- group in the polyene chain), as compared to lutein (2 hydroxyl groups on the β-ionone rings) and β-carotene (no polar functional groups). The decrease in Lpx by the carotenoids can be attributed to their antioxidant properties (McNulty et al., 2007; Sachindra et al., 2007).

Retinol deficiency resulted in altered lipid profile in plasma and liver microsomes as compared to control. Total cholesterol, HDL-cholesterol, LDL+VLDL-cholesterol and phospholipids were lowered in RD group. Feeding carotenoids had a positive effect on the lipid parameters towards control levels. Plasma triglycerides level was significantly increased on feeding carotenoids, indicating the bioavailability of the carotenoids as triglycerides are carriers of carotenoids after their intestinal absorption. Retinol deficiency increased the saturated fatty

acids (SFA) and decreased the unsaturated fatty acids such as monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) in plasma and liver microsomes as compared to the control group. Similar to the results of the present study, Kaul and Krishnakantha (1997) have reported a decrease in MUFA and PUFA as a result of retinol deficiency. They have reported that fatty acids C18:1, C18:2 and C20:4 were decreased by 33.9%, 12.1% and 35.3% respectively. However, they have also reported a decrease in SFA unlike the present study where an increase was observed. In the present study, feeding carotenoids had an ameliorative effect with increased MUFA and PUFA and decreased SFA in plasma and liver microsomes as compared to the RD group. Amongst the carotenoids fed, the ameliorative effect was more pronounced by β-carotene, followed by astaxanthin, fucoxanthin and lutein. The alteration in saturated and unsaturated fatty acids in carotenoid administered groups can be attributed to the fact that carotenoids might inhibit desaturase activity and thereby increase levels of unsaturated fatty acids suggesting that the metabolism of fatty acids is affected (Grolier et al., 1995; Hamm et al., 1987). Murello (1992) has reported elevated total cholesterol and HDL-cholesterol levels on feeding astaxanthin and canthaxanthin to rats. However, β-carotene did not exhibit similar effect as astaxanthin and canthaxanthin in the above study. Oshima et al. (1993) have found that astaxanthin and β-carotene protect the phospholipid layers in unilamellar liposomes against photosensitized oxidation. Asai et al. (1999) have reported lower phospholipid hydroperoxides in RBC, liver lipid peroxidation, on feeding turmeric and capsicum extracts to mice and attributed the effects to antioxidant property of the spices. Takahashi et al. (2004) have also observed incorporation of astaxanthin in HDL (70%). Further, they have suggested that since astaxanthin was incorporated in HDL and LDL, their secretion and levels may be higher in circulation leading to higher cholesterol levels on feeding carotenoids.

RBP is considered as a biomarker for retinol status since its levels are proportional to circulating retinol. In the present study, retinol deficiency showed significantly lowed RBP levels in serum as compared to control, whereas, their levels were increased after a dose of β-carotene. Gamble et al. (2001) have also reported lower RBP levels in serum of VAD rats. In contrast, intubation of a dose of astaxanthin, lutein and fucoxanthin did not show alteration in RBP levels indicating that only β-carotene is cleaved to retinol. This was further supported by enhanced monooxygenase activity in β-carotene group unlike the other groups. In repeated dose and dietary studies, in addition to β-carotene, astaxanthin also exhibited an elevated RBP levels corresponding to increased retinol levels and monooxygenase activity in RD rats. The conversion of astaxanthin to β-carotene and then to retinol was less efficient than retinol from β-carotene.

This may be the reason for lower levels of retinol that was supported by RBP levels. Muto et al. (1972) have reported that the blood RBP level was increased after a single oral dose of vitamin A. The present results and the available reports demonstrate that retinol deficiency results in alteration of various biochemical parameters covered in this study in rats, which were ameliorated by feeding either purified or dietary carotenoids. The ameliorative effect was in the order of astaxanthin > fucoxanthin > lutein > β-carotene.

Carotenoids are believed to provide protection to the membrane lipids, structures and functions. They are localized in membranes based upon their polarity, structure and functional groups attached to β-ionone rings or polyene chain (Woodall et al., 1997). It has been suggested that retinol deficiency may alter membrane bound molecules, which may cause damage to the membrane and its function (Anzulovich et al., 2000). Increased lipid peroxidation (caused by retinol deficiency or otherwise) at the cellular level is associated with pathological abnormalities in tissues (Halliwell and Guteridge, 1989; Anzulovich et al., 2000; Oliveros et al., 2000; Kaul and Krishnakantha, 1997; Melin et al., 1992). In the present study, feeding carotenoids to RD rats resulted in decreased lipid peroxidation that can be attributed to the antioxidant properties of the carotenoids or their metabolites (McNulty et al., 2007; Sachindra et al., 2007; Lakshminarayana et al., 2010). Retinol that is a metabolite of β-carotene, has been known as an essential micronutrient (Olson, 1999). Lakshminarayana et al. (2010) have suggested that oxidized forms/metabolites of lutein may be highly reactive, since oxidation results in radical ions, which can combine with similar reactive oxidative species that could lead to superior antioxidative effect. In this study, xanthophylls resulted in various metabolites, which may have synergistically exerted higher antioxidative effect than β-carotene from which retinol was the major metabolite.

Further, the differences in the antioxidant potential among the carotenoids may be due to their differential arrangement in the membrane of the cells (Britton, 1995). Oshima et al. (1993) have reported that astaxanthin and β-carotene protect the phospholipid layers of the unilamellar liposomes against photosensitized oxidation due to their localization in the phospholipid layers. Asai et al. (1999) have reported similar antioxidant effect on phospholipids in mice fed with turmeric and capsicum extracts. Astaxanthin was reported to exhibit superior protection in rat kidney fibroblasts against UVA light induced oxidative stress compared to lutein and β-carotene in terms of CAT, SOD activity and lipid peroxide levels (O'Connor and O'Brien, 1998). It was reported that astaxanthin was 100-500 times stronger than vitamin E in preventing lipid peroxidation in rat mitochondria (Kurashige, 1990). Whereas, Miki et al. (1991) have reported astaxanthin to be ten times more effective than zeaxanthin, lutein and β-carotene. Previously, it has been reported that astaxanthin furnishes more protection to rat liver microsomes undergoing

radical-initiated lipid peroxidation than β-carotene and vitamin E (Nishigaki, 1994). Goto et al. (2001) have attributed the highly potent antiperoxidative activity of astaxanthin to efficient radical trapping at the surface and inside the phospholipid membrane in liposomes. Cantrell et al. (2003) have reported that β-carotene was most effective in quenching singlet oxygen in liposmomes and lutein was least efficient while astaxanthin and canthaxanthin were intermediate. As evidenced in the present study, spices or their active principles such as curcumin and capsaicin have been reported to ameliorate the changes in lipid profile, ATPases and membrane fluidity caused by hypercholesterolemia in rats (Kempaiah and Srinivasan, 2005).

Carotenoids are found in precise locations and orientations in subcellular structures, and their chemical and physical properties are strongly influenced by other molecules in their vicinity, especially membrane proteins and lipids. In turn, the carotenoids influence the properties of these subcellular structures (Britton, 1995). The effect of retinol deficiency and subsequent feeding of carotenoids was observed at the membrane level with respect to structure and function in liver and brain microsomes in the present study. The activity of membrane bound enzymes such as Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase were significantly higher in RD group while AChE was decreased in brain microsomes as a result of retinol deficiency as compared to control group. The present results, where retinol deficiency resulted in significantly higher activity of Na^+K^+-ATPase in liver microsomes, are in agreement with the results of Kaul and Krishnakantha (1997, 1994 and 1993) who reported increased Na^+K^+-ATPase activity in the liver microsomes of RD rats. Fujiyara et al. (1992) have also reported alteration in the membrane bound enzymes as a result of retinol deficiency in cultured rat hepatocytes. Korichneva et al. (2003) have reported that retinol deprivation enhanced oxidative damage in cardiomyocytes isolated from rats, as indicated by rapid loss of mitochondrial membrane potential.

Feeding a dose of β-carotene (45-60%) and astaxanthin (33-39%) showed greater beneficial effect than lutein (26-35%) and fucoxanthin (31-61%) in this study. Similarly, significant decrease in the activities of the ATPases was found on repeated dose of β-carotene (48-60%) and astaxanthin (30-69%) than lutein (11-42%). In the present study, the AChE in brain microsomes was significantly decreased as a result of retinol deficiency (73-83%) as compared to control and increased in the carotenoid fed groups. Vitamin A is suggested to support acetylcholine synthesis. Choline acetyltransferase, the enzyme promoting acetylcholine synthesis, and the vesicular acetylcholine transporter are modulated by retinoic acid treatment (Roberto et al., 2007). A decrease in acetylcholine content following 12 weeks vitamin A deprivation in the hippocampus and striatum, but not in prefrontal cortex has been reported (Roberto et al., 2007). The protective effect exhibited by β-carotene and astaxanthin in the present study was probably

due to the synergistic antioxidant effect and potential of the newly formed retinol and carotenoids. Lutein and fucoxanthin did not cleave to retinol and yet modulated the activity of these enzymes. Their effect was probably due to their protection against lipid peroxidation by modulation of the activity of SOD, CAT, GST and GSH.

Membrane fluidity is controlled by fatty acid composition and cholesterol content. Thus, the cholesterol: phospholipid ratio, which is an index of the fluidity of the membrane, is inversely proportional to membrane fluidity. Therefore, retinol deficiency affects the membrane structure and makes it less fluid. This may also affect the activity of membrane bound enzymes. A number of membrane bound enzymes have been shown to be sensitive to their fatty acid environments. It has been established that ATPases require a 'fluid' lipid environment for proper functioning (Stubbs and Smith, 1984). In this study, lipid parameters such as cholesterol and phospholipids were decreased as a result of retinol deficiency as compared to control and these were increased on feeding carotenoids as compared to RD group. The cholesterol: phospholipid ratio was significantly increased in retinol deficiency (0.91-0.92) as compared to control (0.69-0.7), indicating altered membrane fluidity. Feeding carotenoids decreased the cholesterol: phospholipid ratio towards control. Of the carotenoids used in this study, β-carotene was most effective followed by astaxanthin and lutein with regard to modulation of the cholesterol: phospholipid ratio at membrane level.

Retinol is an integral part of the membrane and its deficiency alters the fatty acid components of the membrane. This could in turn change the membrane fluidity (Kaul and Krishnakantha, 1997). Kang et al. (2007) have reported that vitamin A deficiency alters lipid catabolic pathways. They also reported that genes involved in peroxisomal fatty acid-oxidation and their transport was differentially expressed in retinol deficiency, leading to changes in fatty acid metabolism. Similar to the present study, cholesterol-phospholipid ratio was significantly increased with the administration of curcumin to RD rats as compared to control (Kaul and Krishnakantha, 1997). Niranjan and Krishnakantha (2001) have observed decreased membrane fluidity in the oxidized ghee fed rats and have speculated that it may have been because of the increased cholesterol: phospholipid ratio, the increased saturated fatty acid/unsaturated fatty acid ratio, or increased lipid peroxidation which leads to increased cross-linked products resulting in reduced membrane fluidity. High cholesterol to phospholipid ratio decreases membrane fluidity, as the cholesterol sterically prevents the movement of phospholipid fatty acyl chains. Whereas, Estornell et al. (2000) reported that vitamin A deficiency did not induce appreciable alterations in the morphology of the mitochondria in rats. Thus, retinol deficiency resulted in altered membrane structure and function as evidenced by changes in biochemical constituents. However, feeding β-

carotene, astaxanthin, lutein and fucoxanthin ameliorated the alterations in biochemical constituents in the membrane caused by retinol deficiency. Although studies of expression of mRNA modulated by retinoid acid on feeding non-provitamin A carotenoids under retinol deficiency is warranted, this study confirms that astaxanthin is converted to β-carotene and retinol under retinol deficiency in murine model. Further, astaxanthin can be considered as a potential alternative for β-carotene, as a precursor of retinol and antioxidant in vitamin A deficiency.

SUMMARY

1. The carotenoid composition of familiar and less familiar leafy greens (n=24), medicinal plants (n=33), vegetables (n=25) and algae (n=18) was determined by HPLC analysis.
2. Amongst the leafy greens, β-carotene levels were higher in *C.album, A.spinosus* and *C.benghalensis* while lutein level was higher in *C.album, C.benghalensis* and *P.sowa*. Among medicinal plants, *B.monnieri* contained higher levels of β-carotene while *C.ternatea* was the richer source of lutein. Amongst the vegetables, β-carotene was higher in *D.carota* while *C.pepo* contained higher levels of lutein. Among algae, *Enteromorpha* was rich in β-carotene, while *S.cristaefolium* contained higher levels of lutein. Fucoxanthin was higher in *D. dichromata* among algae.
3. In general, vitamin A activity (Retinol Equivalent) was higher for leafy greens (lamb's quarters, jio, prickly amaranth, dill leaf, colocasia leaves and kenaf) and vegetables (carrot, yellow zucchini and tomato) as compared to medicinal plants (except for thyme-leaved gratiola, butterfly pea and holy basil) and algae that were rich in xanthophylls.
4. Information on carotenoid profile of many medicinal plants, Indian algae and other greens is very limited. Moreover, no reports are available for nearly 50% of the plants analyzed in this study, many of which are used by the local people as food or medicine. Carotenoid composition of the agri-horticultural produce, medicinal plants and algae analyzed in this study will be of use to improve nutritional awareness and aid health and community workers to recommend these plant materials to ensure vitamin A security.
5. The method for extraction and purification of β-carotene, lutein/zeaxanthin and fucoxanthin was established and >90% purity of the carotenoids was obtained. The HPLC and LC-MS methods for identification and quantification of the carotenoids were relatively simple, reliable and accurate as compared to available procedures in the literature.

6. Gavage of single or repeated doses of β-carotene to RD rats resulted in elevated retinol levels in plasma (>19 fold) and liver (>6 fold) compared to baseline value. No intact β-carotene was detected in plasma after single dose, demonstrating its complete conversion to retinol to meet its requirement. Whereas, after 15-days intubations β-carotene was detected demonstrating saturation of retinol requirement.
7. Single dose of astaxanthin did not result in change in plasma retinol levels while repeated doses resulted in increased retinol levels in plasma (>9 fold) and liver (>1.5 fold). Along with retinol, β-carotene was also detected in the samples indicating that astaxanthin is converted to retinol via β-carotene in retinol deficiency. However, single and repeated doses of lutein did not result in any change in the retinol levels.
8. The time course and absorption kinetics studies revealed that bioavailability of astaxanthin was higher than lutein and fucoxanthin. Since β-carotene was completely converted to retinol, it is considered that it is highly bioavailable and utilized as retinol source in retinol deficient condition. An increase in the triglycerides was observed in carotenoid fed groups, indicating the influx of carotenoids compared to RD group, in the order β-carotene > astaxanthin > lutein > fucoxanthin.
9. Feeding diet supplemented with carrot powder (β-carotene source), dill leaf powder (lutein source) and pure astaxanthin resulted in increased retinol levels in the order carrot powder > dill leaf powder > astaxanthin. The reason for retinol formation in the dill leaf group may be due to the presence of β-carotene in dill leaf. Therefore, orange/yellow vegetables and leafy greens can provide retinol in the diet. Similar to the repeated gavage study, dietary feeding of astaxanthin resulted in the formation of retinol.
10. The increased intestinal monooxygenase activity in β-carotene and astaxanthin fed groups supports the cleavage of these carotenoids to retinol resulting in higher retinol levels in plasma and liver.
11. Studies revealed that absorbed β-carotene was rapidly metabolized to retinol in retinol deficiency. The increased activity of intestinal monooxygenase and serum RBP levels further justifies the metabolism of β-carotene to retinol.
12. Interestingly, retinol was found in RD rats after feeding astaxanthin (except in single dose) along with β-carotene and echinenone indicating that astaxanthin may undergo reduction reactions leading to dehydroxylation with the removal of two hydroxyl groups and one keto group from the β-ionone rings to form echinenone and thereafter β-carotene by the action of reducing enzymes such as reductase, dehydrogenase and dehydroxylase.

The proposed metabolic pathway clearly demonstrates structural features of intermediates. From this study, we could characterize β-carotene, echinenone and retinol by LC-MS. This is the first study to report the formation of echinenone and β-carotene from astaxanthin by reductive pathways, demonstrating the provitamin A activity of astaxanthin in RD rats

13. Lutein was not converted to retinol after single or repeated gavages. However, other metabolites such as zeaxanthin, anhydrolutein and diepoxylutein and their derivatives were characterized. The proposed metabolic pathway clearly demonstrates structural features of intermediates. From this study, we could characterize those metabolites by LC-MS.
14. Fucoxanthin was rapidly metabolized to fucoxanthinol, amarouciaxanthin and their derivatives and characterized in the plasma and liver of RD rats. The proposed metabolic pathway clearly demonstrates structural features of the metabolites. From this study, we could characterize fucoxanthinol, amarouciaxanthin and their derivatives by LC-MS. Even though biological functions of fucoxanthin metabolites need thorough investigation with reference to their health benefits, this is the first detailed report on various fucoxanthin metabolites in rats.
15. Retinol deficiency was associated with increased lipid peroxide levels, decreased activity of antioxidant molecules like SOD, CAT, GST and GSH, increased saturated fats, decreased MUFA and PUFA and decreased levels of cholesterol, phospholipids and triglycerides in plasma and liver over control demonstrating that biochemical constituents and lipid metabolism are affected by retinol deficiency.
16. Carotenoid feeding resulted in decreased lipid peroxidation and increased activities of SOD, CAT, GST and GSH over RD group. The protective effect in terms of antioxidant potential exerted by the carotenoids was in the order of astaxanthin = fucoxanthin > lutein > β-carotene.
17. The fatty acid profile was modulated by carotenoid feeding with decrease in saturated fats and increase in unsaturated fatty acids in the order β-carotene > fucoxanthin = astaxanthin > lutein.
18. Retinol deficiency resulted in significantly increased activity of membrane bound $Na^{+}K^{+}$ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase in liver microsomes and decreased activity of AChE in brain microsomes.

19. Carotenoid feeding resulted in decreased activity of ATPases in liver microsomes and increase in AChE in brain microsomes. The effectiveness of the carotenoids as antioxidants was in the order of β-carotene > astaxanthin > lutein = fucoxanthin.
20. Lipid peroxides levels in liver microsomes were significantly increased as a result of retinol deficiency while activities of antioxidant molecules like SOD, CAT, GST and GSH were significantly decreased as compared to control.
21. A decrease in lipid peroxide levels and increased activity of antioxidant molecules (SOD, CAT, GST and GSH) over RD group was observed in the liver microsomes. Astaxanthin showed maximum protection as an antioxidant at the membrane level, followed by fucoxanthin, lutein and β-carotene.
22. The saturated fats in liver microsomes were increased as a result of retinol deficiency while there was a decrease in the MUFA and PUFA in RD rats as compared to control. The cholesterol: phospholipid ratio was significantly increased in retinol deficiency indicating an alteration in the membrane integrity.
23. Feeding carotenoids resulted in decreased saturated fats while MUFA and PUFA were increased as compared to RD group. There was also decrease in the cholesterol: phospholipid ratio towards control on feeding β-carotene, astaxanthin, lutein and fucoxanthin, in that order.
24. From the results, it is concluded that retinol deficiency altered the biochemical parameters at the tissue and membrane level. Carotenoids can be used as ameliorative components to correct the changes caused by retinol deficiency. Further, astaxanthin can be considered as a potential alternative for β-carotene, as a precursor of retinol and antioxidant in vitamin A deficiency.

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Appendix

List of Publications

1. **Sangeetha Ravi Kumar** and Baskaran V. Retinol deficient rats can convert a pharmacological dose of astaxanthin to retinol: Antioxidant potential of astaxanthin, lutein and β-carotene. *Canadian Journal of Physiology and Pharmacology, 2010, 88: 977-985.*
2. **Sangeetha Ravi Kumar** and Baskaran V. Carotenoid composition and their retinol equivalent in plants of nutritional and medicinal importance: Efficacy of β-carotene from *Chenopodium album* in retinol deficient rats. *Food Chemistry, 2010, 119: 1584-1590.*
3. **Sangeetha Ravi Kumar**, Bhaskar N, Divakar S and Baskaran V. Bioavailability and metabolism of fucoxanthin in rats: structural characterization of metabolites by LC-MS (APCI). *Molecular & Cellular Biochemistry 2010, 333: 299-310.*
4. **Sangeetha Ravi Kumar**, Bhaskar N and Baskaran V. Comparative effects of β-carotene and fucoxanthin on retinol deficiency induced oxidative stress in rats. *Molecular & Cellular Biochemistry, 2009, 331:59-67.*
5. **Sangeetha Ravi Kumar**, N Bhaskar & V Baskaran. Fucoxanthin restrains oxidative stress induced by retinol deficiency in rats through modulation of Na^+K^+-ATPase and antioxidant enzyme activities in rats. *European Journal of Nutrition, 2008, 47: 432-441*.
6. **Sangeetha Ravi Kumar**, Bhaskar N, Divakar S and Baskaran V. Biotransformation Of Fucoxanthin And Structural Elucidation Of Its Metabolites In Retinol Deficient Rats By HPLC and LC-MS (APCI). *Carotenoid Science, 2008, 12: 193* (Proceedings).
7. Mamatha B.S., **Sangeetha Ravi Kumar** and Baskaran V. Provitamin-A and xanthophyll carotenoids in vegetables and food grains of nutritional and medicinal importance. *International Journal of Food Science and Technology, 2011, 46: 305-323.*
8. R Lakshminarayana, G Aruna, **Ravi Kumar Sangeetha**, N Bhaskar, S Divakar & V Baskaran. Possible Degradation Of Lutein In Vitro And In Vivo: Structural Elucidation Of Lutein Metabolites By HPLC And LC-MS (APCI). *Free Radical Biology & Medicine, 2008, 45: 982-993.*
9. R Lakshminarayana, G Aruna, **Ravi Kumar Sangeetha**, N Bhaskar, S Divakar & V Baskaran. Possible Degradation Of Lutein In Vitro And In Vivo: Structural Elucidation Of Lutein Metabolites By HPLC And LC-MS (APCI). *Carotenoid Science, 2008, 12: 192* (Proceedings).
10. **Sangeetha Ravi Kumar** and Baskaran V. Astaxanthin is superior to β-carotene and lutein in modulating lipid peroxidation in retinol deficiency: Provitamin A activity of astaxanthin in rat. (Communicated to *Journal of Physiology*).
11. **Sangeetha Ravi Kumar** and Baskaran V. Absorption kinetics and bioavailability of a pharmacological dose of β-carotene, astaxanthin and lutein in retinol deficient rats. (Communicated to *Phytomedicine*).

List of Presentations at Symposia

International

- **Sangeetha Ravi Kumar** and Baskaran V. Protective effect by modulation of lipid peroxidation, activities of antioxidant and membrane bound enzymes in microsomes by nonprovitamin A carotenoid astaxanthin is greater β-carotene in retinol deficiency (International Conference on Recent Advances in Free radical Research, Natural Products, Antioxidants and Radio protectors in Health and 9th Annual Conference of Society for Free Radical Research-India, 2010).
- **Sangeetha Ravi Kumar** and Baskaran V. β-carotene, lutein and astaxanthin: Comparative antioxidant property in relieving retinol deficiency induced oxidative stress in rats (6th International Food Convention, AFSTI, 2008).
- **Sangeetha Ravi Kumar**, Bhaskar N and Baskaran V. Fucoxanthin has greater potential than β-carotene in modulating retinol deficiency induced lipid peroxidation and activity of antioxidant enzymes in rats (International Congress on Bioprocesses in Food Industries & 5th Annual Convention of the Biotech Research Society of India, 2008).
- **Sangeetha Ravi Kumar**, Bhaskar N, Divakar S and Baskaran V. Biotransformation Of Fucoxanthin And Structural Elucidation Of Its Metabolites In Retinol Deficient Rats By HPLC and LC-MS (APCI). (5th International symposium on Carotenoids, 2008).
- R Lakshminarayana, G Aruna, **Ravi Kumar Sangeetha**, N Bhaskar, S Divakar & V Baskaran. Possible Degradation Of Lutein In Vitro And In Vivo: Structural Elucidation Of Lutein Metabolites By HPLC And LC-MS (APCI). (15th International symposium on Carotenoids, 2008).

National

- **Sangeetha Ravi Kumar** and Baskaran V. Carotenoid composition and vitamin-a activity of leafy greens of nutritional and medicinal importance. (NUTRIFEAST, NSI, 2008).
- **Sangeetha Ravi Kumar,** Bhaskar N, Asha M., Ganesan P. and Baskaran V. Protective Effects Of Fucoxanthin Against Vitamin-A Deficiency Induced Oxidative Stress In Murine Model (77th Annual session and symposium on NOVEL APPROACHES FOR FOOD AND NUTRITIONAL SECURITY, NASI, 2007).
- R Lakshminarayana, G Aruna, **Ravi Kumar Sangeetha**, N Bhaskar, S Divakar & V Baskaran. Possible Degradation Of Lutein In Vitro And In Vivo: Structural Elucidation Of Lutein Metabolites By HPLC And LC-MS (APCI). (77th Annual session and symposium on NOVEL APPROACHES FOR FOOD AND NUTRITIONAL SECURITY, NASI, 2007).